

The Pennsylvania State University

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**LOCAL TO LANDSCAPES: THE INFLUENCE OF LANDSCAPES ACROSS SPATIAL  
SCALES ON BUMBLE BEE COMMUNITIES AND THEIR PATHOGENS**

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## ABSTRACT

Bumble bees (*Bombus* spp) are critical pollinators contributing to native ecosystems as well as agricultural production. At least half of the studied bumble bee species in North America are experiencing population declines and range contractions due to interacting factors such as habitat loss, climate change, invasive species, and pathogens. Pathogens can cause disease, reduce fitness, and shorten lifespans. Moreover, infection rates are increased in bee populations that are stressed by poor nutrition or exposure to pesticides, and thus pathogen prevalence can serve as a broader bioindicator of poor conditions. Given the myriad of factors impacting bumble bees, a comprehensive approach is needed to assess which biotic and abiotic factors are most important for determining the health of bees across diverse landscapes.

Through multi-state and multi-year analysis, I examine how environmental conditions affect bumble bee pathogen levels and communities at different spatial scales. First, I assess whether levels of four bumble bee pathogens and distributions of bumble bee species are driven by variation in habitat at a local scale by leveraging the replicated hill and valley system in Central Pennsylvania. I then evaluate if associations between pathogen levels, bumble bee species, and environmental conditions observed in Pennsylvania are also found in North Carolina. In Pennsylvania, I found that different communities of both pathogens and bumble bees were found between forested hills and developed valleys, with viral loads higher in valleys and loads of one pathogen, *Crithidia bombi*, higher in forests. Valley habitats were dominated by *Bombus impatiens*, a disturbance resilient bee, while the forest habitats hosted more diverse bumble bee communities. Through DNA barcoding I found that there has been significant misidentification of *B. sandersoni* as *B. vagans* or *B. perplexus*, all of which are found primarily in forested landscapes. The North Carolina data, consistent with data from Pennsylvania as well as other

studies in the United States, demonstrate that bumble bee pathogen loads are higher in areas with more honey bees and developed land. However, across the broader datasets, there were variable effects of floral resources and bee community composition on pathogen loads. For example, areas with more floral resources were associated with decreased viral loads in honey bees in North Carolina and in bumble bees in Pennsylvania; but were associated with increased viral loads in North Carolina bumble bees. Moreover, these data show more floral resources support more diverse bumble bee communities, but more diverse communities may facilitate increased loads of some pathogens (e.g., *Crithidia*) for which some bumble bee species are more susceptible.

Ultimately this thesis highlights several outstanding research issues and provides management recommendations. Pathogen transmission and prevalence can clearly be influenced by habitat type but the complexity of landscapes, changing bumble bee communities, and variable susceptibility of different bumble bee species means there is not a single, simple solution to reducing the effects or loads of pathogens for bumble bees. Generally, the presence of honey bees and increased disturbance lead to increased pathogen prevalence. Thus, management efforts should be targeted to improving habitat quality through increased floral and nesting resource quality, which reduces pathogen levels in both honey bees and bumble bees. Higher quality habitat can improve bumble bee community diversity, which can potentially reduce transmission rates, though this depends on the specific species involved. Future studies should focus on species showing declines, to better understand the disease dynamics. While there was partitioning of pathogens at a local scale, the overall loads of pathogens shift over larger regional scales, highlighting the value of examining effects from local to broader landscape scales.

## TABLE OF CONTENTS

LIST OF FIGURES .....	vii
LIST OF TABLES.....	x
ACKNOWLEDGEMENTS .....	x
Introduction .....	1
Chapter 1: Local habitat type influences bumble bee pathogen loads and bee species distributions .....	9
Abstract .....	9
Introduction .....	10
Methods.....	14
Study Area and Field Collection.....	14
Pathogen quantification.....	15
DNA barcoding for specimen identification .....	17
Species abundance and composition.....	18
Landscape and climate variables .....	19
Statistical analysis.....	20
Results .....	21
Landscape composition of habitat types .....	21
Pathogen loads by habitat type and landscape variables.....	22
Effect of habitat type and landscape variables on community composition ..	23
Diversity-related patterns .....	26
Discussion .....	26
Figures.....	33
Supplemental Figures.....	36
Chapter 2: Comparisons of the roles of landscape factors in bee pathogen loads across different geographic regions.....	41
Abstract .....	41
Introduction .....	42
Methods.....	47
Bee and Floral Sampling .....	47
Pathogen and Parasite Screens.....	50
Landscape and Weather Data .....	51
Analysis.....	52
Literature review.....	52
Results .....	54
Pathogen loads in <i>Bombus impatiens</i> populations in North Carolina .....	54

Landscape factors influence pathogen and parasite prevalence in <i>Bombus impatiens</i> populations in North Carolina .....	55
Comparisons of effects of landscape variables between North Carolina and Pennsylvania .....	55
Ecoregional and habitat correlations with pathogen and parasite loads based on literature review .....	58
Discussion .....	57
Figures.....	66
Tables .....	71
 Chapter 3: Declining bumble bee species diversity over time and in more disturbed landscapes .....	 844
Abstract .....	844
Introduction .....	845
Methods.....	867
Landscape Data.....	889
Historical data.....	889
Analysis.....	90
Results and Discussion.....	91
Conclusions .....	96
Figures.....	98
Tables .....	103
 Conclusion.....	 104
 Appendix: Assessing Factors that Explain the Persistence of Crickets and Katydid within Intensive Agricultural Landscapes: The Relative Roles of Pesticide Exposure and Microhabitat Characteristics.....	 109
Abstract: .....	109
Introduction .....	110
Methods.....	113
Study area and site selection.....	113
Point count surveys .....	114
Landscape variables .....	115
Statistical analysis.....	116
Results .....	117
Discussion .....	119
Figures and Tables.....	123
 References .....	 129

## LIST OF FIGURES

Figure 1: Map of sites across central Pennsylvania. Combined landscape cover categories for each habitat. ....	33
Figure 2: Impact of landscape features on pathogen loads. a) Pathogen loads across the valley to forest gradient. b) Correlation matrix of pathogen loads with landscape variables, bumble bee diversity, and species composition. ....	34
Figure 3: Distribution of species by local habitat. a) Catch rate (bees per minute) of <i>B. impatiens</i> , <i>B. bimaculatus</i> , and <i>B. sandersoni</i> across the forest to valley gradient. b) Percent captured of each species in each habitat type. Colors correspond to the those .....	35
Supplemental Figure 1: Box plot of elevation of sites in each habitat type.....	36
Supplemental Figure 2: PCA plot distinguishing Forest, Edge, and Valley sites. ....	37
Supplemental Figure 3: Vector diagram of site PCA. ....	38
Supplemental Figure 4: Complete correlation matrix including all landscape variables, pathogens, and bumble bee species. ....	39
Supplemental Figure 5: Distribution of loads of each pathogen across all sample pools across all sites.....	40
Supplemental Figure 6: Distribution of species by site in the Forest, Edge, and Valley regions. Each row represents a site. ....	40
Figure 4: Map of surveyed sites in North Carolina. Sites coded by overall habitat quality score. ....	66
Figure 5: Histograms of pathogen loads. ....	67
Figure 6: Box plot of <i>C. bombi</i> loads by ecoregion. Coastal Plain and Appalachians are significantly higher on average than the Piedmont ( $p = 0.006$ ).....	67
Figure 7: a) Summer floral Index model for BQCV and <i>Bombus</i> diversity model for <i>C. bombi</i> . The <i>Bombus</i> diversity model for <i>C. bombi</i> is significantly driven by an outlier indicated in red; without this value there are no significant models describing <i>C. bombi</i> loads in <i>B. impatiens</i> . b) Models of BQCV and <i>Crithidia bombi</i> against the spring floral index and percent forest, the two of the main variables initially predicted to effect pathogens loads based on McNeil et al. (2020).. ....	68
Figure 8: Correlation matrix with all landscape and site variables with pathogen loads across Pennsylvania from McNeil et al. (2020) and Chapter 1, and North Carolina 2022 data. 2018	

and 2019 are data from Pennsylvania from McNeil et al. (2020). 2020 are data from Pennsylvania from Chapter 1. 2022 is data from North Carolina. In McNeil et al. (2020), precipitation and growing degree days are from the spring, and the percent natural category does not include grassland/pasture as it does in 2022..... 69

- Figure 9: a) Comparison of *B. impatiens* BQCV and DWV loads at site with and without *A. mellifera*. BQCV at site with *A. mellifera* were significantly higher than sites without ( $p = 0.04$ ). This also compares BQCV of honey bees where present. DWV at sites with *A. mellifera* ( $p = 0.82$ ). b) correlation matrix with *A. mellifera* pathogen loads against all 2022 landscape and site variables, as well as the habitat quality score and *B. impatiens* pathogen loads. .... 70
- Figure 10: Summary of literature review and the significant effect of variables on DWV, BQCV, Trypanosomes, and *Vairimorpha spp.* in *Bombus* in North America. The number of papers addressing a category in the bottom right corner. Please note some papers had more than one variable with in a category, see table 4. .... 71
- Figure 11: Map of surveyed sites across North Carolina colored by habitat quality. . 98
- Figure 12: a) All bees at sites in each ecoregion and all bees at sites for each habitat quality for both 2021 and 2022. b) Bumble bees collected across North Carolina from 1900 to through 2022..... 99
- Figure 13: Correlation matrix describing correlations between percent of species at site and various landscape factors in 2021 and 2022. Asterisks indicates five or fewer bees collected. .... 100
- Figure 14: Comparison of Shannon diversity index compared across habitat quality. 101
- Figure 15: Growing degree days was the top model predicting percent *B. impatiens* at a site. .... 102
- Appendix Figure 1: A map of our Pennsylvania study area highlighting the region where I focused our study. Survey sites are indicated with black triangles. The color scheme of the map indicates insecticide toxic load (LD50/ha) with cool colors representing low values and increasingly warm colors representing higher LD50 values. .... 123
- Appendix Figure 2: Shown are modeled functional relationships between Ensifera species and percent agriculture cover (100m radius, top row) and percent developed cover (100m radius, bottom row). Each species' ecological pattern estimate is shown with a different color of line and the 95% confidence intervals is shown in gray. .... 124
- Appendix Figure 3: Plotted beta ( $\beta$ ) coefficient values for all biologically meaningful logistic regression models (circles), with 85% confidence intervals around each estimate (lines). .... 125



## LIST OF TABLES

Table 1: All primers used for qPCR. ....	71
Table 2: Landscape and site variables used in models. ....	72
Table 3: AIC model selection results. Models were considered significant if delta AIC was below 2, not within 2 delta AIC of null model, and 95% CI of betas did not include zero. .....	73
Table 4: Summary of literature review papers. Describes pathogen studied, variables tested, directionality of effects of variables, and categories used for synthesizing study results in Figure 10 .....	74
Table 5: AIC model selection table for <i>B. impatiens</i> . All competitive models under $\Delta AIC$ 2 are shown. Significant models are indicated with an asterisk.....	103
Appendix Table 1: All habitat covariates used in our analyses. For each variable, I denote the source (Cropland Data Layer [CDL], Chesapeake Bay Watershed dataset [CBW], etc.), mean value, standard deviation (sd). All variables are presented in the form of percentages except distance-to-nearest-building and insecticide toxic load are expressed in meters and LD50/ha, respectively. ....	126
Appendix Table 2: The candidate model sets for each species, ranked in descending order of $\Delta$ Akaike's Information Criterion adjusted for small sample size ( $\Delta AICc$ ). Also shown is the number of model parameters (k), model weight (w) and the $\beta$ parameters for models with covariates (competing models only; $\Delta AICc < 2.0$ ). For each $\beta$ parameter shown, I also indicate whether the $\beta$ 85% confidence intervals include zero (no asterisk) or do not include zero (asterisk). ....	126

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## Introduction

Pollinators are some of the most ecologically and economically significant organisms. Globally, 85% of flowering plants benefit from animal-mediated pollination (Ollerton et al., 2011) and many have coevolved with and depend on pollinators for reproduction (Johnson & Anderson, 2010; Miller-Struttman et al., 2015; Ranta & Lundberg, 1980; Ranta & Vepsäläinen, 1981). Bees can be some of the most effective pollinators (Ballantyne et al., 2017), and there is evidence of plant species declines when their co-evolved bee species decline (Biesmeijer et al., 2006; Burkle et al., 2013; Mathiasson & Rehan, 2020). Additionally, pollinators support production of 75% of major global agricultural crops (Klein et al., 2017), with insect pollinators providing up to \$34 billion in economic value to agriculture in the United States alone (Jordan et al., 2021). Plants such as watermelon, cucumber, and cranberry depend on pollinators to make fruit (Cooley & Vallejo-Marín, 2021). Even in self-pollinating plants, insect pollinations can increase fruit set (Garibaldi et al., 2013) and shelf life (Klatt et al., 2014).

Bumble bees (*Bombus* spp) are especially effective pollinators as they are generalists capable of supporting broader floral communities (Russell et al., 2017; Vaudo et al., 2016; Wood et al., 2021), but yet tend to preferentially pollinate certain plant species on any given day, thus facilitating pollen transfer between conspecifics (Russell et al., 2017). They are also efficient in their floral visitation and sophisticated in their flower handling technique, including using buzz pollination which is essential for some plant species (Russell et al., 2017). There is considerable evidence that bumble bees are often more efficient at pollinating than honey bees (Garibaldi et al., 2013; Winfree et al., 2007) and mechanical pollination methods (Pressman et al., 1999). Buzz pollination, whereby bees use buzz frequencies to release pollen from flowers, makes bumble bees especially adept at collecting and

transferring pollen from plant to plant (De Luca & Vallejo-Marín, 2013) and thus they are a key managed pollinator for many buzz pollinated crops, such as tomato, eggplant, kiwi, and blueberry (Batra, 1993; Cooley & Vallejo-Marín, 2021). They are such effective pollinators that they are commercially reared and imported to improve crop yields (Stubbs & Drummond, 2001; Zhang et al., 2015).

Despite the economic and ecological importance of bumble bees, there is considerable quantitative evidence that many species are in decline. Over the last 120 years at least half of bumble bee species are showing evidence of declines in relative abundance by up to 96% and significant range contractions up to 87% (Cameron et al., 2011; Colla et al., 2012). In North America, species such as *B. affinis* and *B. franklini* have been listed as critically endangered (*FWS-R3-ES-2015-0112*, 2016; *FWS-R1-ES-2018-0044*, 2018). *B. affinis* was once ubiquitous across eastern North America and is now restricted to only a few areas across the East Coast (Smith et al., 2019) and in the upper Midwest. *B. franklini* was common on the West Coast but has not been seen since 2006 (Thorp, n.d.). Many other species have shrinking ranges and fewer observations in places they were historically abundant, including *B. terricola*, *B. pensylvanicus* and *B. fraternus* (Cameron et al., 2011; Colla et al., 2012; Colla, 2016; Colla & Packer, 2008; Colla & Ratti, 2010; Graves et al., 2020; Turley et al., 2022). A few species are maintaining, if not increasing, in relative abundance, with *B. impatiens* being the most common in Eastern North America persisting in most areas where other *Bombus* species are declining (Cameron et al., 2011; Colla et al., 2012; Koch et al., 2015; Koh et al., 2016). There is yet to be a clear reason for the resilience observed in *B. impatiens*, but some argue their broad diet allows them to accommodate changes in habitat conditions that other *Bombus spp.* cannot withstand (Wood et al., 2019).

Climate change is one major factor contributing to some of these declines (Cameron & Sadd, 2020; Goulson et al., 2005). Large-scale, long-term bee record data have found climate shifts to be the

main factor explaining changes in distribution through time, sometimes more so than land use change (Jackson et al., 2022; Sirois-Delisle, 2017; Soroye et al., 2020). These shifts are especially concerning because studies have found that some bumble bee species are expatriated from the southern half of their range, but are not moving north at the same rate, meaning that ranges are shrinking not just moving to correct climates (Kerr et al., 2015). Additionally, we see alpine or mountainous bumble bees moving up in elevation to avoid warmer temperatures (Biella et al., 2017; Miller-Struttman et al., 2022); the concern is that at some point the bees will reach the top of their mountain and no longer be able to avoid warming conditions (Fourcade et al., 2019). Climate change can also impact the floral communities that bees depend on for their nutrition, and/or their ability to forage to collect resources (Höfer et al., 2021; Kuppler et al., 2021; Powers et al., 2022). There is increasing evidence for phenological mismatch and loss of co-evolved relationships between some bumble bees and plants likely due to shifts in local climate (Miller-Struttman et al., 2015).

Habitat loss and degradation has occurred at a large scale over the last century and has long been considered as a major stressor to these bees through loss of sufficient cross-season food resources (Cameron & Sadd, 2020; Goulson et al., 2008). Bumble species have varying habitat requirements; however, they all need consistent floral resources to persist (Licznar & Colla, 2020). Agriculture is often the driving force behind the habitat conversion associated with bumble bee declines both in North America and in other parts of the world (Feon et al., 2010; Grixti et al., 2009). Even within agricultural systems, the loss of landscape heterogeneity, i.e., the loss of small diverse farming practices to monoculture crops has been associated with reduced bumble bee diversity through removal preferred habitats, food resources, and also nesting resources, as edge habitats and slopes are considering leading areas for bumble bee nesting (Carvell et al., 2007; Grixti et al., 2009; Hemberger et al., 2021; Öckinger & Smith, 2007). The availability of natural areas in and around farms increase bee abundance and

colony production by providing nesting and alternative food sources, implying that bees must have access to non-agricultural habitat to provide their ecosystem service of pollination (Carvell et al., 2007; Crone & Williams, 2016; Greenleaf & Kremen, 2006; St. Clair et al., 2020). While bees may persist in some agricultural landscapes, they can be exposed to pesticides which, while sometimes lethal (Raimets et al., 2018), can also reduce fecundity (Laycock et al., 2012), weaken their immune system (Czerwinski & Sadd, 2017), and reduce foraging ability (Gill et al., 2012). In the long term, habitat loss and degradation can lead to isolated populations (Bhattacharya et al., 2003), reduced abundance, and potentially reduced genetic diversity (Lozier et al., 2011).

Resource competition from invasive species also may impact bumble bee declines. When resources are scarce honey bees have been found to outcompete bumble bees (Thomson, 2016) and honey bees are correlated with reduced species richness and abundance in bumble bee communities (Su et al., 2022). Some bumble bee species themselves are the invasives such as *B. terrestris* in Patagonia, which has taken over where *B. dahlombii* used to be the most dominant pollinator (Morales et al., 2013). While not explicitly invasive, there is increasing concern of the impact of commercial bumble bees that are used in greenhouses or agricultural production to improve yields (Ratti & Colla, 2010). Commercial bumble bees *B. impatiens* in North America and *B. terrestris* in the rest of the world have been linked to spillover of novel or highly lethal pathogens to native bumble bee communities (Colla et al., 2006; Otterstatter & Thomson, 2008).

Pathogens, such as viruses or gut macroparasites, are often cited as a major reason for decline of some bumble bee species (Cameron & Sadd, 2020; Goulson et al., 2015). *Vairimorpha bombi* has been implicated as a major player, as it is often lethal (Otti & Schmid-Hempel, 2007) and is often most prevalent in rare species of bumble bees (Cordes et al., 2012; Levenson & Tarpy, 2022; Malfi & Roulston, 2014), but there are many other pathogens infecting bumble bees. For example, Black Queen



Cell Virus and Deformed Wing Virus both occur in relatively high loads in honey bees and have since spilled over in to bumble bees (Alger, 2018; Burnham et al., 2021; Tantillo et al., 2015). One of the most common pathogens in bumble bees is *Crithidia bombi*, a trypanosome gut parasite (Brown et al., 2000; Mockler et al., 2018). Transmission and levels of these pathogens are influenced by the physiology and immunocompetence of the host bumble bee, as well as environmental conditions (Meeus et al., 2018). For example, *C. bombi* is usually transmitted between bees on flowers, making the structure of the flower and the microclimate around the flower critical in determining transmission potential (Adler et al., 2018; Figueroa et al., 2019; McArt et al., 2014). While there are other pathogens that have been found in bumble bees besides these four, the other pathogens tend to be sporadic and rare in the Eastern United States (Dolezal et al., 2016; Ezray, 2019). Importantly, there is evidence for compounded effects of stressors on bees: while a pathogen may not be lethal on its own, when the bee is also exposed to increased stress in the form of starvation (e.g. *C. bombi*; Brown et al., 2000), pesticides (e.g., *Vairimorpha*; Calhoun et al., 2020), or degraded landscapes (e.g. *Vairimorpha spp.*, *Crithidia spp.*, *Apicystis bombi*; Piot et al., 2019) pathogen load, prevalence, or severity can increase and become debilitating or lethal.

Given the myriad of factors impacting these bees, a comprehensive approach is needed to assess which factors are more important for the health of these bees and the degree to which different abiotic and biotic factors contribute to prevalence and levels of these pathogens in wild bumble bees in diverse habitats and landscapes. Two studies have been exemplary in leading the way for such analyses. McNeil et al. (2020) examined pathogen loads (DWV, BQCV, *V. bombi*) in bumble bees collected across Pennsylvania, and found that increased floral resources in the landscape were associated with reduced pathogen loads, which increased abundance of managed honey bees was associated with increased pathogen loads. However, there were also significant effects of latitude and longitude in this study,

suggesting a strong influence weather, climate or other ecoregional conditions (for example, northwestern Pennsylvania is more heavily forested than southeastern Pennsylvania). Thus, questions remained about the role of local habitat in influencing pathogen loads. McArt et al. (2017) studied the broadest landscape scale thus far, using data from Cameron et al. (2011) collected across 40 states and modeling the effect of broad land use and management factors on *Vairimorpha bombi* and *Crithidia bombi* prevalence in four declining and four stable *Bombus spp.* They found a significant increase in *Vairimorpha bombi* prevalence in declining *Bombus spp.* with increasing development, latitude, and the pesticide chlorothalonil, but no significant relationships with stable *Bombus spp.* or *C. bombi* levels. Notably, McArt et al (2017) found a strong regional effect with higher pathogen prevalence at more northern latitudes. Regional fluxes are likely influenced by the accumulated effects of habitat and climate, but also history of land use. The variation by region further highlights the need to examine patterns across pathogens that integrates across different spatial scales and to consider management needs at a more regional scale.

In this thesis I examine how biotic and abiotic factors in the landscape affect bumble bee communities and pathogens, by addressing the local effects of habitat on bumble bee pathogen loads and community, as suggested in McNeil et al 2020, and by comparing pathogen-landscape effects between regions, as suggested in McArt et al 2017, in an effort to better understand how to support declining bumble bee populations.

In Chapter One I seek to better understand whether landscape patterns in Pennsylvania inferred by McNeil et al. (2020) are driven by local habitat with a more targeted sampling regime. Central Pennsylvania is a unique system with repeated hills and valleys providing the opportunity to decipher the effect of habitat on both bumble bee communities and their pathogen loads. The valleys are mostly developed and home to towns and agriculture, while the mountain ridges are forested and relatively

undisturbed. I collected bumble bees at 31 sites across central Pennsylvania in 2020, characterized their bumble bee communities, and assess the loads of four leading bumble bee pathogens (BQCV, DWV, *Vairimorpha bombi*, as well as *Crithidia bombi*, which was not assayed in McNeil et al. (2020) in *Bombus impatiens*, the Common Eastern Bumble Bee, across this forest to valley gradient. This chapter discusses local differences between bumble bee pathogen and communities, and how habitats contribute to potential differences in pathogen transmission and community composition.

In Chapter Two I seek to evaluate if patterns observed across Pennsylvania are upheld more broadly across the eastern United States, with the goal of providing management recommendations for mitigating pathogen effects in bumble bee communities. I quantified and modeled the relationships between bumble bee pathogens, bumble bee communities, honey bees, and landscapes across the state of North Carolina. This study takes advantage of the distinct ecoregional found within the state of North Carolina as well as habitats of varying quality to understand what landscape factors most influence pathogen loads. As in Chapter One, I screened *B. impatiens* for BQCV, DWV, *Vairimorpha bombi*, and *Crithidia bombi*, and I used mixed fixed effects models to determine which landscape variables best described the patterns in pathogen loads. I also screened *A. mellifera* for pathogens, which was not done in previous studies, assessing the effects of landscapes on these bees and comparing disease loads by site between honey bees and bumble bees. In addition to our pathogen models, I conducted a literature review to better understand the effects of landscapes on bumble bee pathogens across different ecoregions and landscape types in North America. This chapter explores the multi-faceted effects of landscapes on bumble bee pathogens and highlights broader geographic trends.

In Chapter Three I seek to assess historical and current *Bombus* community composition in North Carolina in response to abiotic and biotic factors in the landscapes, in an effort to provide management recommendations to support diverse bumble bee species in North Carolina. I assessed *Bombus*

communities across ecoregions and across habitats of varying quality in 2021 and 2022. As in Chapter Two, I used mixed fixed effect models to determine which landscape variables best described *Bombus* species diversity and proportion of *B. impatiens* in the community in 2022. Using historical data from North Carolina, I compared past *Bombus* communities to the communities observed in 2021 and 2022. This chapter describes patterns of *Bombus* community change over time and discusses potential landscape factors driving of those changes.

This thesis evaluates and integrates the relative roles of habitat, weather, climate, bee community, and ecoregion on landscapes features, climate, and habitats on parasite prevalence and transmission in bumble bees and on bumble bee community composition. As bumble bees are critical pollinators in both natural and agricultural ecosystems, this research assists in providing a more holistic understanding of how best to support declining bumble bee populations and species and identifying programs and locations where management resources can be most beneficial. In addition to providing information as to how to mitigate pathogens in bumble bee populations, these studies use pathogens as bioindicators of poor-quality landscapes. Moreover, these studies highlight significant gaps in our knowledge about bumble bee pathogen infection and transmission dynamics and how they are influenced by anthropogenic changes.

## Chapter 1: Local habitat type influences bumble bee pathogen loads and bee species distributions

### Abstract

Bumble bees (*Bombus* spp.) perform important ecological services in both managed and natural ecosystems. Anthropogenically-induced change has altered floral resources, climate, and insecticide exposure, factors which impact health and disease levels in these bees. Habitat management presents a solution for improving bee health and biodiversity, but this requires better understanding of how different pathogens and bee species respond to habitat conditions. I take advantage of the washboard of repeated ridges (forested) and valleys (mostly developed) in central Pennsylvania to examine whether local variation in habitat type and other landscape factors influence bumble bee community composition and levels of four leading pathogens in the common eastern bumble bee, *Bombus impatiens*. Loads of viruses (DWV and BQCV) were found to be lowest in forest habitats, whereas loads of a gut parasite, *Crithidia bombi*, were highest in forests. Ridgetop forests hosted the most diverse bumble bee communities, including several habitat specialists. *B. impatiens* was most abundant in valleys, and showed higher incidence in areas of greater disturbance, including more developed, unforested, and lower floral resource sites, a pattern which mirrors its success in the face of anthropogenic change. Additionally, DNA barcoding revealed that *B. sandersoni* is much more common than is apparent from databases. Our results provide evidence that habitat type can play a large role in pathogen load dynamics, but in ways that differ by pathogen type, and point to a need for consideration of habitat at both macro-ecological and local spatial scales.

## Introduction

Bumble bees (*Bombus* Latr.; Hymenoptera; Apidae: *Bombus* spp.) perform critical pollination services for flowering plants in both agricultural and natural landscapes, thus, conserving their populations is a priority for both economic and ecological reasons (Goulson et al., 2008). Many bumble bee species are in decline, with climate change, habitat loss/degradation, and pathogens implicated as leading explanatory stressors (Cameron & Sadd, 2020). While some bumble bee species are showing declines, others are stable or increasing in distribution, likely as a result of differences in natural history, physiology, and habitat preferences and requirements (e.g., Colla & Packer, 2008; Jackson et al., 2022; Williams et al., 2009). Moreover, there is growing evidence that certain pathogens are more associated with declining bumble bee populations (Cordes et al., 2012) and that physiological stress, including that imposed by poor nutrition as a result of habitat degradation, can increase pathogen loads (Brown et al., 2003; Meeus et al., 2018). Assessing how local environmental conditions, including habitat type and quality, influences bumble bee species abundance and pathogen loads is important for developing strategies for managing landscapes to curtail bumble bee diseases and maintain healthy populations and communities.

Bumble bee habitat requirements are influenced by multiple aspects of their life cycles, which vary by species. Important habitat components include queen overwintering sites, colony nest sites, and floral resources (*i.e.*, nectar and pollen) for developing brood and adults (reviewed in Liczner & Colla, 2019). Bumble bee species-specific floral resource preferences are influenced by tongue length, degree of specialization on certain flowers, and nutritional content (Harder, 1985; Miller-Struttman et al., 2015; Somme et al., 2015; Vaudo et al., 2016). Flowers need to be available to support all stages of the bumble bee life cycle of a given species. While certain bumble bees have been noted to prefer specific

habitats (e.g., Williams et al., 2014), quantitative data on species-specific habitat preferences are limited (cf. Colla, 2016). A better understanding of how species partition themselves by habitat will provide the information needed to understand the influence of anthropogenic change.

The pathogens that infect *Bombus* spp. vary substantially in their epidemiological properties. For example, *Crithidia bombi* (Trypanosomatida: Trypanosomatidae), a trypanosome gut parasite, is usually benign but can impose deadly disease on host bees when combined with other stressors, such as starvation (Brown et al., 2000). In contrast, *Vairimorpha bombi* (Microsporidia: Nosematidae), a *Bombus*-specific microsporidium, is almost always highly virulent (Otti & Schmid-Hempel, 2007; Rutrecht & Brown, 2008) and has been implicated as a leading driver of bumble bee population declines (Cameron et al., 2011). Deformed wing virus (DWV; Picornavirales: Iflaviridae: *Iflavivirus*) is a commonly occurring, broad-range insect virus that usually spreads through the digestive system to infect many tissues throughout a host. Although it can reach high levels in the honey bee (*Apis mellifera*), DWV does not typically reach high infection levels in wild bumble bee populations (Alger et al., 2019a; McNeil et al., 2020). Black queen cell virus (BQCV; Picornavirales: Triatovirus), in contrast, can be very prevalent, reaching infection frequencies greater than 50% in some wild bumble bees communities (Alger et al., 2019a; McNeil et al., 2020), although usually not with as high titers as honey bees (Alger et al., 2019a; Welch et al., 2009). As in honey bees, BQCV infections are most problematic at the larval bumble bee stages, where it can kill developing larvae and seems to be specifically problematic for queen larvae; however, high loads of BQCV can also be found in adult workers and drones (Peng et al., 2011; Tantillo et al., 2015). All these pathogens are thought to be spread between bumble bees either within social colonies or through foraging on a shared floral community (Alger et al., 2019b; Burnham et al., 2021; Durrer & Schmid-Hempel, 1997). Given the varying epidemiology of these pathogens, I

may expect these pathogens to differ in the environmental and community variables impacting their prevalence and transmission.

The severity and prevalence of disease can be amplified by a wide variety of environmental stressors. For example, physiological stress caused by inadequate forage (i.e., poor nutrition) reduces immune responses in wild bees, leaving them vulnerable to disease (Branchiccela et al., 2019; Brunner et al., 2014; Figueroa et al., 2021). Inclement weather (e.g., precipitation) can lead to high prevalence of disease infection in bumble bee populations (Chen et al., 2012; Gardner et al., 1977; Gisder et al., 2010; McNeil et al., 2020; Neidel et al., 2017; Palmer-Young et al., 2018, 2019). Furthermore, toxins introduced into the environment by humans (e.g., fungicides [McArt et al., 2017] and insecticides [Czerwinski & Sadd, 2017]) can play a large role in reducing immune performance of wild bumble bees (Czerwinski & Sadd, 2017). Beyond abiotic factors, heterospecific interactions (direct or indirect) also impact bumble bee disease ecology, e.g., honey bees often harbor higher levels of pathogens that can spread to *Bombus* spp (Dolezal et al., 2016; Graystock et al., 2016; Mallinger et al., 2017; Murray et al., 2019). Additionally, studies have found that declining and thus rare bumble bee species (e.g., the federally endangered *B. affinis*) harbor higher pathogen loads than more widespread common species, suggesting that these species might be more susceptible (Cordes et al., 2012). Given the species-level differences in potential to act as pathogen reservoirs, the species composition of bees in the community should impact landscape-level pathogen dynamics.

While there are environmental correlates identified that impact certain pathogens, data on which habitat types improve or disproportionately promote transmission of pathogens are limited. Habitats vary in their floral resources, temperature, and rainfall conditions, as well as in the types of bee communities that they support: all of these could influence pathogen dynamics in a selected bumble bee species



(Meeus et al., 2018). Studies on the role of habitat on pathogen loads in Europe showed that *C. bombi* levels increased in forested sites (Bosmans et al., 2018), and prevalence of both *C. bombi* and *V. bombi* increase with urban habitat (Goulson et al., 2012; Mráz et al., 2021; Theodorou et al., 2016). Given that habitats are key units of conservation, improved knowledge of which habitats harbor more pathogens and why will help guide management solutions.

In an effort to tease apart the leading factors and stressors in the landscape that drive pathogen loads and thus better understand how to manage and model pathogen transmission in real landscapes, McNeil et al. (2020) assayed pathogen loads of DWV, BQCV, and *Vairimorpha bombi* in bumble bees (*Bombus impatiens*, Cresson) across numerous sites across Pennsylvania, USA, and found disease prevalence in the landscape was best explained by managed honey bee colony density, spring forage, and availability of nesting habitat. However, in landscapes, environmental and habitat variables can be highly intercorrelated (Lichstein et al., 2002; Petracca et al., 2018): for example, in McNeil et al. (2020) forested habitats are predicted to have more spring forage and nesting sites, which in turn correlates with lower pathogen loads. McNeil et al (2020) also found a correlation between latitude and longitude that aligned with different habitat regions in the state: the more forested higher altitude northern sites exhibited lower pathogen loads, especially in BQCV and DWV, than the more southeastern agricultural and non-forested valley landscapes.

Here, I extend the work of McNeil et al. (2020) to evaluate the impact of habitat types on both wild bumble bee pathogen loads and bumble bee species composition using a controlled, more local design. I collected wild bumble bees from forested ridges, agricultural valleys, and ecotonal habitats within the replicate-rich hill and valley system in central Pennsylvania (Figure 1). I examine how habitat as well as other landscape variables influences loads of four pathogens – BQCV, DWV, *Vairimorpha*

*bombi* and *Crithidia bombi* – in *B. impatiens*, as well as the diversity of bumble bee species across these zones. Our study provides insight into the role of habitat type and quality on bee diversity, community composition, resilience, and disease loads. Overall, our study demonstrates that both the abundance of different pathogens with the same bee species (*Bombus impatiens*) and the distribution of different bumble bee species varies significantly with habitat type even at a more local scale.

## Methods

### *Study Area and Field Collection*

The Central Appalachian region of Pennsylvania has a series of ridges and valleys laid out in a repeated and linear fashion caused by a collision of continents ~270 million years ago. These ridges include a mix of deciduous- and mixed coniferous forests and are higher elevation (200 m – 660 m for sampled sites). The valleys are of mixed land use but dominated largely by agricultural and suburban/urban habitat and are lower elevation (130 m - 400 m in sampled sites). To assess the role of habitat on pathogen loads, I chose sites across three distinct habitat types sampled across this region: 13 forest ridgetop sites, 12 valley sites, and 12 edge sites that were intermediate in elevation and land cover composition. Sites were a minimum of 1.6 km apart (Figure 1a).

Using a fairly random walk across each site, I searched across all flowers, and collected and recorded species identification of the first 24 bumble bees at each of the 37 sites (for 4 sites I obtained less than 24 due to a shortage of bumble bees [2 at site 20, 2 at site 23]) and I recorded the time it took to collect at each site. For each bee captured, I recorded its species identity, caste, and species of flower upon which it was captured. Bee species were visually identified *in situ* although I retained individuals

that could not be identified in the field. I retained all *B. impatiens* workers obtained from this survey for pathogen screens (31 sites: 11 valley, 9 edge, 11 forest). While it may not be representative of the response of all regional bumble bee, *B. impatiens* is the most common species at all sites, thus providing enough individuals to compare between sites. I did not collect on days with rain, wind over ~24 kmph, or where the temperature was below 15.5°C or above 29.5°C. Collections occurred from 22 June - 8 July, 2020, which spans the period of peak worker production for most bumble bee species and encompasses the phenological window for all bumble bees in the region (Williams et al., 2014). Bees for pathogen screens were placed, alive, in a cooler on wet ice for transport to Pennsylvania State University where they were euthanized and stored in a -80°C freezer.

#### *Pathogen quantification*

To detect pathogen levels in sampled bees, I performed quantitative PCR (qPCR) for four pathogens: DWV, BQCV, *Vairimorpha bombi*, and *Crithidia bombi*, along with a control gene *Elongation factor - 1 $\alpha$*  (*EF-1 $\alpha$* ). These screens were performed on pooled abdomens of three *B. impatiens* workers per sample and I analyzed one to three pools per site, depending on how many *B. impatiens* I collected (6 sites had no pools, 3 sites had 1 pool, 8 sites 2 pools, 20 sites 3 pools; valley: 30 pools, edge: 24 pools, forest: 25 pools). Using pools of *B. impatiens* ensured that there is enough pathogen in a sample that it can be detected.

To quantify pathogen loads I performed qPCR on these pooled extracts. RNA was extracted using the Zymo Direct-zol™ RNA extraction kit and protocols. For the lysis step, three bee abdomens were homogenized in 1500  $\mu$ l TRIzol in a 2 ml vial with three metal beads using an Omni Bead Ruptor for two 35-second cycles on ‘low’ intensity. RNA was quantified using a Nanodrop (ThermoFisher

Scientific) and diluted in molecular grade water to obtain a 500 ng, 10 µl sample for reverse transcription. RNA was reverse transcribed using the Applied Biosystems High-capacity cDNA Reverse Transcriptase Kit following recommended reagent ratios and thermocycler conditions. Following cDNA synthesis, the cDNA was diluted in 8 µl of water for 2 µl of cDNA for each of four diluted 96 well plates that were kept in a -20°C freezer until use in quantitative PCR within five days of synthesis to avoid using degraded cDNA.

Quantitative PCR was performed using a standard 10 µl SybrGreen-based reaction (Applied Biosystems). Each sample was run in triplicate and each plate had three non-template-controls. Plates were run on an Applied Biosystems 7900 instrument (ThermoFischer Scientific) using standard conditions, annealing at 60°C. Amplifications were performed using the following primers: black queen cell virus (BQCV) 5'-TTTAGAGCGAATTCGGAAACA-3' and GGCGTACCGATAAAGATGGA (McNeil et al., 2020; vanEngelsdorp et al., 2009), deformed wing virus (DWV) (A & B) 5'-GTT TGT ATG AGG TTA TAC TTC AAG GAG-3' and 5'-GCC ATG CAA TCC TTC AGT ACC AGC -3' (Ryabov et al., 2014), *Vairimorpha bombi* 5'- GGCCCATGCATGTTTTTGAAGATTATTAT-3' and 5'-CTACACTTTAACGTAGTTATCTGCGG-3' (BOMBICAR ;(McNeil et al., 2020; Plischuk et al., 2009) , *Crithidia bombi* 5'-GGCCACCCACGGGAATAA-3' and 5'-CAAAGCTTTCGCGTGAAGAAA-3' (Ezray, 2019), *EF-1α* 5'- CCGACAAGGCTCTTCGTTTA-3' and 5'-ATGCCTGGCTTCAGAATACC-3' (McNeil et al., 2020; Tian et al., 2019). Each gene was run on a separate plate. Each set of primers has been used previously and found to double linearly as expected with cycles, thus a standard curve was not used.

All C<sub>T</sub> values above 35 as well as those with no amplification were considered 'no detection' and given a value of 35. I used the standard conversion for normalization with 2 delta-delta Ct as was done

in McNeil et al. (2020) to obtain log<sub>2</sub> fold differences from no detection (0). If a sample did not amplify, it was assigned a value of 0 regardless of *EF-1 $\alpha$*  normalization.

### *DNA barcoding for specimen identification*

Due to the difficulty in correctly separating the mimetic (Ezray, 2019; Williams et al., 2014) bumble bee species *B. sandersoni*, *B. perplexus*, and *B. vagans* via morphological means (Milam et al., 2020), I used DNA barcoding to identify each specimen in this complex to species. A leg was removed from each specimen and extracted using the E.Z.N.A tissue DNA kit and associated protocols with 100  $\mu$ l of elution buffer. Following extraction, each sample was amplified using a standard PCR procedure using a 15  $\mu$ l reaction with 1  $\mu$ l DNA, 7.5  $\mu$ l Taq Mastermix, 0.3  $\mu$ l each of the primers BF1 (Bombus\_F1; 5'-GCYATATGATCAGGAATAATTGG-3') and BR1 (Bombus\_R1; 5'-GGATCACCTCCTCCTATTGGATC-3') and the following PCR conditions: 95°C for 2 min 30s; 94°C for 30s, 48°C for 30s, 72°C for 40s, repeated 38 times; 72°C for 5 min, 4°C (hold). Following successful amplification, each sample was purified using a 5.6  $\mu$ l Exosap reaction (Applied Biosystems) and sequenced using Sanger sequencing at the Penn State Genomics Core Facility (University Park, PA). I edited the resulting chromatograms and constructed a neighbor-joining phylogeny in Geneious 8.1.9 (Dotmatics, 2021) along with reference sequences from *B. sandersoni* (NCBI accession MW339904), *B. sandersoni* sister species *B. mixtus* (NCBI accession MK529979), *B. vagans* (NCBI accession OK044465), and *B. perplexus* (NCBI accession MT951475) from NCBI GenBank (Clark et al., 2016) which allowed straightforward identification of each to species, with the exception of *B. sandersoni* which in about 5% of cases came out more allied to sister species (Cameron et al., 2007) *B. mixtus*. Although there may be *B. mixtus*, for this study I lump these all into *B. sandersoni* until further

work is done on the group. Specimens were pinned and stored as vouchers. I cross-validated our DNA barcode identification with wing morphometric analysis (unpublished data), which also separated the three groups with marginal overlaps between them (e.g., Kozmus et al., 2011; Milam et al., 2020), and morphology (Milam et al., 2020). Given that barcode data improves understanding of the occurrence of these ambiguous species, I compared the total number of observations of *B. sandersoni*, *B. vagans*, and *B. perplexus* I obtained with records from Pennsylvania on iNaturalist (all records, not only research grade level observations, [all dates available; 2009 – May 13<sup>th</sup>, 2022]) and the Global Biodiversity Information Facility (GBIF.org, 2022). These databases represent current perceptions of the relative abundance of these species. iNaturalist data is intended to represent the “common knowledge” regarding the identification of these three species including identifications from A.I., the general public, as well as expert identifiers. Species whose identities are less clear in this database may remain unidentified or may be falsely identified, both sources of biased perception regarding the real abundance of these species. GBIF data represents the morphological identification of these species by scientists, and thus is more likely aligned with a scientific perspective on the relative abundance of these species. These IDs could also be limited by bias of what can be reliably identified, as bees that are uncertain may also not be assigned to species.

### *Species abundance and composition*

I assessed overall species composition in each habitat type by pooling counts of each species across all sites (n=294 valley, n=298 edge, n=331 forest). As another metric of habitat type assessment, I assessed bees-per-minute collected for *B. impatiens*, *B. bimaculatus* (Cresson), and combined *B. perplexus* (Cresson) + *B. sandersoni* (Franklin) + *B. vagans* (Smith) (referred to hereafter as *B.*

*sandersoni* complex); while all three species are within *Pyrobombus*, they are not closely related and are analyzed together because they tended to co-occur, because of their historic morphological ambiguity, and for more power in the analysis. Other species were not analyzed using this metric due to low overall catch numbers which limited statistical power. Bees-per-minute was calculated by dividing the number of *Bombus* individuals collected at each site by the time in minutes spent collecting at each site multiplied by the number of observers. In addition, to understand habitat preferences by species, I examined the correlation of each species with individual landscape variables outlined below, using the proportion of collected individuals per site. For this, I analyzed only the bee species that occurred at four or more sites (excludes *B. terricola*, *B. flavidus*, and *B. ternarius*). To compare diversity of bumble bee species between sites, I calculated Shannon diversity index (Shannon, 1948) on all of the bees captured at that site.

### *Landscape and climate variables*

I created a buffer at each site of 500 m (estimated standard foraging distance for several bumble bee species (Osborne et al., 2008; Redhead et al., 2016), and extracted landscape metrics from the National Land Cover Database (Homer & Fry, 2020) using the program R (R Core Team, 2022). Specifically, I generated data for each site for the following variables: percent forest (sum of deciduous, mixed, and coniferous classes), percent developed (sum of high, medium, and low development categories), percent developed open, percent non-forest open (sum of percent shrubland, percent woody wetlands, percent herbaceous wetlands, and percent grassland/pasture), and percent crop (sum of all agricultural categories). “Non-forest open” was used as a category to represent natural or semi-natural landcover that was not tied to forests. “Developed open” was kept separate as it included areas such as city parks and

cemeteries where bees were often surveyed and could be a key habitat feature separate from other forms of development. I extracted elevation for each site using ArcGIS Pro's elevation tool using a 30 m DEM (ESRI, 2021; USGS, 1999).

I also calculated the following specialized landscape indices for each site: spring floral availability, summer floral availability, nest habitat availability, and insecticide toxic load use. These indices were extracted using the same protocol described by McNeil et al. (2020) using the Integrated Valuation of Environmental Service and Tradeoffs crop pollination model (Tallis & Polasky, 2009) alongside the reclassification tables from (Koh et al., 2016) for the nesting and floral indices, and (Douglas et al., 2020) reclassification tables for the insecticide index. I modified the nesting index to only include the resources for ground nesting bees, which was the most representative option for bumble bees. I also quantified honey bee colony density at each site obtained from the Pennsylvania Department of Agriculture registered apiary database (K. Roccasecca, Pennsylvania Department of Agriculture, unpublished data), using the same buffer and scale as McNeil et al. (2020) (5 km buffer, scaled by the number of colonies in each apiary) since this corresponds with the foraging range of honey bees (Steffan-Dewenter & Tscharntke, 2000). Finally, I quantified the following weather and climatic factors: April, May, and June precipitation, precipitation from the three months combined, and growing degree days (GDD) based on 10°C base, extracted from PRISM data (PRISM Climate Group, 2014).

### *Statistical analysis*

To better relate habitat type to landscape variables, I examined how landcover types were distributed across our three major habitat types and performed a principal component analysis to determine the extent to which these major variables differ in these three habitats, including forest, non-forest open,



developed, developed open, and crop land cover classes, as well as April precipitation, May precipitation, June precipitation, summer precipitation, spring floral index, summer floral index, nesting index, pesticide index, honey bee density, elevation, latitude, and longitude. To determine whether bee densities varied among species (i.e., bees/minute) I used a Kruskal-Wallis test (Ostertagová et al., 2014) because our data were not normally distributed, followed by a Dunn post-hoc test among habitat types using a Bonferroni correction to adjust the p-value for multiple comparison (Jafari & Ansari-Pour, 2019). I likewise compared pathogen loads among habitat types (forest/valley/edge) using the same procedure. Finally, I analyzed the relationship between specific landscape variables and both pathogen levels and bumble bee community composition using a Pearson's correlation matrix.

## Results

### *Landscape composition of habitat types*

The three major habitat types (valley, edge, and forest) were distinct with respect to land cover and elevation (Figure 1B, Supplemental Figure 1). Forest sites were dominated by 'forest' cover at the 500 m radius scale (total: 93.6%) with the remaining small portion being largely 'open developed' (total: 6.67%). Valleys, in contrast, were comprised of cropland (total: 42.5%) or developed (total: 24.4%), with smaller percentages of forest (total: 10.2%) and grassland/pasture (total: 8.83%). Edge sites were intermediate in composition regarding both forest cover and developed cover (Fig. 1B). Both valley and edge sites occurred at similar elevation distribution and  $\bar{x}$  that was lower (valley = 300.7 m, edge = 327.61 m; Supplemental Figure 1) than the forest sites (490.27 m; Supplemental Figure 1). A principal component analysis shows the clear transition between these habitats and that these habitat distinctions were a strong predictor of land cover variance (loading: 37.9%, PC1; Supplemental Figure 2). For

example, % forest, spring and summer floral index, nesting index, and to a lesser extent elevation and precipitation (Supplemental Figure 3) are all highly correlated and found predominately in forest sites. Conversely % developed, % crops, pesticide toxic load, growing degree days and honey bee density are correlated (Supplemental Figure 3) and occur predominantly in the valley sites. The second principal component (loading: 24.61%) primarily distinguishes geographical position (the hill-and-valley system falls on a diagonal and are, thus, correlated) and vary in the degree of urban development vs. cropland along this diagonal. Urban development in particular is correlated with honey bee incidence and higher growing degree days.

#### *Pathogen loads by habitat type and landscape variables*

BQCV was commonly detected but showed considerable variance in load across samples, whereas DWV levels were usually low and thus had less power for discriminating across sites (Supplemental Figure 4). Levels of both viruses (BQCV and DWV) were highest in *B. impatiens* bees collected in the valley sites, lowest in the forest sites, and intermediate in the edge sites. There was a significant effect of habitat type on BQCV loads (Figure 2a;  $\chi^2 = 9.82$ ,  $df = 2$ ,  $p = 0.007$ ) with significant differences between valley ( $\bar{x} = 7.48$ ) and forest ( $\bar{x} = 4.59$ ;  $\chi^2 = 9.82$ ,  $z = -3.13$ ,  $p\text{-adj} = 0.003$ ), but edge was not different from either habitat type. Similarly, there was a significant effect of habitat type for DWV (Figure 2a;  $\chi^2 = 6.12$ ,  $df = 2$ ,  $p = 0.047$ ) with higher loads in the valleys ( $\bar{x} = 2.20$ ) than in the forest ( $\bar{x} = 0.88$ ) sites ( $\chi^2 = 6.12$ ,  $z = -2.45$ ,  $p = 0.021$ ), but edge was not different from either habitat type. In contrast to the two viruses, I observed no effect of habitat type of the parasite *V. bombi* levels (Figure 2a;  $\chi^2 = 3.45$ ,  $df = 2$ ,  $p = 0.171$ ), with very low levels across most sites and just a few samples with high loads (Supplemental Figure 4), thus limiting power for inference of

landscape influence. *C. bombi* was commonly detected but showed considerable variance in our samples (Supplemental Figure 4). For *C. bombi*, levels were the inverse trend to that found in viruses, with an overall highly significant effect of habitat (Figure 2a;  $\chi^2 = 20.39$ ,  $df = 2$ ,  $p < 0.001$ ) and significantly more *C. bombi* in forest sites ( $\bar{x} = 12.57$ ) than in edge ( $\bar{x} = 9.58$ ;  $\chi^2 = 20.39$ ,  $z = -2.80$ ,  $p = 0.076$ ) and valley ( $\bar{x} = 5.90$ ;  $\chi^2 = 20.39$ ,  $z = 4.49$ ,  $p < 0.001$ ) sites, but no difference between edge and valley sites ( $\chi^2 = 20.39$ ,  $z = 1.69$ ,  $p = 0.135$ ).

There were no strong correlations between pathogen loads and landscape and climate variables. BQCV was most correlated with nest ( $r = -0.43$ ) and spring floral resources ( $r = -0.40$ ), showing higher levels when these are low (Figure 2b), and levels were highest in developed areas ( $r = 0.35$ ) and areas with more pesticides ( $r = 0.35$ ). DWV was most negatively correlated with amount of forest ( $r = -0.24$ ), thus being lower when forest is high (Figure 2b) and most positively correlates with non-forested open (grassland/pasture) areas ( $r = 0.24$ ) and developed areas ( $r = 0.35$ ). *V. bombi* had no landcover correlations above  $|r| = 0.19$  (non-forest open). *C. bombi* was most and positively correlated with nest ( $r = 0.50$ ) and spring floral availability ( $r = 0.47$ ) (Figure 2b), and most negatively correlated with areas of high urban development ( $r = -0.36$ ) and pesticides ( $r = -0.37$ ).

#### *Effect of habitat type and landscape variables on community composition*

Bumble bee capture rate was higher in the forests ( $\chi^2 = 22.61$ ,  $df = 2$ ,  $p < 0.001$ ). Patterns of capture rate by species follow patterns of relative diversity by habitat. The *B. sandersoni* complex had a significantly higher capture rate in the forest (Figure 3a;  $\chi^2 = 28.70$ ,  $df = 2$ ,  $p < 0.001$ ). There was no difference in *B. impatiens* capture rate between habitat types (Figure 3a,  $\chi^2 = 1.69$ ,  $df = 2$ ,  $p = 0.428$ ). *B. bimaculatus* capture rate was on average highest in the forest, but only significantly lower in the

valley (Figure 3a;  $\chi^2 = 25.34$ ,  $df = 2$ ,  $p < 0.001$ ). Capture rate was impacted by site characteristics and ease of capture by habitat thus is not an ideal proxy for abundance.

Across all sites, *B. impatiens* was the most collected species, comprising 39% of all bees collected. *B. impatiens* was relatively most abundant in valley sites (67% of all *Bombus* collected) and progressively declined in relative abundance in edge (37% of all *Bombus* collected), and forest sites (30% of all *Bombus* collected; Figure 3b). The relative patterns of species composition are fairly consistent among sites and are not driven heavily by any one site (Supplemental Figure 5), as suggested by capture rate statistics. When examining individual landscape variables, *B. impatiens* has higher relative abundance in more developed ( $r = 0.52$ ), agricultural ( $r=0.40$ ), and warmer (based on GDD;  $r=0.31$ ) areas and is proportionally less abundant in natural and forested areas and in areas with more floral resources (Figure 3c). The second most common species, *B. bimaculatus*, comprised 33% of all bees collected. It reached highest relative capture levels within edge habitats but occurred relatively evenly across all habitats, occupying 25% of valley sites, 42% of edge sites, and 38% of forested sites (Figure 3b). As such, *B. bimaculatus* had low correlation with any landscape variable.

The “*B. sandersoni* complex” comprise 19% of all bumble bees collected including 10% of bees at valley sites, 21% of bees at edge sites, and 32% of bees at forest sites (Figure 3b), and thus are most prevalent in forest regions. The barcode data for these bees allowed clear assignment of members of this complex to species (although see note on *B. mixtus* in the Methods). These data revealed that *B. sandersoni* is the most encountered member of this complex in central Pennsylvania (10% of all bees observed), followed by *B. perplexus* (5%), and *B. vagans* (4%). This is contrary to the relative proportions of these three species for Pennsylvania on iNaturalist and GBIF. While *B. sandersoni* comprised 53% of individuals observed among these three species in our data, iNaturalist and GBIF had

no to very few (3%) occurrence records for *B. sandersoni*, respectively (Figure 3d). In iNaturalist records, the majority of bees in this complex were identified as *B. perplexus*, while in GBIF, the majority of bees in this complex were identified as *B. vagans* (Figure 3d).

*B. sandersoni* and *B. vagans* were both more common in forests, with *B. sandersoni* showing the highest affinity for forest sites, as it reaches its highest proportions in ridgetop forest sites (20%), and makes up only 5% of bees collected at valley sites and 6% of bees collected at edge sites (Figure 3b). *B. vagans* occupies just 1% of bees collected at valley sites, but 6% of both edge and forest sites (Figure 3b), and was collected at a higher percent of forest sites than edge sites (forest: 53% or 7/13 sites; edge: 41% or 5/12 sites; Supplemental Figure 4). *B. perplexus* showed less preference within this complex, being distributed more similarly to *B. bimaculatus*, occupying 4% of the species collected at valley sites, 7% at edge sites, and 3% at forest sites (Figure 3b). When examining specific landscape variables, *B. sandersoni* was least correlated with croplands ( $r = -0.37$ ) and areas with more pesticides ( $r = -0.47$ ) and, in line with its forest site association, is most correlated where there is more nesting habitat ( $r = 0.54$ ), spring floral resources ( $r = 0.50$ ), and forests ( $r = 0.52$ ). *B. vagans* follows similar patterns. *B. perplexus* within this complex does not follow these landscape preferences and showed only weak correlations with any variables.

As for the less commonly encountered bees, *B. griseocollis* (De Geer) was in all habitats but with decreasing relative capture from valley (7%) to edge (6%) to forest (4%) and thus it showed most similar landscape variable preferences to *B. impatiens*. The remaining bumble bee species comprise just 3% of all bumble bees collected and include *B. fervidus* (Fabricius), *B. terricola* (Kirby), and *B. ternarius* (Say). *B. fervidus* (Fabricius), a bee known to nest in grassy substrates, was most positively associated with cropland ( $r = 0.43$ ) and non-forested open areas (grasslands/pasture;  $r = 0.43$ ).

### *Diversity-related patterns*

*B. impatiens* abundance was the strongest explanatory variable for diversity patterns, being negatively and significantly correlated with the Shannon Diversity Index ( $r = -0.76$ ) as communities dominated by this species show lowered species richness and evenness (Figure 3c). The only deviation of diversity patterns from patterns matching *B. impatiens*, is that diversity otherwise appears to be more strongly negatively impacted by urban development ( $r = -0.60$ ) and high temperatures (GDD) ( $r = -0.50$ ). There were no strong associations between honey bee density and pathogen loads when compared to other landscape effects; however, there is a slight negative correlation with bumble bee diversity ( $r = -0.37$ ). Bumble bee community diversity also had weak positive associations with pathogen loads in *B. impatiens* for *C. bombi* ( $r = 0.30$ ). There were no clear strong associations with the presence of a particular species and loads of particular pathogens that is not better explained by or confounded by stronger shared associations with habitat, with the exception that *B. griseocollis* is more abundant in sites with *Vairimorpha*, being the most correlated factor with *Vairimorpha* incidence ( $r = 0.22$ ).

## **Discussion**

The Ridge-and-Valley ecoregion of central Pennsylvania provides a unique setting within which to disentangle the roles of local habitat and geography on pathogen loads and bumble bee species distributions. Herein, I provide one of few empirical demonstrations that local habitat can play a major role in shaping bumble bee community composition and the disease loads present therein. Ecological characteristics are frequently spatially auto-correlated which means making inferences about the effects of *local* habitat from geographically-extensive studies is challenging (Lichstein et al., 2002; Petracca et al., 2018). Our study design employed an approach where I sampled disparate habitats from a relatively

constrained study area which allowed us to quantify habitat effects with minimal confounding effects of larger scale geography. Although recent work by McNeil et al. (2020) demonstrated that more northerly portions of Pennsylvania, which are more forested, hosted less disease, our work builds upon their findings in demonstrating that, even within a constrained geography, disease and habitat have similar relationships (especially for BQCV and DWV). As conservationists in Pennsylvania (Pennsylvania Pollinator Health Task Force, 2017) and beyond (Schweitzer et al., 2012) are tasked with the conservation of native bumble bee communities, our results point to a need for consideration of habitat at both broad spatial scales (*sensu* Gillespie et al., 2022; Koh et al., 2016; McNeil et al., 2020) as well as intermediate/local scales (Conflitti et al., 2022; Mola et al., 2021).

I found levels of two viruses (BQCV and DWV) in sampled *B. impatiens* increased along gradients from forest to valley. This aligns with the more broad-scale results of McNeil et al. (2020), which found a trend of increased virus loads in non-forested regions of Pennsylvania. Our more fine-scale results show that these viruses partition by habitat type even at a local scale, suggesting that habitat *per se* plays a key role in virus loads. What remains unknown, however, are the proximate mechanisms driving this pattern of which several are plausible. For example, it is possible that forest ecosystems have more floral resources than valleys (Koh et al., 2016). This might lead to starvation-induced reduction in immune function (Branchiccela et al., 2019; Brunner et al., 2014; Figueroa et al., 2021) and, thus, higher viral loads, in valleys. Alternatively, given the heightened exposure to a variety of stressors in valleys (e.g., honey bees, pesticides, etc.; Figures 2-3; McNeil et al. 2020), there may be synergistic impacts to valley bees that I was unable to detect with our modest sample size. While it may be predicted that BQCV and DWV, viruses abundant in honey bees (Alger et al., 2019a; Ezray, 2019), may be driven by honey bee density, our data did not find honey bees to be a better predictor than other landscape variables. This is in contrast to McNeil et al. (2020), which found a positive correlation

between pathogen loads and honey bee colony density. Beyond the ecological patterns, our observation that BQCV loads were generally higher than those of DWV is consistent with prior studies that found DWV to have higher prevalence and infection levels in honey bees, and lower levels in bumble bees (McNeil et al., 2020; Olgun et al., 2020) and other solitary bees (Dolezal et al., 2016).

Unlike BQCV, our study observed that most bees had low levels of *Vairimorpha bombi* punctuated by occasional individuals that had high *Vairimorpha* levels, a pattern also reported by other studies (Gillespie, 2010; McNeil et al., 2020). This infection pattern makes finding correlations with any environmental variables difficult due to limited variation in the dataset. Interestingly, our work contrasts with the findings of McNeil et al (2020) and multi-year field data of (Manlik et al., 2022), who found *Vairimorpha bombi* loads to be positively correlated with precipitation, presumably because *Vairimorpha* thrives in moist conditions. Although our study area was included within the larger extent sampled by McNeil et al. (2020), they sampled in different years than I did (2018-19 vs 2020) and, although loads in that study were similarly small, they were higher than I observed. Given that both studies used identical field and laboratory protocols, I believe the lack of a precipitation effect observed here was due to the lower overall rainfall observed during the summer of our study (The Pennsylvania State Climatologist, 2020). These study-specific differences driven by between year variation in rainfall are likely to become more pronounced as the effects of climate change increase (Kunkel et al., 2013), thus highlighting the need for longer-term studies that encompass multiple sampling years. A recent molecular study in Pennsylvania revealed that *V. bombi* in bumble bees is less common than other species, especially *V. apis* (Jones et al., 2022), which would explain why higher loads have been detected in surveys of *Vairimorpha* in bumble bees in this region using morphology (Cameron et al., 2011; Malfi & Roulston, 2014), thus future work should examine the relative impacts of different *Vairimorpha* on these bees as well.



Unexpectedly, *Crithidia bombi* was more prevalent in bees from forest habitats and showed a general decline in more developed landscapes (Figure 2a). This observation is counterintuitive because forest ecosystems are expected to be among the highest quality bee habitats in the region due to their heightened floral resources, low anthropogenic disturbance, reduced thermal stress, and low pesticide loads (McNeil et al., 2020). One explanation for this is that *Crithidia bombi* is less deadly in areas where bumble bees are less stressed (Brown et al., 2000) thus habitats with few stressors, or increased floral availability like forests, could lead to increased survival of bees infected with *Crithidia bombi*. Alternatively, persistence and transmission of *Crithidia bombi* can be negatively affected by exposure to sunlight (Figuroa et al., 2019); in this case, more shaded environments in forests would facilitate *Crithidia bombi* transmission. Previous work on the influence of agricultural chemicals on *Crithidia* found no clear effects (Straub et al., 2022), however other aspects of agriculture or urbanization could reduce these loads. There is also evidence to suggest that other insects can vector *Crithidia bombi* transmission such as flower flies (Davis et al., 2021) or solitary bees (Figuroa et al., 2021), and those insects may be more abundant in forested ecosystems. Thus, addressing vector diversity and drivers of community transmission among bee/other insect species could shed additional light on the high *Crithidia* loads in forests (Davis et al., 2021; Nicholls et al., 2022).

Not only did disease loads vary along habitat gradients but I observed clear evidence that bumble bee community composition varied along similar ecological gradients. For instance, *B. impatiens*, *B. griseocollis*, and *B. fervidus* were proportionately more abundant in valleys which contained little forest cover, high urban development, and high agricultural cover. In contrast, *B. sandersoni* and *B. vagans* seem to be primarily restricted to forest sites (Figure 3). This finding agrees with reports that *B. vagans* and *B. sandersoni* may be forest specialists (Colla et al., 2012; Williams et al., 2014). With that in mind, the understanding of distribution patterns of these species have been limited by the difficulty in

distinguishing between *B. sandersoni*, *B. perplexus*, and *B. vagans*, resulting in many studies combining these three species into one category in field surveys. Moreover, combining these species into a single category has limited conservationists' ability to determine which may require conservation action and which remain common. It has been suggested that *B. vagans* and *B. perplexus* are the more abundant species with *B. sandersoni* making up the smallest proportion of the community to the point of recommendation for "immediate conservation attention" (Colla et al., 2012; Koch et al., 2015). This is reinforced by GBIF data which supports much fewer identified bees belonging to *B. sandersoni* in Pennsylvania than the other species (Figure 3d). iNaturalist data mostly just recognizes *B. perplexus*, a result that likely differs from GBIF because *B. perplexus* is easier to identify by sight using color traits, resulting in the other two species not being determined to species (Milam et al., 2020). In contrast to perception among the public and scientists, our data using both barcode data and morphological validation supports *B. sandersoni* being substantially more abundant than the other two species in Pennsylvania (Figure 3d). Milam et al. (2020) obtained barcodes of these three bee species sampled from across their eastern U.S. range and also identified *B. sandersoni* more often than the other two species in the northeastern U.S., although not in the upper Midwest (Wisconsin). This highlights the need to improve criteria for identification (cf. Milam et al., 2020) in these species to better understand their ranges and reconsider their conservation priorities.

In our study, as with most studies on wild bumble bees conducted in the Northeastern U.S. (Koch et al., 2015), *B. impatiens* was the most common bumble bee species (39% of all observations in this study). The relative percent caught was lower in the forests than in the valley and the proportion of *B. impatiens* was positively correlated with more disturbed/agricultural habitats. Taken together, our data support a hypothesis that *B. impatiens* thrives in systems where other species are declining and where habitat may be marginal for others (Conflitti et al., 2022; Koh et al., 2016). In many regards, *B.*

*impatiens* is a ‘super generalist’ bumble bee species that has extraordinary dietary breadth (Wood et al., 2019) and even behaves as an invasive species in some places (Looney et al., 2019; Ratti & Colla, 2010). Indeed, specimen data have shown that *B. impatiens* represents an increasing proportion of bumble bee communities in the eastern Nearctic over the last 50 years (Cameron et al., 2011; Colla et al., 2012), the inverse of the pattern exhibited by many other North American *Bombus* species. Ultimately, our work and that of others indicates that, as habitat quality (e.g., forage and nesting quality) continues to degrade in many areas, the relative proportion of *B. impatiens* will continue to increase as other species decline (Albrecht et al., 2012; Biesmeijer et al., 2006; Brosi & Briggs, 2013).

While the habitat-specific disease loads in *B. impatiens* workers could be explained by a variety of proximate factors such as floral community composition, nesting conditions, and microclimate characteristics, our data indicate that forests and valleys support very different bumble bee communities in the Ridge-and-Valley region of Pennsylvania. Thus, another hypothesis behind habitat-specific transmission might be an interaction between pathogen loads and bumble bee community composition. Indeed, different bumble bee species are characterized by different susceptibility to pathogens (Cordes et al., 2012) and the habitat preferences suggested here may contribute to habitat-specific variation in disease transmission dynamics. For example, Malfi and Roulston (2014) found higher *Crithidia* levels in *B. perplexus* (~2X higher) and *B. bimaculatus* (~3X higher) than in *B. impatiens*, species which are more abundant in forests in our study. Although habitat could have driven their results, if these species were indeed more susceptible to *Crithidia*, they would promote higher levels in forests. It is also recognized that species vary considerably in propensity to be infected by *Vairimorpha*, with *B. impatiens* less susceptible than most and species in decline most susceptible (Cameron et al., 2011; Malfi & Roulston, 2014), thus spread of this pathogen could be highly community dependent and better inferred in more vulnerable species. Future studies in which pathogen levels are monitored in bumble

bee species beyond this model species and which compares loads of different species within each habitat type would provide additional insights. Work like that presented here, assessing how landscape characteristics and bee communities interact to influence pathogen loads will provide more informed guidance for supporting healthy pollinator populations and focusing conservation aims (Pennsylvania Pollinator Health Task Force, 2017; Schweitzer et al., 2012).

Figures

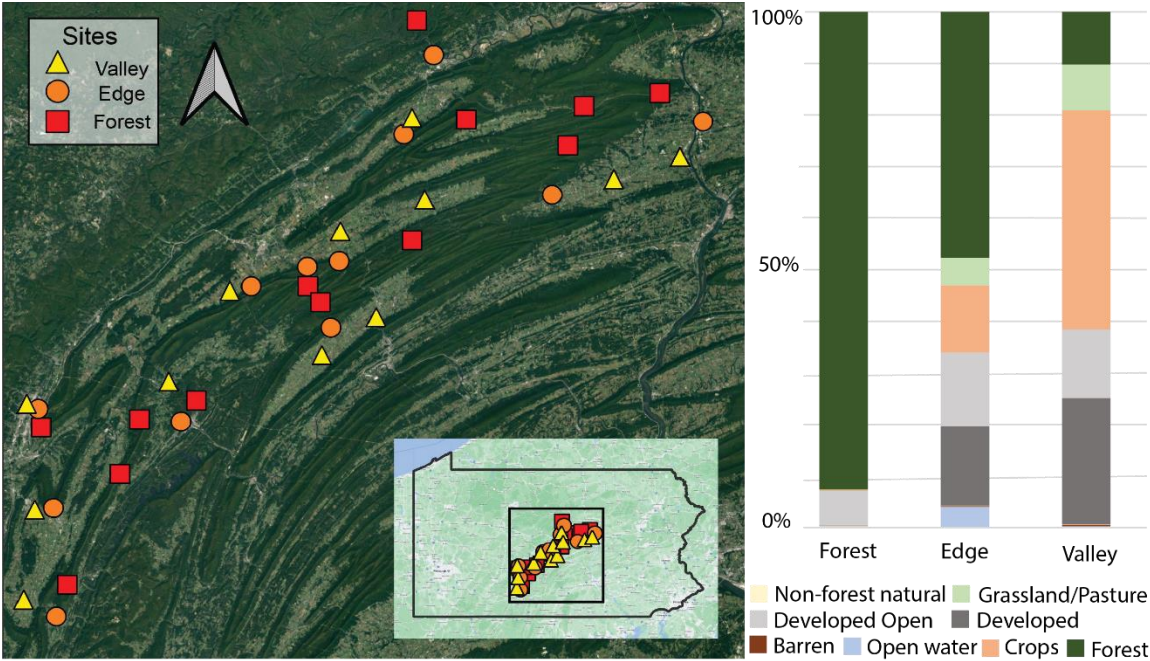


Figure 1: Map of sites across central Pennsylvania. Combined landscape cover categories for each habitat.

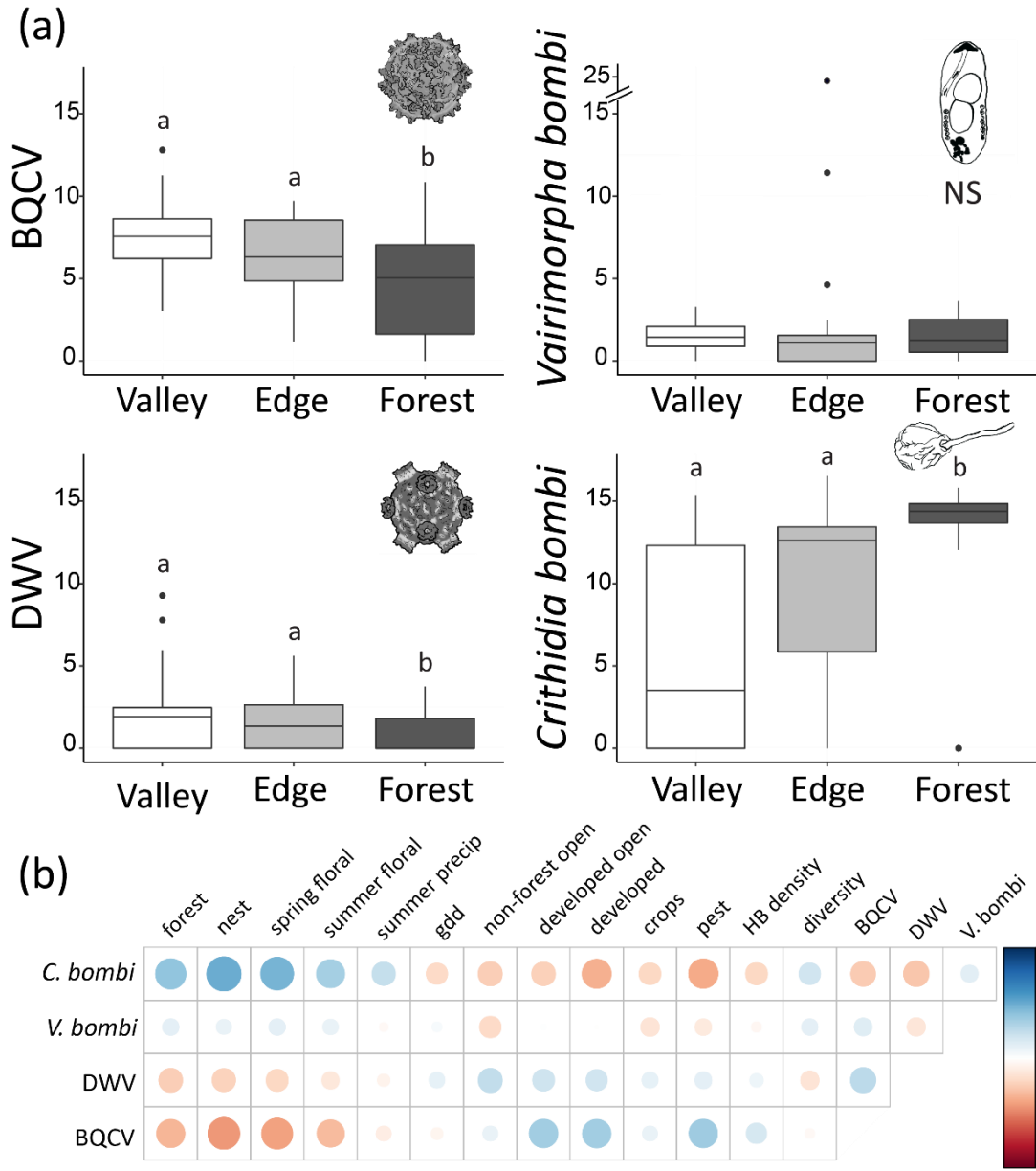


Figure 2: Impact of landscape features on pathogen loads. a) Pathogen loads across the valley to forest gradient. b) Correlation matrix of pathogen loads with landscape variables, bumble bee diversity, and species composition.

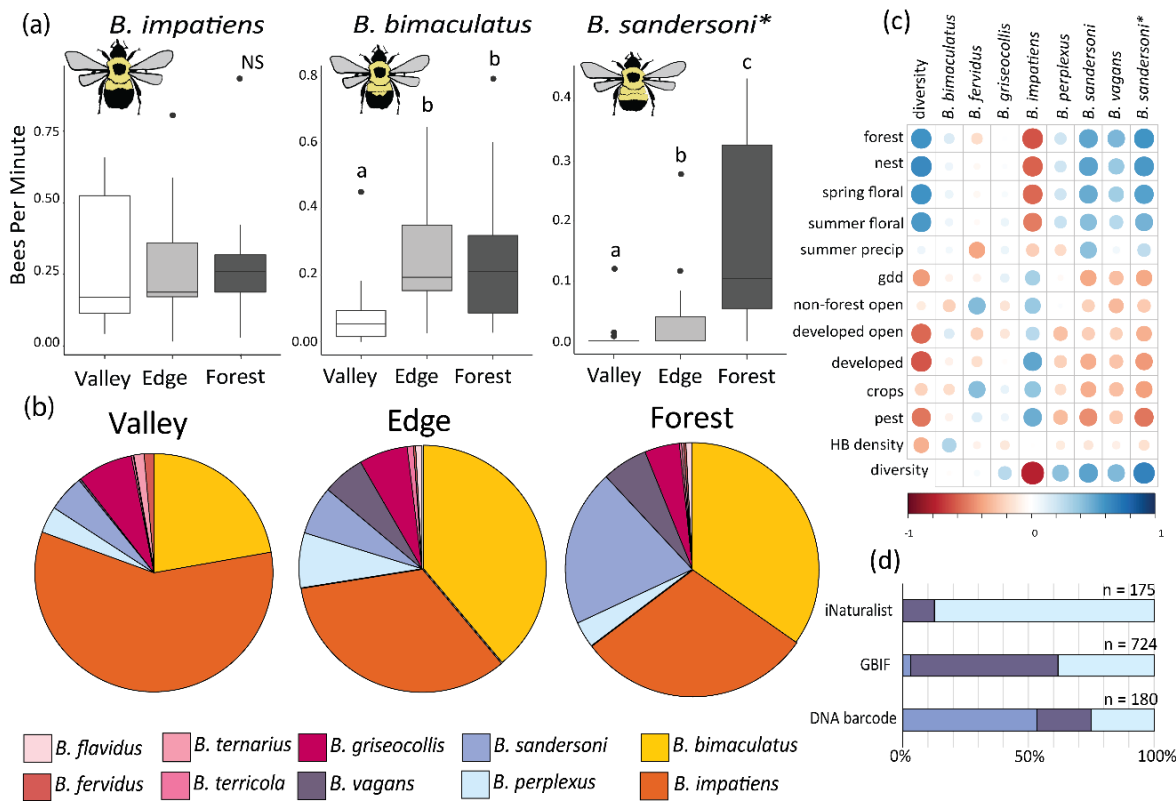
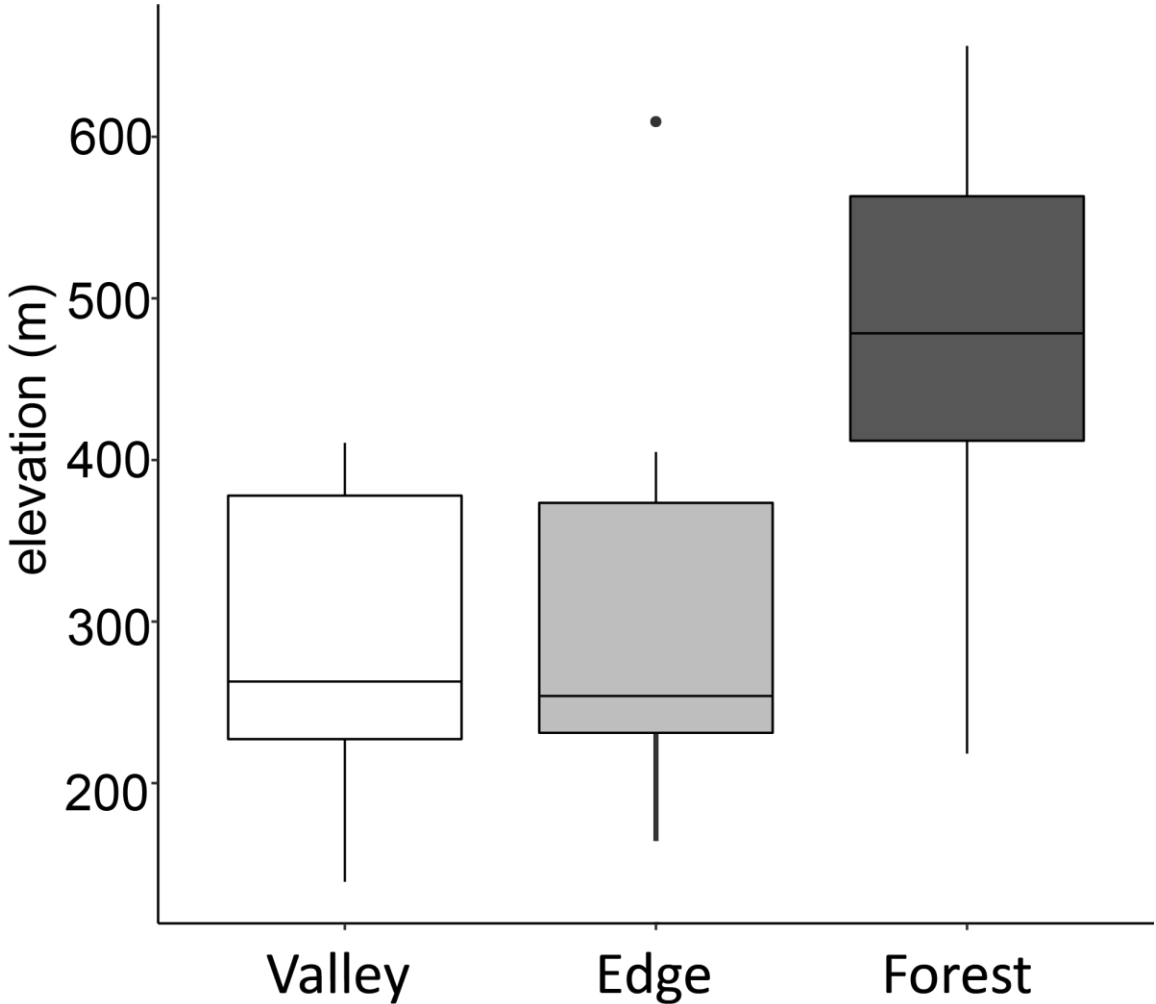


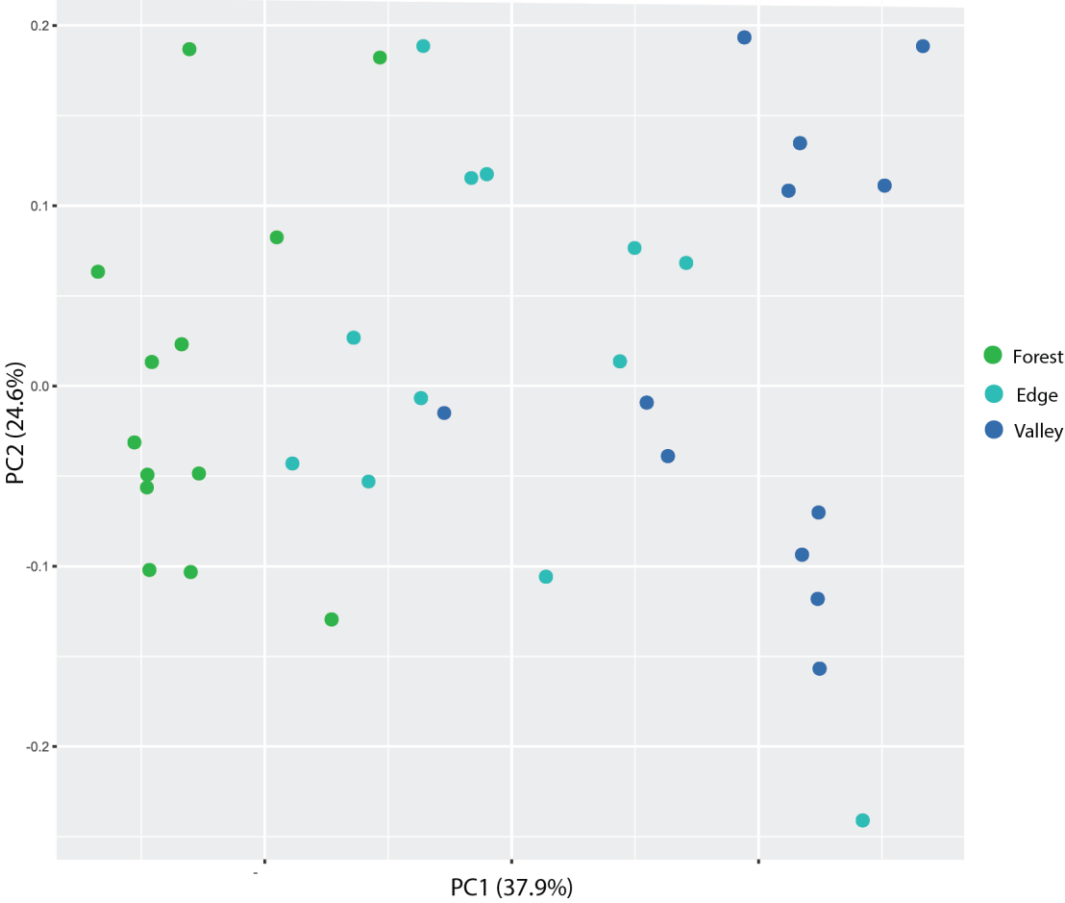
Figure 3: Distribution of species by local habitat. a) Catch rate (bees per minute) of *B. impatiens*, *B. bimaculatus*, and *B. sandersoni* across the forest to valley gradient. b) Percent captured of each species in each habitat type. Colors correspond to the those

Supplemental Figures

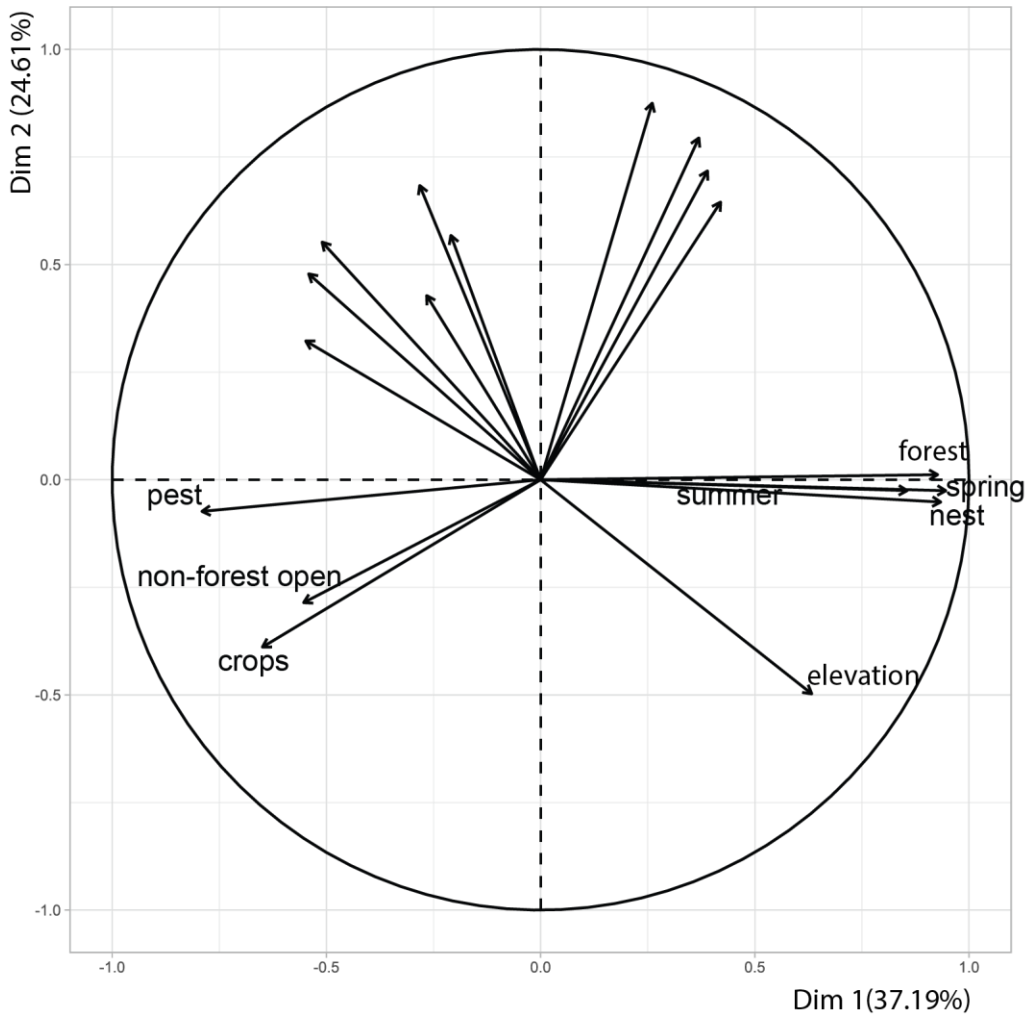


Supplemental Figure 1: Box plot of elevation of sites in each habitat type.

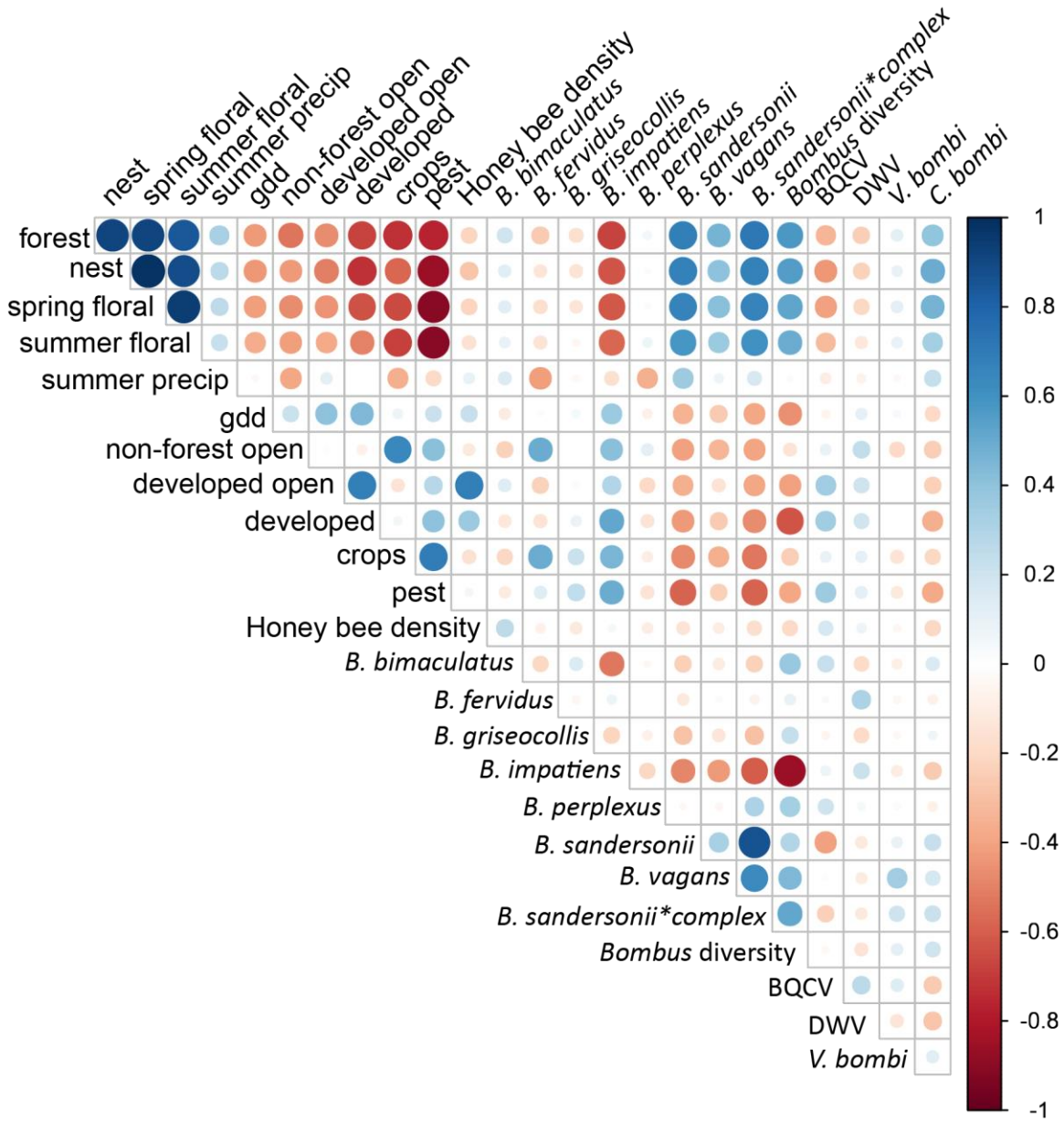




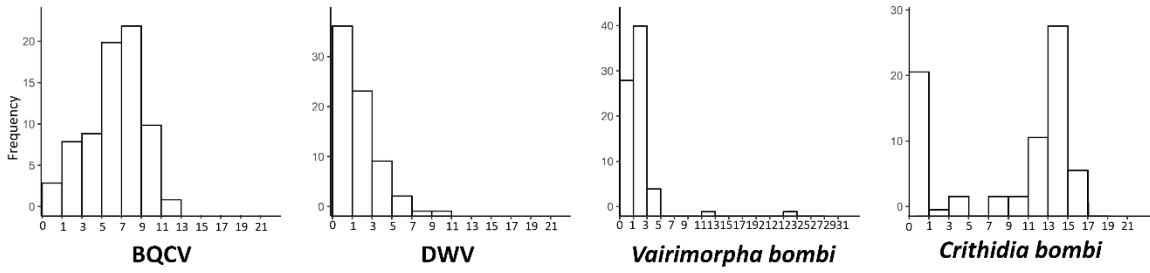
Supplemental Figure 2: PCA plot distinguishing Forest, Edge, and Valley sites.



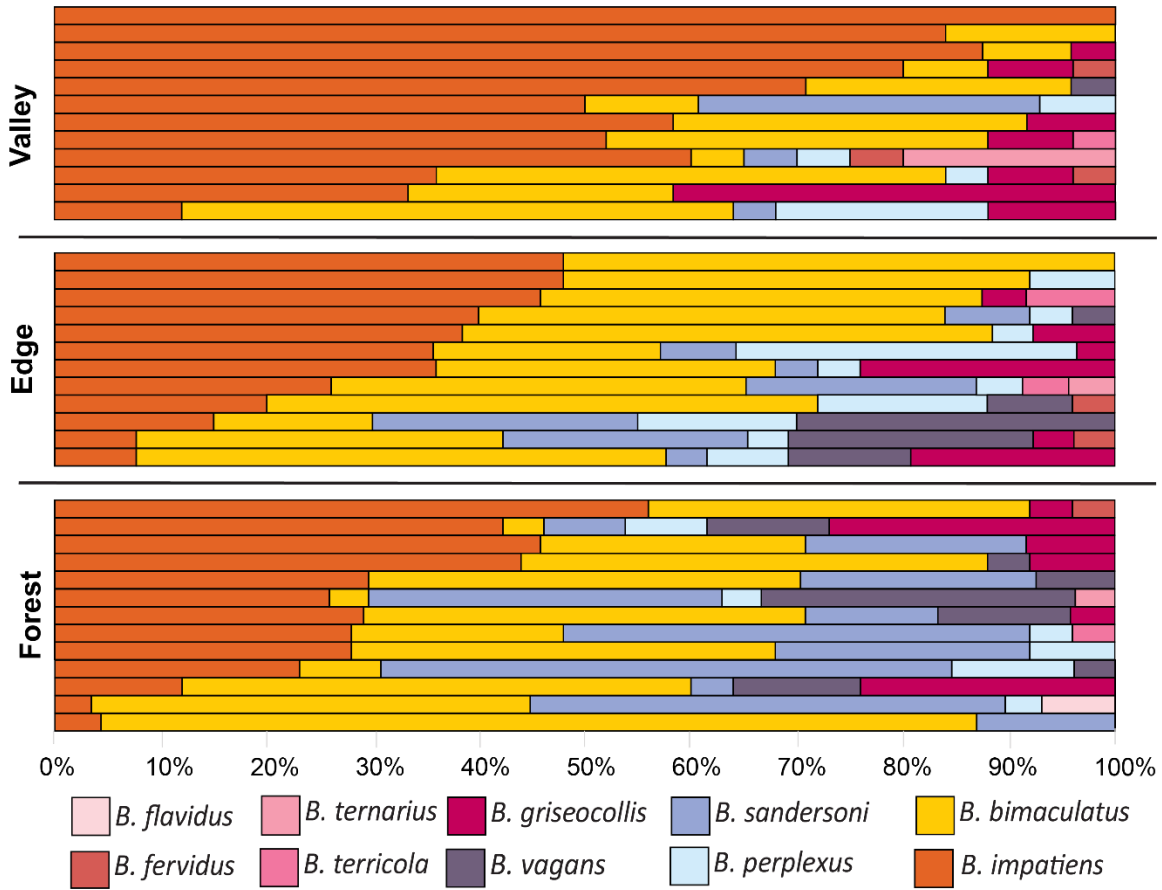
Supplemental Figure 3: Vector diagram of site PCA.



Supplemental Figure 4: Complete correlation matrix including all landscape variables, pathogens, and bumble bee species.



Supplemental Figure 5: Distribution of loads of each pathogen across all sample pools across all sites.



Supplemental Figure 6: Distribution of species by site in the Forest, Edge, and Valley regions. Each row represents a site.

## **Chapter 2: Comparisons of the roles of landscape factors in bee pathogen loads across different geographic regions**

### **Abstract**

Pathogens and parasites are considered primary drivers of declines of wild bumble bee populations. Pathogen and parasite transmission is influenced by abiotic and biotic factors in the environment, and a bee's susceptibility to infection is similarly influenced by multiple factors, including nutrition and exposure to other stressors. Thus, identifying the factors influencing pathogens and parasite prevalence, at a landscape scale, in order to inform land management strategies to reduce disease burden in bumble bees, is uniquely challenging. Here, we evaluated the pathogen and parasite loads of the Common Eastern Bumble Bee (*Bombus impatiens*) from sites representing different habitat types across the state of North Carolina. We examined levels two viruses, Black Queen Cell Virus (BQCV), and Deformed Wing Virus (DWV), and two parasites *Vairimorpha bombi* and *Crithidia bombi*: these vary in their epidemiology and are commonly found in wild bumble bees. We evaluated land use, habitat quality, bee community, and weather variables using mixed effect models to determine which variable could best predict levels of these different pathogens and parasites. Very low prevalence of DWV and no *Vairimorpha* were detected in samples, and thus these were not evaluated in our models. BQCV loads were positively correlated with a summer floral availability index; this was surprising since our previous studies in Pennsylvania found lower viral levels when spring floral availability was high. *C. bombi* showed consistent infection and thus had low variance in loads but showed some signs of being related to bee diversity patterns. We then conducted a literature review to better understand the broadscale patterns of pathogen/parasite – landscape relationships. Ten studies

addressed similar questions, all showing varying and sometimes opposite effects of landscape variables. Most consistently however, honey bees and increasing amounts of developed land are associated with increased pathogen loads. Floral availability and community composition had mixed effects on pathogens, sometimes increasing prevalence and sometimes decreasing prevalence, depending on the context, and likely resulting from differences in pathogen transmission and maintenance at different spatial scales. Overall, our studies and others indicate that increasing flowering plant species diversity, increasing pollinator species diversity, and reducing spillover from honey bees should reduce wild bumble bee pathogen and parasite loads. One finding from our study was that honey bee pathogen loads were reduced in areas with high spring floral resources, indicating that landscape level management practices may reduce pathogen loads in managed bees as well, and thereby limit spillover into wild bee communities.

## **Introduction**

Bumble bees are important pollinators of agricultural and wild flowering plant species (Klein et al., 2007; Ollerton et al., 2011). However, many populations and species of bumble bees are experiencing concerning declines across the world due to factors such as pesticides, climate change, and habitat loss (Cameron & Sadd, 2020; Goulson et al., 2008). Since some of the most threatened bumble bee species have been found to have the highest pathogen prevalence (Cameron et al., 2011; Cordes et al., 2012; Levenson & Tarpy, 2022; Malfi & Roulston, 2014), it is hypothesized that pathogens and parasites are significant drivers of bumble bee declines (Cameron et al., 2011). Moreover, these infections may also serve as bioindicators of other bee stressors, since disease levels are affected by physiological and environmental conditions (Meeus et al., 2018). Landscape quality thus should

modulate bee pathogen and parasite loads through effects on overall bee resilience to disease as well as influencing the maintenance and spread of these organisms. Understanding the role of landscapes on pathogen and parasites loads can inform land management recommendations and potentially improve outcomes for bumble bees.

Bumble bees can harbor many pathogens and parasites that each have different epidemiological properties, and which therefore may be affected by different landscape factors (Cameron & Sadd, 2020). Two abundant and often studied *Bombus*-specific parasites are *Crithidia bombi* and *Vairimorpha bombi*. *Vairimorpha bombi*, a microsporidian fungal relative, is one of the most threatening *Bombus* parasites (Cameron et al., 2011), and is often lethal (Otti & Schmid-Hempel, 2007; Rutrecht & Brown, 2008). A landscape study across the United States found *Vairimorpha bombi* levels in declining *Bombus spp.* to be most correlated with fungicide use (McArt et al., 2017). Prior work also suggests that this parasite is more abundant in wetter, more humid landscapes (Manlik et al., 2022; McNeil et al., 2020), which is consistent with other *Vairimorpha spp.* (Neidel et al., 2017). *Crithidia bombi*, a trypanosome, is generally benign and occurs broadly and with high incidence across some *Bombus spp.* (Jones et al., 2022; Levenson & Tarpy, 2022; Malfi & Roulston, 2014). Infections in *Crithidia* are sensitive to the environment, as it becomes lethal when bees are exposed to extreme stress (Brown et al., 2000). Because *C. bombi* is a gut parasite, it is heavily influence by bee gut microbiota (B. Mockler, 2016) and diet (Figuroa et al., 2019, 2020; Folly et al., 2020; Palmer-Young et al., 2016). Prior work in urban and agricultural plantings has found its prevalence is greater in areas with more impervious surfaces and low floral diversity (Ivers et al., 2022; Levenson & Tarpy, 2022). Both *Crithidia bombi* and *Vairimorpha bombi* are generalist parasites within the genus *Bombus* (Kissinger et al., 2011).

Bumble bees are also infected by viruses that occur more broadly in bee and insect communities. Black queen cell virus (BQCV), a bee virus, and Deformed wing virus (DWV), a broad range insect virus (Nanetti et al., 2021), both are more abundant in honey bee (*A. mellifera*) populations than in bumble bees, suggesting a spillover effect (Alger et al., 2019a; Dalmon et al., 2021; Gusachenko et al., 2020; Manley et al., 2015; Nanetti et al., 2021). BQCV can cause death of brood (Siede & Büchler, 2003), but also infects adult bees (Nanetti et al., 2021; Peng et al., 2011). While DWV and BQCV are highly infective in *A. mellifera*, only BQCV reaches similar infection levels in wild bumble bees (around 50%-100%; Alger et al., 2019a; Ezray, 2019; McNeil et al., 2020), and DWV is often found at only low levels in non-*Apis* bees (Dalmon et al., 2021; Dolezal et al., 2016; Ezray, 2019; Chapter One; McNeil et al., 2020). Studies in Pennsylvania have found that levels of both viruses are lower in landscapes with forests (Chapter One; McNeil et al., 2020), and in landscapes with fewer honey bees (McNeil et al., 2020). Given their varying levels of severity, sensitivity to the environment, and effects on bumble bees, BQCV, DWV, *C. bombi*, and *V. bombi* can be surveyed in populations across landscapes and help answer conservation questions about how to best support healthy bumble bee communities.

The insect community may also play an important role in shaping parasite and pathogen levels within bumble bee populations (Cohen, 2022; Fearon et al., 2023; Ivers et al., 2022; Malfi & Roulston, 2014). In an urban garden study, increased pollinator richness was correlated with reduced *Apicystis bombi* prevalence in bumble bees (Ivers et al., 2022). In a similar urban garden study, the rate of honey bee parasitism had a positive correlation with pathogen richness (observed *A. borealis*, ABPV, *Apicystis spp.*, BQCV, CBPV, *Crithidia spp.*, DWV, SBPV, and SBV) in bumble bees, but the diversity of bumble bee species was not correlated with pathogen richness in honey bees (Cohen, 2022). Not only is there potential spillover from managed honey bees, (Alger et al., 2019a; Gusachenko et al., 2020; McNeil et al., 2020), but there is concern the managed bumble bees may increase *V. bombi* prevalence



in wild bumble bees (Colla et al., 2006; Graystock et al., 2013, 2016). In some US states, honey bee apiaries are managed and tracked by local agriculture departments (e.g. Pennsylvania; K. Roccasacca, Pennsylvania Department of Agriculture, unpublished data), but most states do not have such programs (e.g. North Carolina). There is no such tracking for managed bumble bee colonies. This lack of information makes understanding the impact of managed bees more difficult to elucidate. Furthermore, given different susceptibility of bumble bee species to these pathogens (Averill et al., 2021; Blaker et al., 2014; Cordes et al., 2012; Gillespie, 2010; Levenson & Tarpy, 2022; Malfi & Roulston, 2014), composition of bumble bees in a community, a variable that is impacted both by floral quality and by habitat type (Carvell et al., 2011; Chapter One; Jha & Kremen, 2013), is likely to impact pathogen and parasite levels. In fact, a “habitat-mediated dilution effect” was observed by Fearon et al. (2023), where natural habitat increased pollinator species richness which in turn decreased BQCV and DWV. Lastly, these pathogens can be transmitted by insects other than bumble bees or managed bees; for example, syrphid flies were found to be mechanical vectors for *Crithidia bombi* (Davis et al., 2021).

Pathogen loads can be affected by landscape factors from small the large scales, but how these factors integrate with each other to determine pathogen diversity and abundance at a local scale remains poorly understood. At a small scale, individual flowers can facilitate or hinder pathogen transmission depending on structure, and exposure to light can impact pathogen survival (Figueroa et al., 2019; McArt et al., 2014). At a slightly larger scale, the size of the urban garden can influence pathogen richness; indeed, Cohen et al. (2016) found that large urban gardens with more perennials increased pathogen richness. Bumble bees can travel up to two kilometers from their nest during foraging trips (Redhead et al., 2016), while honey bees can travel between five to ten kilometers (Beekman & Ratnieks, 2000). Thus, pathogens from a single bee can be spread at a multi-kilometer spatial scale. In chapter 1, I saw distinct differences in pathogen loads across the mountain-valley region of Central

Pennsylvania, with forested ridge tops hosting more *C. bombi* and developed valleys hosting more BQCV. Additionally, there can be a regional effect, driven by differences in ecoregional climate and habitat conditions. McNeil et al. (2020) showed that, in Pennsylvania, the more forested and cooler northwestern parts of the state had less viral pathogens than the more developed, open, and warmer southeastern portion of the state. The fact that land use and climate can impact bumble bee pathogens at multiple scales can lead to complexity in finding consistent patterns: while Tsvetkov et al. (2021) found that in the declining species *B. terricola*, BQCV and Sac Brood Virus increased at agricultural sites alongside transcriptomic evidence of pesticide stress, Olgun et al. (2020), found no significant differences between pathogen prevalence in natural, agricultural, and urban habitats.

Previous studies in Pennsylvania thus provided strong evidence for effects of local habitat type and quality on abundance of pathogens in wild bumble bee populations, as well as an indication that overall differences in regional ecosystems and climate might influence pathogen levels. To determine if patterns associated with habitat type and quality were consistent in other ecoregions, I evaluated the role of habitat type, habitat quality, and ecoregion on bee pathogen loads in North Carolina. North Carolina is the native home to fifteen species of bumble bees (H. Levenson & Youngsteadt, 2019), thus there is a similar diversity and species composition to Pennsylvania and most of the eastern Nearctic (Strange & Tripodi, 2019). It however represents a more southern end of this faunistic zone. It also harbors a variety of habitats that allow for testing the effects of landscape variables and habitat quality. In particular, the eastern end is composed of coastal grasslands, its central region is largely developed agricultural piedmont, and its western region includes largely natural forests. Here I assess the effect of landscape habitat type, habitat quality (density and diversity of floral resources, nesting resources, and surrounding landscape disturbance), and honey bee and bumble bee communities on *B. impatiens* BQCV, DWV, *C. bombi*, and *V. bombi* pathogen loads. I also evaluate loads of the BQCV and DWV in honey bees to

examine the impact of landscapes on honey bee pathogen levels, and, in turn the impact of infected honey bees on bumble bee loads. I compare these results to those of McNeil et al. (2020) in Pennsylvania as well as other landscape studies on bumble bees in the United States to make more general recommendations regarding landscape management for bumble bees.

## Methods

### *Bee and Floral Sampling*

I collected bumble bees at 31 sites across North Carolina from June 7 to July 7, 2022 (Figure 4). These sites were selected to span a diversity of habitat quality and types and with the aim to cover all portions of the state. North Carolina has three broad distinct ecoregions (NC Department of Natural and Cultural Resources, 2020). The Appalachians in western North Carolina are similar to the Appalachians in Pennsylvania making for a distinct comparison between the two states. The Piedmont represents more urban land as well as intensive agriculture, and Coastal Plains highlights one of the more unique native ecosystems on the east coast (Figure 4). At each site, two trained technicians conducted an unlimited-length visual encounter survey for *Bombus spp.* of all species. Surveys were conducted under conditions of low wind speed and when temperatures were above 15.5°C and below 36°C. During each survey, I examined all available flower species for foraging bumble bees, until I captured 20 *Bombus spp.* workers. These 20 bees were used for an initial assessment of bumble bee diversity at the site, which was calculated using Shannon-diversity index using the vegan package in R (Oksanen et al., 2022), as our community composition metric. Each visual encounter survey consisted of one to two highly trained field researchers searching each site, making a conscious effort to avoid biasing sampling on a single

floral species or patch and to cover all blooming species at that site. Our goal for pathogen analysis was to collect 15 *B. impatiens* from each site; if our initial random sample of 20 *Bombus spp.* included fewer than 15 *B. impatiens* workers, we continued sampling until 15 *B. impatiens* workers were collected. During this extra time, we also sampled up to 15 *Apis mellifera* honey bees at each site to use for pathogen analysis. For some sites no honey bees could be found during or after our initial survey and thus no honey bees were obtained, and for some sites with very few honey bees we obtained less than 15 honey bees for the pathogen analysis. At one site we did not collect *A. mellifera* even when present because it was a private farm that had several managed *A. mellifera* colonies. Additionally, we recorded the start- and end times for the initial 20 bee survey at each site using a stopwatch to keep track of time spent searching. If a site yielded less than three *B. impatiens* workers across four hours of sampling, it was not included in the study. *B. impatiens* were gathered into vials and stored immediately onto dry ice. Samples were transferred to  $-80^{\circ}\text{C}$  for long-term storage.

Floral availability at each site was assessed using floral species richness, floral abundance, and floral density to quantitatively understand the specific impacts of floral availability. During or immediately following the survey, one observer would record all species of blooming flowers and estimate the number of flowers of each species within two meters of the path walked (floral abundance). During this time the observer responsible for the survey would use a GPS tracking app (Strava, Inc., 2022) to record where the observer surveyed. If the floral survey was done after the bumble bee survey, the observer would retrace their steps using the GPS app. The stop watches were stopped while the observer was counting and recording the floral data. The GPS routes were imported into ArcGis pro (ESRI, 2021), where the total floral survey area, including the 2m buffer, was calculated. The floral density was calculated by dividing the abundance of all flowers by the total area surveyed. This was done for the total number of flowers at a site, which was used for the floral density metric. Floral species richness

was calculated using the Shannon diversity index in the package *vegan* in R (Oksanen et al., 2022) using number of flowers per floral species.

At each site an overall habitat quality score was qualitatively assigned after the surveys. This was based on floral species richness, abundance, and density of flowers which were each separately categorized with a score of one through four. For floral species richness, a score of four including high numbers of 15 or above blooming floral species, three was 10-20 blooming floral species with moderate number of all blooming flowers, two was 5-15 blooming floral resources dominated by few species, and one was 1-10 blooming floral resources dominated by one or two species. For floral abundance, four was extremely abundant (too many flowers to easily survey them all), three was abundant (almost too many flowers to easily survey them all), two was less abundant (some flowers, and easy to survey all), and one was extremely low abundance (very easy to survey all flowers, need to resurvey some flowers). For floral density, four was extremely high density of blooming floral resources across the entire site, three was large patches of high-density floral resources, two was medium-small patches of medium density floral resources, one was few – small patches of low-density floral resources. The scores of each category were averaged for each site (rounding up above 0.5 and down below 0.5) and this score is referred to as “habitat quality”; one is low quality, two is medium-low quality, three is medium-high quality, and four is high quality. This score qualitatively describes each site in a combined metric and helped to avoid over sampling higher or lower quality sites.

From the floral data I calculated several other floral interaction variables to help illuminate some of the specific relationships at a site. The number of floral species supporting bumble bee foraging was summed up by recording every flower a bee was collected from at a site. The percent of floral species foraged at a site was calculated by dividing the number of floral species foraged by the total species

richness. Percent clover was calculated by dividing the number of clovers at a site by the total floral abundance. These data are used in the modeling analysis and correlation matrices.

### *Pathogen and Parasite Screens*

*B. impatiens* and *A. mellifera* samples were pooled in groups of three individuals (up to 5 pools/site) and screened for four pathogens and parasites using RNA extraction and quantitative PCR (qPCR). Abdomens were dissected from worker bees while frozen. Three frozen abdomens were placed in a 5 ml tube with 4 metal beads and 2.0 ml of Trizol and homogenized using an Omni Bead-ruptor (Omni International, Kennesaw, Georgia) for 70 seconds on low speed. After brief centrifugation to remove particulate matter, 350  $\mu$ l of homogenate was used for RNA extraction. RNA was extracted using the standard protocol for the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, California) including incorporated DNA removal steps with DNaseI. RNA samples eluted in water were quantified and quality was assessed using a Nanodrop One (ThermoFisher Scientific, Waltham, Massachusetts). For each sample, 500 ng of RNA was converted to cDNA using standard protocols of the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific; 10  $\mu$ l RNA + 10  $\mu$ l Master-mix reaction, proportional to recommended quantities), followed by a 1(cDNA):4(water) dilution.

Quantitative PCR was performed using this cDNA to evaluate levels of the following pathogens and parasites: Black Queen Cell Virus, Deformed Wing Virus, *Crithidia bombi*, and *Vairimorpha bombi* (primers in table 1). Elongation factor 1 $\alpha$  (EF-1 $\alpha$ ; primers in table 1) was used as a control gene for normalization. For qPCR, I used a standard 10  $\mu$ l reaction in triplicate with no template controls, run on a QuantStudio 5 RT-PCR system machine (40 cycles, 60°C annealing temperature; ThermoFisher Scientific, Waltham, Massachusetts) using dissociation curves to ensure no non-specific products are

generated. Within-site samples were randomized across extraction and cDNA runs and plates. Gene expression levels were calculated from qPCR  $C_T$  values by first adjusting  $C_T$  values based on EF-1 $\alpha$ . All included samples had EF-1 $\alpha$  values within two  $C_T$  of one another, thus this normalization did not have strong impact on resulting numbers. I considered any  $C_T$  value above 35 a non-detection (no pathogen) and I calculated the fold difference in expression for each sample from this baseline level for analysis and presentation. Samples confirmed to have no pathogen prior to normalization were adjusted to have no pathogen after (a 0 value). For quality control, I re-tested ten samples of varying loads (including a non-detection and the highest load) along with a positive control from a previous study (Chapter One) to confirm our results were not due to undetected contamination, primer degradation, or a spurious result. In particular, I confirmed that the low variation in  $C_T$  values for *Crithidia* and lack of detection of *Vairimorpha* was not an artifact of contamination and faulty primers, respectively. I detected no issues with any of our controls and their  $C_T$  values gave results consistent with prior runs (e.g., positive values for *Vairimorpha*, more variable values for *Crithidia*).

### *Landscape and Weather Data*

Landscape and weather characteristics were extracted from different data sets to compare pathogen loads to the broader landscape. Using the Crop Land Data Layer (CLDL; Boryan et al., 2011) with a 2 km buffer, I extracted percent agriculture (sum of all crop variables), percent natural (sum of shrubland, grassland, woody wetland, herbaceous wetlands categories), percent forest (sum of deciduous, evergreen, and mixed forest categories), percent developed (sum of high development, medium development, and low development categories), and percent developed open for each site. The 2 km buffer was used to represent the maximum foraging range of most bumble bee species (Redhead et al.,

2016) and was used in McNeil et al (2020). Percent developed and percent developed open are separate elements of a larger “developed” category that account for open areas such as parks and cemeteries that were often our survey sites as opposed to urban development involving roads or housing. Using the CLDL, I calculated spring and summer floral resource indices using the Koh et al. (2016) reclassification tables and calculated the insecticide index as in McNeil et al (2020) using the protocol from Douglas et al. (2022). The floral and nesting indices use opinions of pollinator professionals to model the availability of resources across various landcover classes in the CLDL, considering differences between ecoregions across the country (Koh et al., 2016). To account for geographic correlation, I included latitude, longitude, and elevation (calculated in ArcGIS; ESRI, 2021; USGS, 1999). For weather variables, I used PRISM data (PRISM Climate Group, 2014) to extract total precipitation for April, May, and June of 2022, the average dew point of April, May, and June of 2022, and calculate growing degree days, a metric for heat that accounts for the number of degrees and days above 10°C, between April 1<sup>st</sup> and August 1<sup>st</sup> of 2022.

### *Analysis*

I created histograms and obtained median levels for each pathogen in our study and compared these values to those obtained in comparable works from Pennsylvania. Pathogen loads for BQCV, DWV, and *Crithidia bombi* were compared by North Carolina ecoregion (Coastal Plains, Piedmont, Appalachian) and by a qualitative habitat quality metric (high, medium-high, medium-low, low; described above) using Kruskal-Wallis to account for non-normalcy in the data and a post-hoc Dunn’s test when necessary to compare between groups ( $\alpha = 0.05$ ). To test for an effect of presence of honey bees on bumble bee pathogen loads and bumble bee diversity, I used Kruskal-Wallis to compare BQCV and



DWV loads (the two pathogens of the four tested that are found in honey bees) as well as *Bombus spp.* diversity at sites with and without honey bees. I modeled relationships between loads in bumble bees using BQCV, DWV, and *C. bombi* response variables as functions of the above-mentioned landscape, floral and bee diversity, and climate variables (Table 2) along with BQCV, DWV, and *C. bombi* loads at each site as predictors using mixed-effects models, implemented in the lme4 package in R (Bates et al., 2017). I did not analyze correlations with *Vairimorpha* given that this species was not detected across all samples. Ecoregion was used as a random effect to account for non-independence due to large-scale patterns across ecoregions. I also included site as a random effect to account for potential pseudo-replication because multiple samples were collected from each site. Candidate models were made from single variables, single quadratic variables, and all two non-correlated variables (Pearson's Correlation Coefficient < 0.7). AIC model selection was used to determine which model best described our data. I included a null model, with no predictor variables, as a control for comparison. Any models within two delta AIC were considered equivalent. The effect of a predictor variable was considered non-significant if the 95% confidence interval included zero or if it was within 2 AIC units of the null model. Additionally, I created a Pearson's correlation matrix with the same variables as above, with BQCV and DWV loads in *A. mellifera*, to assess any general patterns between pathogen loads and landscape variables.

### *Literature review*

To compare our results to broader research I conducted a literature review of all studies in North America examining correlations between landscape variables and bumble bee pathogen loads. Using Google Scholar and Web of Science I searched for any published scientific manuscripts that included

evaluations of bumble bee pathogen and parasite prevalence across a landscape scale. I then summarized the key landscape characteristics that influenced pathogen load. For simplicity I reduced the synthesis in this paper to the four pathogens from the present study: BQCV, DWV, *Vairimorpha spp.*, and *Crithidia spp.* I categorized the variables observed in each study into broader categories to better compare studies to each other. For the purposes of this study, I focused our assessments on the papers that addressed how pathogen levels relate to amount of agriculture, amount of developed land, amount of natural/forest land (also including grassland and pasture in this category), honey bee presence or abundance, bumble bee community (any measure of community including abundance, diversity, or richness), floral resources (any measure of floral resources, often richness, diversity, or density), nesting availability (any measure of nesting habitat used) elevation, latitude and longitude, and weather (temperature and precipitation), as those were the categories most related to our work, the topics studied, and represent broader categories of potential effectors. I only included variables that exceeded a statistically significant threshold reported in the study. This review includes the data from McNeil et al. (2020) and Chapter One, but not the data collected in North Carolina.

## Results

### *Pathogen loads in Bombus impatiens populations in North Carolina*

Pathogen and parasite loads varied in their occurrence and distribution across sites. All samples had at least one pathogen detected above our threshold. I detected no *Vairimorpha bombi* in any of our samples (Figure 5) and thus did not use *V. bombi* in any further statistical testing. There were low loads of DWV (mean: 0.67, sd: 1.70; Figure 5). All samples except for one tested positive for *Crithidia bombi*. *C. bombi* levels were relatively high, with little variation among samples (mean: 10.20, sd: 1.71; Figure

5). Given the low variation in *C. bombi*, there was less power to detect patterns across landscape characteristics. Most samples tested positive for BQCV, but there was high variation of load within the samples (average: 6.57, sd:4.21; Figure 5). The high variation in BQCV improved the ability to detect patterns of this pathogen across landscape characteristics.

*Landscape factors influence pathogen and parasite prevalence in Bombus impatiens populations in North Carolina*

There was little effect of habitat quality on parasite and pathogen loads. In high quality habitat, 34% of the samples had BQCV, 3.6% had DWV, and 61.4% had *Crithidia bombi*. In the low-quality habitat, 30.4% of the samples had BQCV, 6.3% had DWV, and 63.2% had *Crithidia bombi*. There was no significant difference in pathogen loads between the designated low, medium, and high quality habitats (BQCV:  $p = 0.86$ ; DWV:  $p = 0.72$ ; *Crithidia bombi*:  $p = 0.51$ ). There were no significant differences between ecoregions for BQCV ( $p = 0.61$ ) and DWV ( $p = 0.2$ ) but there was a difference between ecoregions for *Crithidia bombi* ( $p = 0.006$ ; Figure 6), with the Coastal Plains and the Appalachians having significantly higher levels than the Piedmont (Coastal Plains - Piedmont:  $p = 0.012$ ; Appalachian - Piedmont:  $p = 0.0024$ ) but not differing from each other (Coastal Plains - Appalachian:  $p = 0.9$ ; figure 3).

In examining relationships between pathogens and landscape variables, BQCV and *Crithidia bombi* had some significant models, albeit not many. The BQCV top model included floral abundance and summer floral, however summer floral alone was within 2 AIC units of the top model. While the top model (with both variables) technically performs better, the difference of 2 delta AIC suggests that floral abundance is a “pretender variable” (Arnold, 2010; MacKenzie et al., 2017) and given that summer floral availability is in all the top 5 models for BQCV, that is likely the driving variable for BQCV

(Figure 7a; Table 3). The *Crithidia bombi* top model was quadratic bumble bee species diversity (Table 3), whereby loads are lowest for the less diverse and most diverse sites, and highest for those with intermediate diversity. However, this result is dependent on an outlier site which had the highest bumble bee diversity but was the only sample with no *C. bombi*. When this value is removed the null model is within the two AIC units and diversity is not within the range of significance. For DWV the top model includes a positive relationship with BQCV (Table 3). All other models for DWV were within two delta AIC of the null model.

In examining correlations among our variables (Figure 8), there were several notable trends. While no correlations were particularly strong, *C. bombi* loads were lower when floral diversity was higher ( $r = -0.25$ ) and in warmer areas (GDD,  $r = -0.16$ ), and was most positively correlated with the number of floral species *Bombus spp.* were collected on ( $r = 0.27$ ). BQCV loads were higher in places with higher summer floral resources ( $r = 0.54$ ) and in warmer (GDD,  $r = 0.18$ ) and more developed (developed,  $r = 0.36$ ; developed open,  $r = 0.44$ ) areas, and most negatively correlated with forest ( $r = -0.28$ ) and areas with more nesting habitat ( $r = -0.23$ ), which are more forested/mixed habitat areas. DWV has no strong trends, but patterns generally mirror those of BQCV.

#### *Pathogen loads in honey bees and impacts of honey bees' presence on bumble bees*

BQCV loads were higher in bumble bees at sites with honey bees than sites without (Figure 9a; *A. mellifera* absent,  $\bar{x}$ : 4.13; *A. mellifera* present,  $\bar{x}$ : 6.17;  $p = 0.028$ ) but were not significantly different between honey bees and bumble bees at sites with honey bees (Figure 9a; *A. mellifera*:  $\bar{x} = 7.45$ ;  $p = 0.69$ ). Over all, honey bees had higher loads of BQCV than *B. impatiens* ( $p = 0.014$ ). While 78% of bumble bees had detectable BQCV, 99.98% of honey bee samples did, thus honey bees have higher incidence.

DWV loads were much higher in honey bees than bumble bees (Figure 9a;  $p < 0.05$ ). There was no difference in *B. impatiens* DWV loads at sites with and without honey bees (Figure 9a; *A. mellifera* absent,  $\bar{x}$ : 0.58; *A. mellifera* present,  $\bar{x}$ : 0.62;  $p = 0.81$ ) or between honey bees and bumble bees at the same site given high variance in honey bees (Figure 9a; *A. mellifera*:  $\bar{x} = 5.42$ ; *B. impatiens*:  $\bar{x} = 0.60$ ;  $p = 0.57$ ). DWV loads were lower than BQCV in honey bees ( $p = 0.002$ ). There were no differences in *Bombus* diversity at sites with and without honey bees (*Bombus spp.* diversity:  $p = 0.82$ ).

Honey bee pathogen loads correlated with several landscape variables (Figure 9b). The landscape factors correlating with these viruses are similar between honey bees and bumble bees, matching bumble bee trends in both North Carolina and Pennsylvania, but correlations tend to be stronger in honey bees than bumble bees, especially for BQCV. BQCV was most negatively correlated with the nesting index ( $r = -0.60$ ), forest ( $r = -0.56$ ), and spring floral index ( $r = -0.45$ ); loads were most positively correlated with longitude ( $r = 0.51$ ) and amount of developed land ( $r = 0.42$ ). DWV in honey bees had similar patterns to BQCV, being most negatively correlated with spring floral resources ( $r = -0.36$ ), elevation ( $r = -0.36$ ), and growing degree days ( $r = -0.28$ ); loads were most positively correlated with longitude ( $r = 0.48$ ) and amount of developed land ( $r = 0.19$ ). BQCV and DWV were positively correlated with each other ( $r = 0.35$ ), and negatively correlated with *Bombus* diversity (BQCV:  $r = -0.20$ ; DWV:  $r = -0.28$ ).

#### *Comparisons of effects of landscape variables between North Carolina and Pennsylvania*

Compared with the McNeil et al (2020) and Chapter One, for the most part the correlations and patterns with the landscape variables were similar, but with smaller magnitudes (Figure 8). For example, spring floral index and forests are most often negatively correlated with BQCV and DWV across all

years and localities, but Pennsylvania correlations appear to be much stronger than in North Carolina. Similarly, growing degree days, and percent development is positively correlated with BQCV and DWV across studies. *C. bombi* tends to show the opposite trends from virus levels in both North Carolina and Pennsylvania, where it is more abundant in forests and less abundant in warmer more developed areas (Figure 8; 2020: percent developed:  $r = -0.36$ ; percent forest  $r = 0.41$ ; 2022: percent developed:  $r = 0.04$ ; percent forest:  $r = 0.03$ ). The biggest difference between results from North Carolina and Pennsylvania is the relationship with the summer floral index; between 2018-2020 in Pennsylvania there is a negative correlation between quality of summer floral resources and BQCV and DWV loads, and a positive correlation with *C. bombi* (2020). In 2022 in North Carolina, the opposite trend was found, where summer floral has a positive correlation with BQCV and DWV and a negative correlation with *C. bombi*.

#### *Ecoregional and habitat correlations with pathogen and parasite loads based on literature review*

The overall body of literature evaluating bumble bee pathogen and parasite prevalence at the landscape scale in North American covers a broad array of species of both bumble bees and pathogens/parasites. In total I identified 10 published papers, and one unpublished paper (Table 4) that covered landscape – pathogen comparisons. Four papers used only *B. impatiens* for pathogen work (Fearon et al., 2023; Figueroa et al., 2020; Chapter One; McNeil et al., 2020), two used only *B. vosnesenskii* (Cohen, 2016; Ivers et al., 2022), and the remaining six used a mixture of bees from their study area including *B. occidentalis*, *B. pensylvanicus*, *B. affinis*, and *B. terricola* (Blaker et al., 2014; Levenson & Tarpy, 2022; McArt et al., 2017; Olgun et al., 2020; Tsvetkov et al., 2021). For our analysis, I focused on studies reporting landscape results regarding BQCV (n=6 studies), DWV (n=5),

*Vairimorpha spp.* (n=8), and Trypanosome (n=8). Other pathogens observed include but are not limited to conopids, *Apicystis spp.*, parasitic flies, nematodes, and various mite species.

Across studies there are a wide array of potential landscape factors addressed, but most papers focus their study on just a few variables, reducing their comparisons to a few specific landcover classes such as urban, agriculture, or forest (Table 4). Other papers used more site-scale variables like local floral resources (Table 4). Seven papers included at least one measure of bee community composition (often a diversity index, abundance, or richness) as an explanatory variable for pathogen prevalence (Figure 10; Table 4). Seven papers included at least one measure of floral resources (Figure 10; Table 4).

The literature reviewed found mixed effects of landscapes on pathogen loads, however there are certain variables that leaned more towards negative effects and others towards positive effects. In general, more floral resources and more nesting resources are associated with lower levels of pathogens, whereas sites with more developed land, more agricultural land, and with honey bees are associated with increased in pathogen levels (Figure 10). Interestingly, floral resources, measured in diversity, richness, or density, has mixed effects depending by study, sometimes associated with significantly increased and sometimes decreased pathogen prevalence in DWV, BQCV, and Trypanosomes (Cohen, 2016; Fearon et al., 2023; Figueroa et al., 2020; Levenson & Tarpay, 2022; McNeil et al., 2020), while more floral resources, when significant, were associated with reduced *Vairimorpha* loads. Similarly, sites with natural areas and forests have no significant effects on *Vairimorpha spp.*, but are associated with increased *C. bombi* (Chapter One), decreased DWV (Chapter One) and are associated with either increased (Fearon et al., 2023) or decreased (Chapter One; Tsvetkov et al., 2021) BQCV prevalence. Bumble bee community also has mixed effects, both increasing or decreasing prevalence depending on the pathogen, or in the case of Trypanosomes, depending on the study. The presence of honey bees was

relatively consistently associated with increased bumble bee pathogen loads, for viruses (BQCV and DWV), and for *V. bombi*, a bumble bee parasite. Similarly, available nesting habitat was consistently associated with decreased pathogen prevalence.

## Discussion

Across studies, states, and regions there were some consistent, but not equally strong, relationships between landscapes and bumble bee pathogens. In both North Carolina and Pennsylvania, DWV and BQCV levels in bumble bees were positively correlated with amount of developed land, growing degree days, and honey bee abundance or presence, and levels were negatively correlated with amount of forest and nesting availability. The degree to which these patterns were seen, however, differed, with North Carolina showing weak patterns in most cases, thus few of these trends reached significance in models. Part of this was due to lower loads or variance of pathogens in North Carolina versus Pennsylvania: North Carolina bee samples had lower levels of DWV, no detectable *Vairimorpha*, and less variance in *Crithidia*, and while there was variance in BQCV it still showed weaker trends. Interestingly, in North Carolina, there was a stronger association of landscape variables and pathogens in honey bees than in bumble bees, though some overall patterns were the same: this suggests that some bee species are responding similarly to the landscape conditions and habitat quality, but the ability to detect these effects is dependent on the amount of variance available in pathogen loads and dependent on the variables observed. Examination of the literature showed considerable variance in directionality and significance of these relationships. Overall, this demonstrates that landscape impacts on bumble bee parasite loads are complex and that there is not one key factor but likely many interacting factors driving pathogen epidemiology in these bees.



Floral resources were key factors in determining pathogen loads, however when compared across studies, the directionality of these relationships appears context dependent and complex, and perhaps ultimately less of a driver than other factors. In North Carolina, summer floral resources were related to increased BQCV loads in bumble bees and spring floral resources were correlated with decreased BQCV loads in honey bees. In a similar analysis in Pennsylvania, spring floral resources are associated with lower BQCV loads in bumble bees as well. However, across the literature BQCV prevalence has shown inconsistent patterns with floral quality features. It has been found to be higher with more abundance of perennials (Cohen, 2016), floral density (Fearon et al., 2023). and floral richness (Fearon et al., 2023), but decreasing with increased diet breadth (Figueroa, et al., 2020). My data suggests sites with more floral diversity decrease loads but increased floral species visited increases pathogen transmission (Figure 8). While manipulating floral resources is a direct management tool for bumble bee landscape conservation, floral availability has mixed effects on pathogen loads and prevalence due to the benefit of flowers as a food source and the transmission potential of flowers. Additionally, floral resources can provide more pollen and nectar to support healthy bees, but the more visits' flowers get, the more pathogen transmission can occur (Alger et al., 2019b; Burnham et al., 2021; Durrer & Schmid-Hempel, 1997; Figueroa et al., 2019).

The presence of honey bees may complicate the effects of floral resources on bumble bee pathogen loads as well, as BQCV loads in bumble bees tend to be higher where honey bees are present. *Bombus* DWV prevalence was not increased by the presence of *A. mellifera*, as DWV loads were consistently low in *B. impatiens*. This is consistent with prior evidence showing low infection rates of DWV in non-*Apis* bees (Dolezal et al., 2016). Additionally, honey bee pathogen loads were negatively correlated with bumble bee diversity which supports the hypothesis that increased biodiversity can reduce infection levels (Civitello et al., 2015). This may be because sites with more bumble bee

diversity have fewer honey bees (MacInnis et al., 2023), or diverse bumble bee communities reduce transmission for honey bees (i.e., transmission reduction; Keesing et al., 2006), or perhaps that the myriad factors promoting healthier environments for bumble bee communities also lead to lower pathogens in honey bees (e.g., Dolezal et al., 2019). While honey bees are likely to have different disease dynamics than bumble bees given their intense but variable management, different sociality, and more generalist foraging, we found the BQCV infection patterns to be similar between honey bees and bumble bees. For example, the strong negative correlation between BQCV and percent forest cover and spring floral availability was observed in bumble bees in Pennsylvania, honey bees in North Carolina, to a certain extent bumble bees in North Carolina, as well as in other literature, which supports clear feedback between nutritional availability and honey bee pathogen effects (Dolezal et al., 2019; Dolezal & Toth, 2018). However, the negative correlation between BQCV and total precipitation found in honey bees was not observed in bumble bees, perhaps indicative of varying bee response to weather and climate (e.g., Quinlan et al., 2022; Rowland et al., 2021). Agriculture is seen to generally increase prevalence of various pathogens in honey bees, either through nutritional stress or pesticide exposure (Alburaki et al., 2018), however, while land development has a more negative effect, we do not see a clear correlation with agriculture in North Carolina. Overall, however, these results suggest that one mechanism for reducing spillover of viruses from managed honey bees to wild bees is to reduce pathogen levels in managed bees by to improving floral resources and habitat conditions.

Given *Vairimorpha bombi* is one of the most lethal bumble bee parasites (Otti & Schmid-Hempel, 2007; Rutrecht & Brown, 2008) and the leading cause of some population declines (Cameron et al., 2011), it is one of the most studied bumble bee parasites. Nonetheless, the main driver of *V. bombi* prevalence and loads in bumble bee populations remains unclear. Broadly in the literature, *V. bombi* is less prevalent when there are abundant floral and nesting resources, suggesting that bumble bee

populations in high quality habitats should harbor less *Vairimorpha spp.* (Figure 10). This is mirrored by the increase in *Vairimorpha spp.* in areas with increased stressors, such as increase amount of agriculture and honey bees (Figure 10). Our study did not detect any *V. bombi* in North Carolina, so could not reflect on these patterns, and other landscape studies as well are hampered by low loads, thus limiting power (McArt et al., 2017). Levels may have been particularly low in our study because *Vairimorpha* has been found to be most associated with the rarest bumble bee species and to have low levels in *B. impatiens*, which dominate our communities (Chapter Three; Cameron et al., 2011; Cordes et al., 2012; Gillespie, 2010; Levenson & Tarpy, 2022; Malfi & Roulston, 2014). To better understand this highly lethal parasite for conservation, surveys and pathogen screens on susceptible *Bombus spp.*, like *B. occidentalis* and *B. pensylvanicus* (Gillespie, 2010; Levenson & Tarpy, 2022), at a landscape scale would be more effective for understanding dynamics of this pathogen. Additionally, there is little research on other *Vairimorpha spp.* in *Bombus* and a recent paper from Jones et al. (2022) found 98% of *B. impatiens* in squash fields infected with *Vairimorpha apis* but that very few were infected with *V. bombi*. Since past studies mostly focus on morphology, they may have incorrectly classified *V. apis* as *V. bombi*. This suggests a need for future research to examine *Vairimorpha*, and the landscape factors driving it, across a broader geographic range.

*Crithidia bombi* is most often influenced by bumble bee community composition, suggesting that transmission dynamics between individual bees, which can be influenced by the number of bee species in the community, may explain prevalence and load. In Chapter One there was a positive correlation between *C. bombi* and forests, which also vary in community, and in North Carolina due in part to low variation in pathogen loads, the relationship between *C. bombi* and community diversity was significant but dependent on an outlier. The relationships suggest *C. bombi* is highest at intermediate levels of species diversity. Species may vary in *Crithidia* loads; thus, the composition and diversity of

communities alone may explain this pattern. Our lowest diversity communities were typically dominated by *B. impatiens*. Previous studies suggest that *B. impatiens* have lower loads than *B. bimaculatus* and *B. griseocollis* (Malfi & Roulston, 2014) and these are the two species are second and third most abundant in our data set (Chapter Three). Thus, our intermediate diversity communities likely harbor more of these two species. The most diverse communities would harbor additional, but rarer, bumble bee species, which were previously found to have lower *Crithidia* loads (Cordes et al., 2012; Malfi & Roulston, 2014). Thus, the susceptibility of different bumble bee species to *Crithidia* and their prevalence at our studies sites may have explained a possible quadratic relationship. Furthermore, given that *Crithidia* is impacted by bee nutritional state (Brown et al., 2000), the reduction of *C. bombi* at higher levels of *Bombus* diversity could be a result of better habitat resources in more diverse bumble bee communities.

Given that *C. bombi* is not usually transmitted from bee to bee directly, it would be important to consider the transmission mechanism, which is defecation on flowers. Avoiding encounters with an infected individual would not be enough to reduce pathogen loads, and the structure of the environment and the potential external factors influencing transmission would be critical as well. Based on our North Carolina data compared to the Pennsylvania 2020 data, loads of *C. bombi* can vary greatly across geographic regions, making *C. bombi* appropriate for addressing questions from a classical disease ecology framework in a broader scale. However, *C. bombi* alone is often lethal only in high stress conditions (Brown et al., 2000), thus may be of less conservation concern. Perhaps the best way to mitigate any threat of *C. bombi* would be to reduce the stressors that make it lethal, which is ultimately supporting diverse pollinator communities and high-quality habitats.

Across North Carolina and the continental United States, the disease dynamics of bumble bee pathogens are context dependent, multifactorial, and still not adequately understood. Developing effective strategies to consistently reduce pathogen loads in bumble bees will require additional research that is conducted across spatial scales, from gardens to geographic regions. General strategies, such as increasing the diversity and abundance of pollinator plantings and thereby increasing pollinator species diversity (Blaauw & Isaacs, 2014), will likely reduce overall transmission of most pathogens. As pollinator populations decline and there is increased stress on the remaining populations, a strong understanding of how local habitat, pollinator communities, landscape conditions and ecoregional factors influence pathogen dynamics will provide critical information to develop local and generalized practices and pathogens and landscapes both support and harm bees will provide better context and tools to conserve our native pollinators.

### Figures

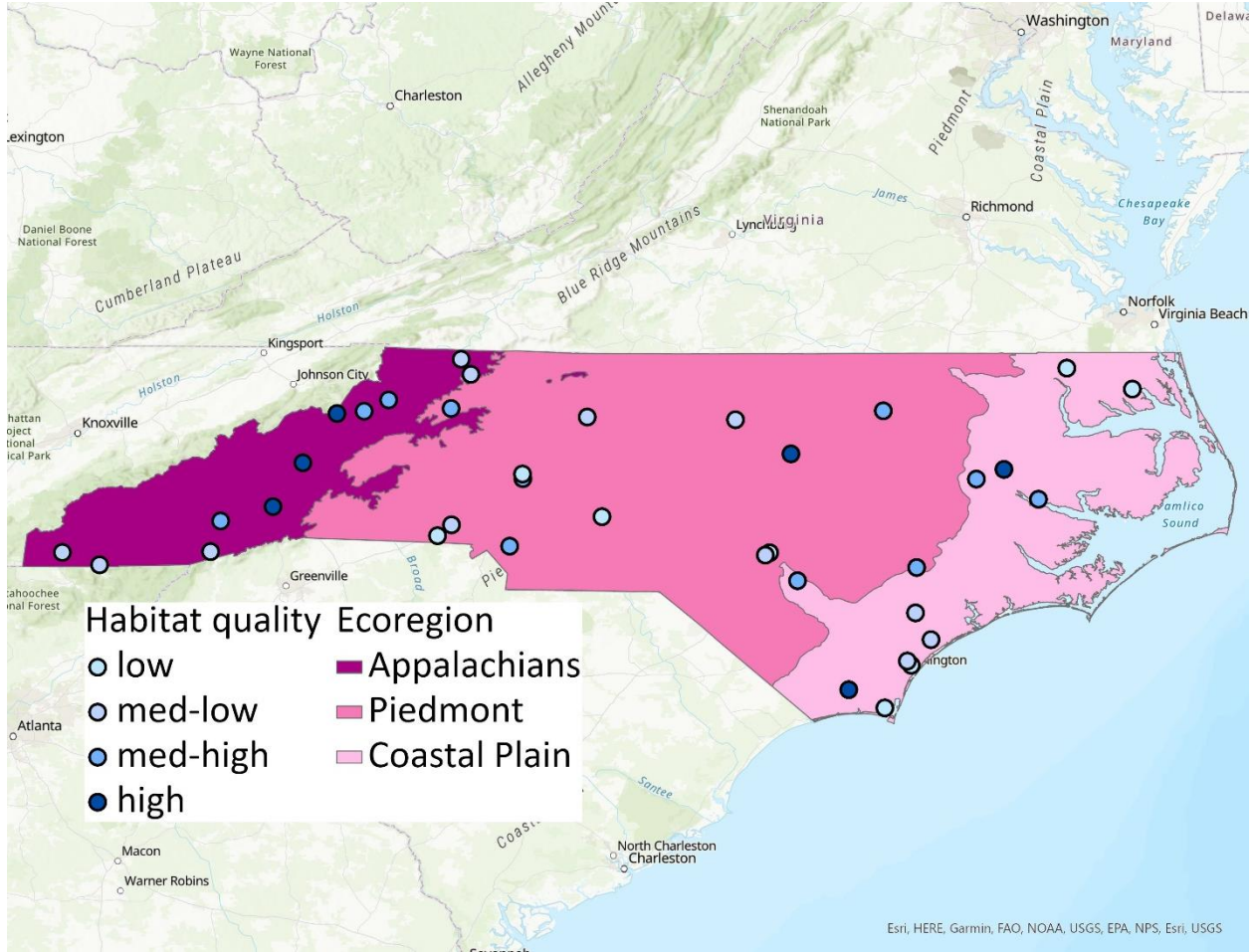


Figure 4: Map of surveyed sites in North Carolina. Sites coded by overall habitat quality score.

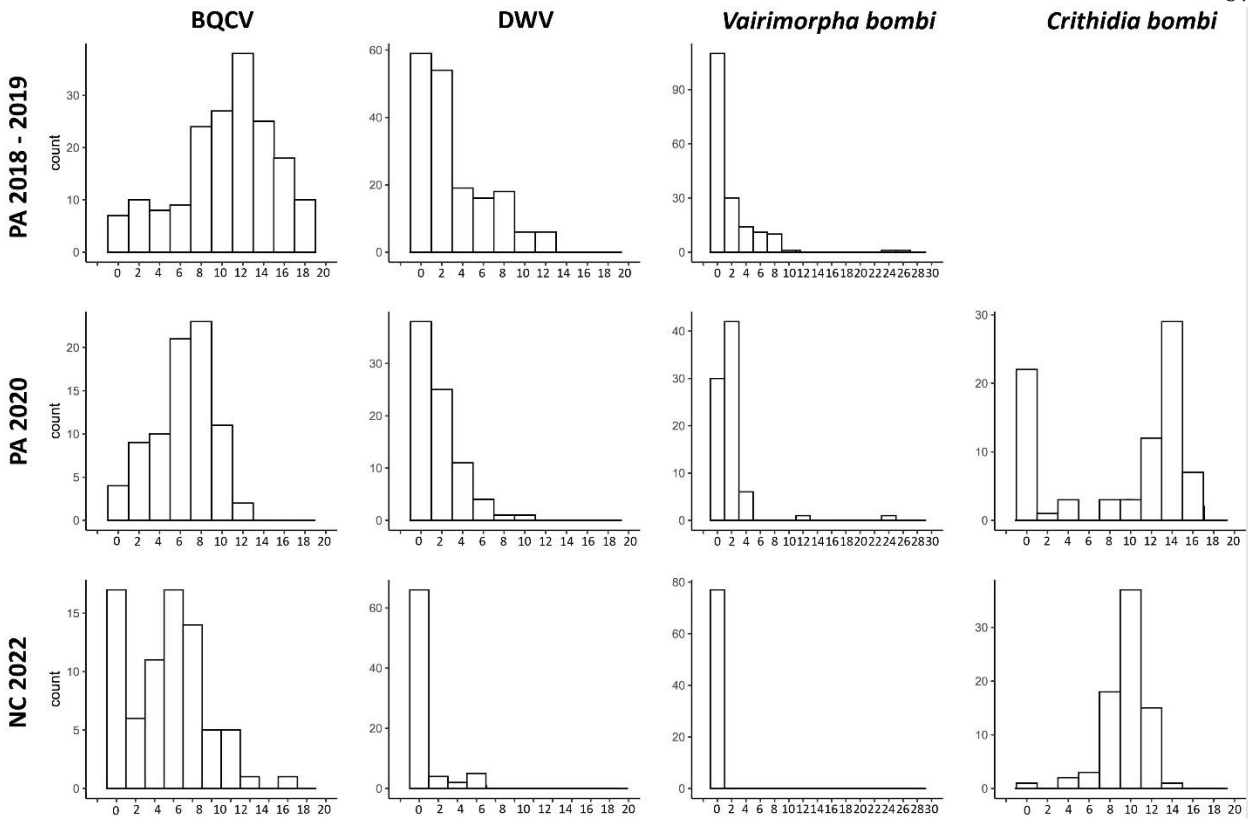


Figure 5: Histograms of pathogen loads.

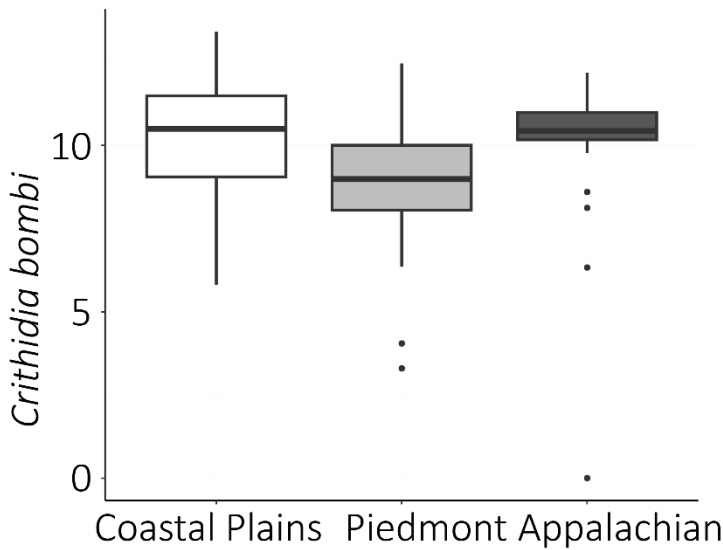


Figure 6: Box plot of *C. bombi* loads by ecoregion. Coastal Plain and Appalachians are significantly higher on average than the Piedmont ( $p = 0.006$ ).

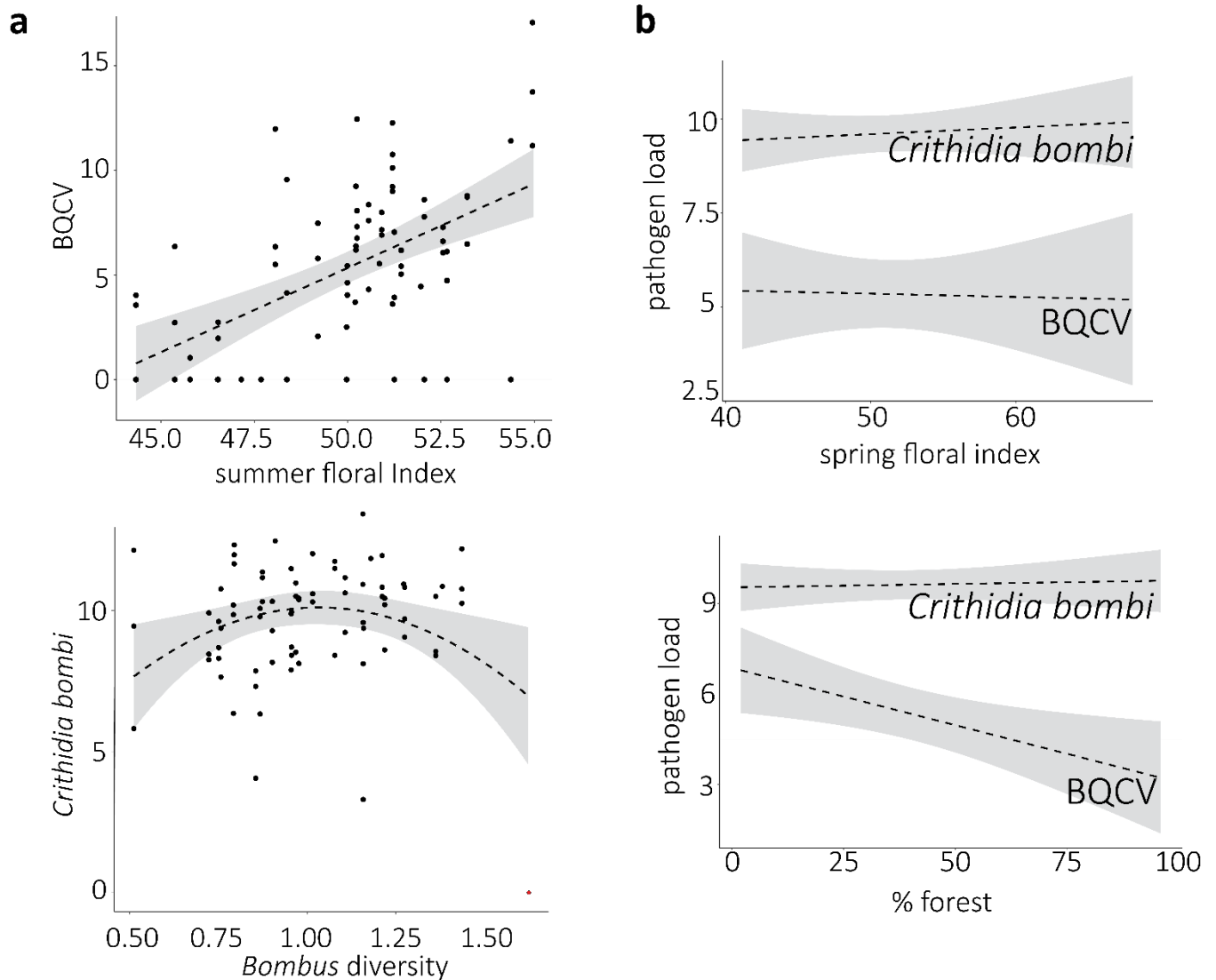


Figure 7: a) Summer floral Index model for BQCV and Bombus diversity model for *C. bombi*. The Bombus diversity model for *C. bombi* is significantly driven by an outlier indicated in red; without this value there are no significant models describing *C. bombi* loads in *B. impatiens*. b) Models of BQCV and *Crithidia bombi* against the spring floral index and percent forest, the two of the main variables initially predicted to effect pathogens loads based on McNeil et al. (2020).



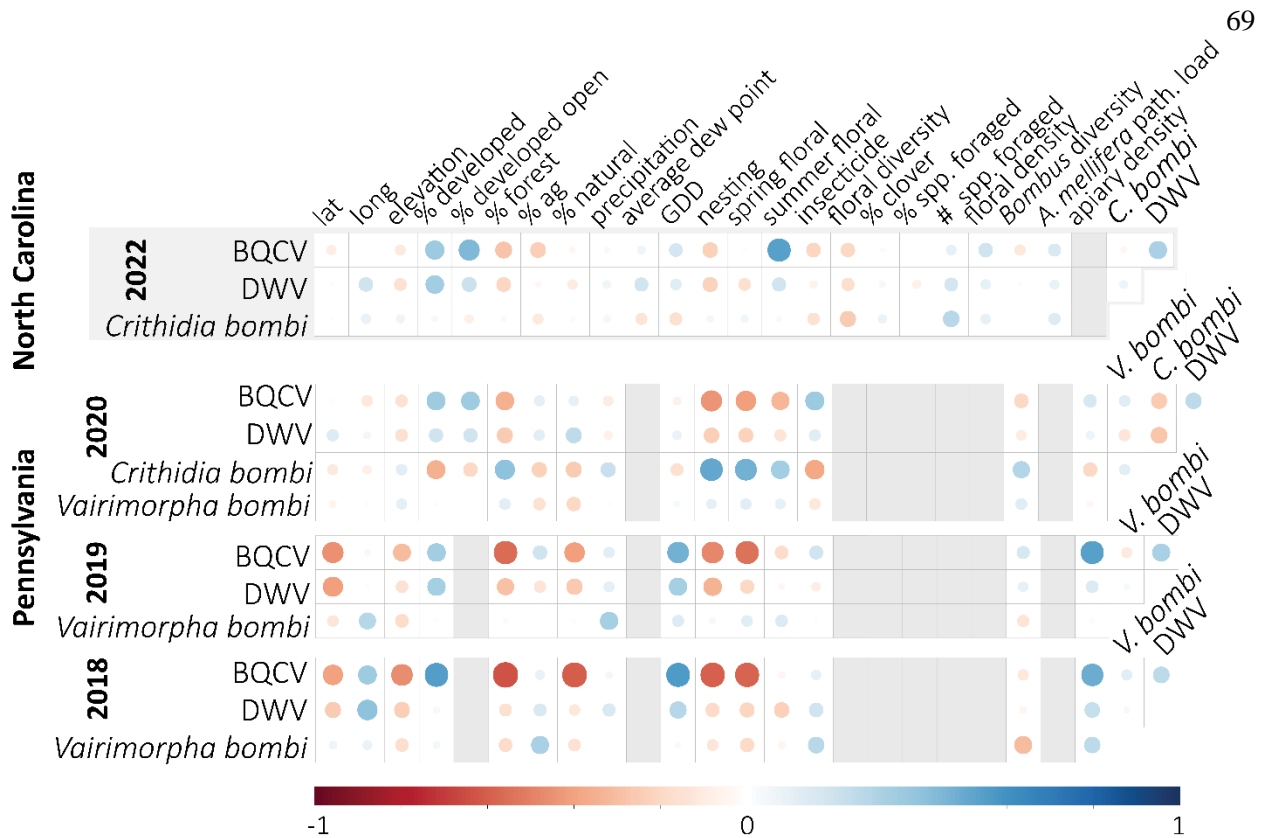


Figure 8: Correlation matrix with all landscape and site variables with pathogen loads across Pennsylvania from McNeil et al. (2020) and Chapter 1, and North Carolina 2022 data. 2018 and 2019 are data from Pennsylvania from McNeil et al. (2020). 2020 are data from Pennsylvania from Chapter 1. 2022 is data from North Carolina. In McNeil et al. (2020), precipitation and growing degree days are from the spring, and the percent natural category does not include grassland/pasture as it does in 2022.

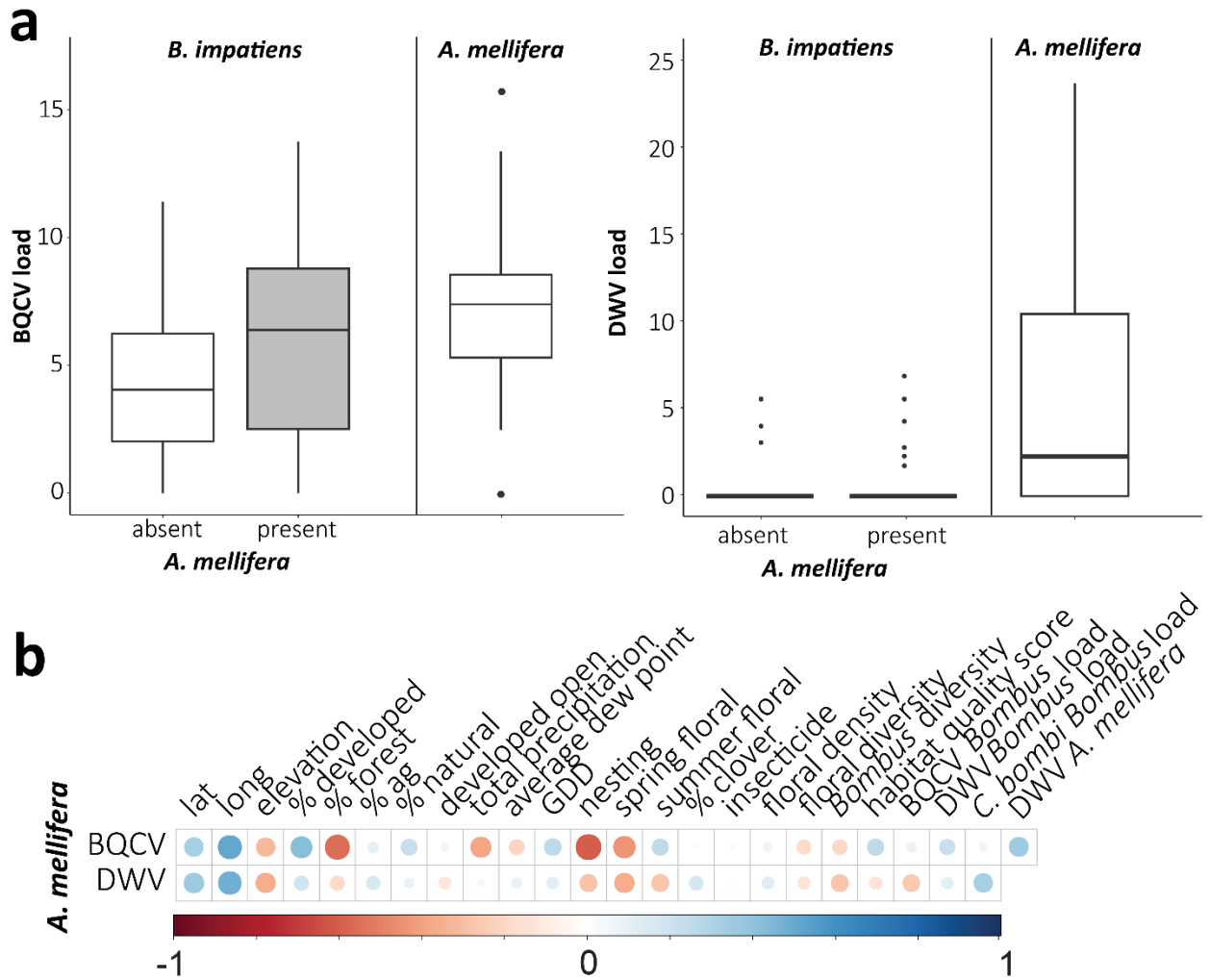


Figure 9: a) Comparison of *B. impatiens* BQCV and DWV loads at sites with and without *A. mellifera*. BQCV at sites with *A. mellifera* were significantly higher than sites without ( $p = 0.04$ ). This also compares BQCV of honey bees where present. DWV at sites with *A. mellifera* ( $p = 0.82$ ). b) correlation matrix with *A. mellifera* pathogen loads against all 2022 landscape and site variables, as well as the habitat quality score and *B. impatiens* pathogen loads.

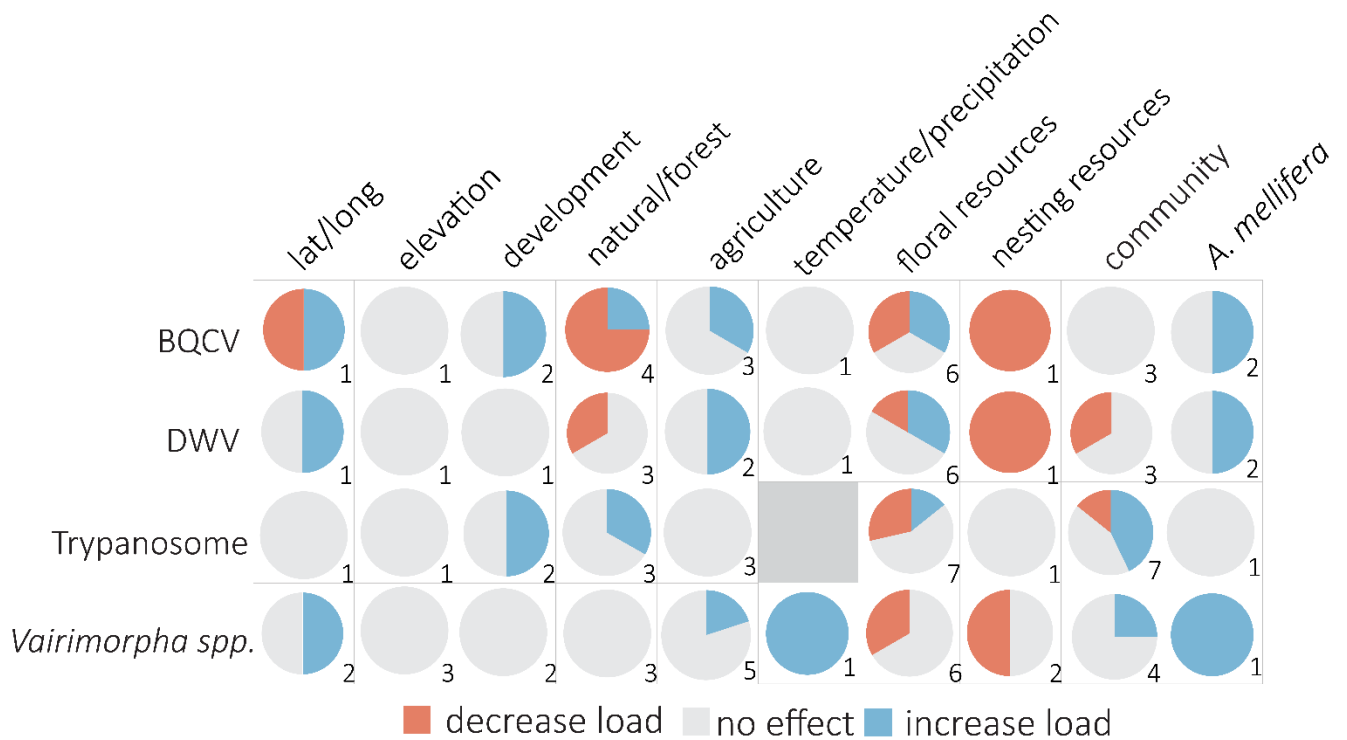


Figure 10: Summary of literature review and the significant effect of variables on DWV, BQCV, Trypanosomes, and Vairimorpha spp. in *Bombus* in North America. The number of papers addressing a category in the bottom right corner. Please note some papers had more than one variable with in a category, see table 4.

## Tables

Table 1: All primers used for qPCR.

Gene	Forward primer	Reverse primer
Ef-1a	CCGACAAGGCTCTTCGTTA	ATGCCTGCCTTCAGAATACC
BQCV	TTTAGAGCGATTTCCGGAAACA	GGCGTACCGATAAACATGGA

DWV	TGAATTCAGTGTTCGCCATA	GAGATTGAAGCGCATGAACA
<i>V. bombi</i>	GGCCCATGCATGTTTTTGAAG ATTATTAT	CTACACTTTAACGTAGTTATCTGCGG
<i>C. bombi</i>	GGCCACCCACGGGAATAA	CAAAGCTTTCGCGTGAAGAAA

Table 2: Landscape and site variables used in models.

Site and landscape characters		
variable	scale	source
floral abundance estimate	site	collected at site
floral density	site	collected at site
floral richness	site	collected at site
honey bee presence	site	collected at site
bumble bee diversity - Shannon diversity index	site	collected at site
floral diversity - Shannon diversity index	site	collected at site
% flowers used in foraging	site	collected at site
% clover	site	collected at site
latitude	site	collected at site
longitude	site	collected at site
elevation	site	ArcGIS pro DEM
% forest	2 km radius	CLDL
% ag	2 km radius	CLDL
% development	2 km radius	CLDL

% developed open	2 km radius	CLDL
% natural	2 km radius	CLDL
spring floral index	2 km radius	CLDL (Koh et al 2016)
summer floral index	2 km radius	CLDL(Koh et al 2016)
nesting index	2 km radius	CLDL(Koh et al 2016)
insecticide index	2 km radius	CLDL (Douglas et al 2020)
total precipitation from April, may June	2 km radius	PRISM
average dew point form April, may, June	2 km radius	PRISM
Growing Degree Days between April 1 <sup>st</sup> – August 1 <sup>st</sup> (based on 10 C)	2km radius	PRISM

Table 3: AIC model selection results. Models were considered significant if delta AIC was below 2, not within 2 delta AIC of null model, and 95% CI of betas did not include zero.

<b>BQCV</b>						
<b>Model</b>	<b>ΔAIC</b>	<b>ModelLik</b>	<b>w</b>	<b>variable</b>	<b>beta</b>	<b>95% CI</b>
summer floral + floral abundance	0.00	1.00	0.32	floral abundance	0.32	0.1 - 0.54
				summer floral	0.46	0.24 - 0.67
summer floral + DWV	2.23	0.33	0.10	summer floral	0.50	0.27 - 0.73
				DWV	0.21	0.04 - 0.38
summer floral	2.32	0.31	0.10	summer floral	0.54	0.31 - 0.78
summer floral + <i>A. mellifera</i> presence	3.91	0.14	0.04	summer floral	0.49	0.25 - 0.72
				<i>A. mellifera</i> presence	0.40	-0.07 - 0.86
summer floral + spring floral	4.02	0.13	0.04	summer floral	0.61	0.37 - 0.85
				spring floral	-0.21	-0.45 - 0.02
<b><i>Crithidia bombi</i></b>						
<b>Model</b>	<b>ΔAIC</b>	<b>ModelLik</b>	<b>w</b>	<b>variable</b>	<b>beta</b>	<b>95% CI</b>
<i>Bombus</i> diversity ^2	0.00	1.00	0.17	<i>Bombus</i> diversity^2	-0.36	-0.56 - -0.15
null	2.52	0.28	0.05	-	-	-

# flowers spp. foraged	2.64	0.27	0.04	# flower spp. foraged	0.29	0.03 - 0.55
floral diversity	3.64	0.16	0.03	floral diversity	-0.27	-0.56 - 0.02
<i>A. mellifera</i> presence	4.98	0.08	0.01	<i>A. mellifera</i> presence	0.17	-0.46 - 0.80
<b>DWV</b>						
<b>Model</b>	<b>ΔAIC</b>	<b>ModelLik</b>	<b>w</b>	<b>variable</b>	<b>beta</b>	<b>95% CI</b>
BQCV	0.00	1.00	0.31	BQCV	0.14	0.07 - 0.21
nesting ^2	3.47	0.18	0.06	nesting^2	0.19	0.09 - 0.29
BQCV + % natural	3.59	0.17	0.05	BQCV	0.13	0.06 - 0.21
				% natural	-0.11	-0.20 - 0.03
BQCV + average dew point	3.74	0.15	0.05	BQCV	0.13	0.06 - 0.21
				average dew point	0.07	0.00 - 0.14
BQCV + % developed	4.40	0.11	0.03	BQCV	0.11	0.04 - 0.19
				% developed	0.06	- 0.01 - 0.14

Table 4: Summary of literature review papers. Describes pathogen studied, variables tested, directionality of effects of variables, and categories used for synthesizing study results in Figure 10.

<b>Blaker et al 2014</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
<i>Vairimorpha spp.</i>	Presence of other bees infected with <i>V. bombi</i>	increase	bee specific variables
<i>Vairimorpha spp.</i>	elevation	not significant	elevation
<i>Vairimorpha spp.</i>	increase over season	increase	season
<b>Cohen et al 2022</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
BQCV	bee abundance, Shannon diversity index	not significant	community

BQCV	abundance of perennials	increase	floral
BQCV	abundance of flowering annuals, garden size, landscape floral resources/natural habitat within 1 km	not significant	floral
BQCV	parasite and pathogen prevalence/infection rate of honey bees	not significant	honey bee
DWV	bee abundance, Shannon diversity index	not significant	community
DWV	garden size	increase	floral
DWV	abundance of flowering perennials, abundance of flowering annuals, landscape floral resources/natural habitat within 1 km	not significant	floral
DWV	parasite and pathogen prevalence/infection rate of honey bees	not significant	honey bee
Trypanosome	Bee abundance	increase	community
Trypanosome	Shannon diversity index	not significant	community
Trypanosome	Garden size	decrease	floral

Trypanosome	abundance of flowering perennials, abundance of flowering annuals, landscape floral resources/natural habitat within 1 km	not significant	floral
Trypanosome	parasite and pathogen prevalence/infection rate of honey bees	not significant	honey bee
<b>Chapter 1</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
BQCV	valley	increase	habitat
BQCV	edge	not significant	habitat
BQCV	forest	decrease	natural / forest
DWV	valley	increase	habitat
DWV	edge	not significant	habitat
DWV	forest	decrease	natural/forest
Trypanosome	valley	decrease	habitat
Trypanosome	edge	not significant	habitat
Trypanosome	forests	increase	natural/forest
<i>Vairimorpha spp.</i>	forests	not significant	natural/forest
<i>Vairimorpha spp.</i>	edge, valley	not significant	habitat
<b>Fearon et al 2022</b>			



<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
BQCV	<i>Bombus spp.</i> richness	not significant	community
BQCV	floral density	decrease	floral
BQCV	floral richness	increase	floral
BQCV	landscape richness	not significant	landcover
BQCV	natural habitat	increase	natural/forest
DWV	<i>Bombus spp.</i> richness	decrease	community
DWV	floral richness	increase	floral
DWV	floral density	not significant	floral
DWV	landscape richness	not significant	landcover
DWV	natural habitat	not significant	natural/forest
<b>Figuroa et al 2020</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
Trypanosome	ag cover	Not significant	ag
Trypanosome	network connectedness	decrease	community
Trypanosome	bee species richness, bee abundance, similarity in foraging patterns, module membership, betweenness centrality, similarity in foraging patterns	not significant	community
Trypanosome	<i>B. impatiens</i> diet breadth	decrease	floral

Trypanosome	flower species richness, floral abundance,	not significant	floral
Trypanosome	landscape simplification	decrease	landcover
Trypanosome	site	not significant	site
<i>Vairimorpha spp.</i>	ag cover	not significant	ag
<i>Vairimorpha spp.</i>	bee species richness, module membership, betweenness centrality, similarity in foraging patterns	not significant	community
<i>Vairimorpha spp.</i>	<i>B. impatiens</i> diet breadth	decrease	floral
<i>Vairimorpha spp.</i>	flower species richness	not significant	floral
<i>Vairimorpha spp.</i>	landscape simplification	decrease	landcover
<i>Vairimorpha spp.</i>	network connectedness	decrease	network
<b>Ivers et al 2022</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
Trypanosome	pollinator community composition	not significant	community
Trypanosome	proportion of mulch, proportion of urban cover	increase	developed
Trypanosome	floral resources	not significant	floral
Trypanosome	landscape composition	not significant	landcover
Trypanosome	nesting resources	not significant	nesting
<i>Vairimorpha spp.</i>	pollinator community composition	not significant	community

<i>Vairimorpha</i> spp.	floral resources	not significant	floral
<i>Vairimorpha</i> spp.	landscape composition	not significant	landcover
<i>Vairimorpha</i> spp.	nesting resources	not significant	nesting
<b>Levenson &amp; Tarpay 2022</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
Trypanosome	medium and low flower diversity	increase	floral
Trypanosome	flower cover	not significant	floral
<i>Vairimorpha</i> spp.	<i>B. pensylvanicus</i>	increase	community
<i>Vairimorpha</i> spp.	flower cover, flower diversity	not significant	floral
<i>Vairimorpha</i> spp.	season	not significant	season
<b>McArt et al 2017</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
Trypanosome	cultivated crops, ag, total predicted pesticides, total predicted insecticides, total predicted fungicides, total predicted herbicides, three most used insecticides, fungicides, herbicides	not significant	ag
Trypanosome	spp. composition	increase	community
Trypanosome	human population density	not significant	developed
Trypanosome	elevation	not significant	elevation

Trypanosome	latitude, longitude	not significant	lat/long
Trypanosome	forest, natural, clumpiness of natural habitat	not significant	natural/forest
<i>Vairimorpha spp.</i>	chlorothalonil	increase	ag
<i>Vairimorpha spp.</i>	cultivated crops, ag, total predicted pesticides, total predicted insecticides, total predicted fungicides, total predicted herbicides, three most used insecticides, fungicides, herbicides	not significant	ag
<i>Vairimorpha spp.</i>	human population density	not significant	developed
<i>Vairimorpha spp.</i>	elevation	not significant	elevation
<i>Vairimorpha spp.</i>	latitude	increase	lat/long
<i>Vairimorpha spp.</i>	forest, natural, clumpiness of natural habitat	not significant	natural/forest
<b>McNeil et al 2020</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
BQCV	insecticide index, arable	not significant	ag
BQCV	spp. diversity, Catch per effort	not significant	community
BQCV	developed	increase	developed
BQCV	elevation	not significant	elevation

BQCV	spring floral resources	decrease	floral
BQCV	summer floral index	not significant	floral
BQCV	honey bee colony density	increase	honey bee
BQCV	latitude	decrease	lat/long
BQCV	longitude	increase	lat/long
BQCV	shrubland, natural, grass/pasture, forest	decrease	natural/forest
BQCV	nesting habitat quality	decrease	nesting
BQCV	sum. GDD, spring precipitation, summer precipitation	not significant	weather
DWV	insecticide index, arable	decrease	ag
DWV	spp. diversity, Catch per effort	not significant	community
DWV	elevation	not significant	elevation
DWV	spring floral resources,	decrease	floral
DWV	summer floral index	not significant	floral
DWV	honey bee colony density	increase	honey bee
DWV	longitude	Not significant	lat/long
DWV	latitude	decrease	lat/long
DWV	shrubland, natural, grass/pasture, forest	not significant	natural/forest
DWV	nesting habitat quality	decrease	nesting

DWV	sum. GDD, spring precipitation, summer precipitation	not significant	weather
<i>Vairimorpha spp.</i>	insecticide index, arable	not significant	ag
<i>Vairimorpha spp.</i>	spp. diversity, Catch per effort	not significant	community
<i>Vairimorpha spp.</i>	developed	not significant	developed
<i>Vairimorpha spp.</i>	elevation	not significant	elevation
<i>Vairimorpha spp.</i>	spring floral resources	decrease	floral
<i>Vairimorpha spp.</i>	summer floral index	not significant	floral
<i>Vairimorpha spp.</i>	honey bee colony density	increase	honey bee
<i>Vairimorpha spp.</i>	latitude	not significant	lat/long
<i>Vairimorpha spp.</i>	longitude	not significant	lat/long
<i>Vairimorpha spp.</i>	shrubland, natural, grass/pasture, forest	not significant	natural/forest
<i>Vairimorpha spp.</i>	nesting habitat quality	decrease	nesting
<i>Vairimorpha spp.</i>	sum. GDD, spring precipitation, summer precipitation	increase	weather
<b>Olgun et al 2020</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
BQCV	ag	not significant	ag
BQCV	host spp.	not significant	bee specific variables
BQCV	urban	not significant	developed

BQCV	open	not significant	open
BQCV	season	not significant	season
DWV	ag	not significant	ag
DWV	host spp.	not significant	bee specific variables
DWV	urban	not significant	developed
DWV	open	not significant	open
DWV	season	Not significant	season
<b>Tsvetkov et al 2021</b>			
<b>pathogen</b>	<b>variable</b>	<b>Effect direction</b>	<b>category</b>
BQCV	ag	increase	ag
BQCV	natural	decrease	natural/forest
Trypanosome	ag, pesticides	not significant	ag
Trypanosome	spp. composition	increase	community
Trypanosome	natural	not significant	natural/forest
<i>Vairimorpha spp.</i>	ag, pesticides	not significant	ag
<i>Vairimorpha spp.</i>	natural	not significant	natural/forest

### Chapter 3: Declining bumble bee species diversity over time and in more disturbed landscapes

#### Abstract

Bumble bees are a diverse group of critical pollinators, but many species have been facing population declines over the last 120 years. These declines are often due to habitat loss, climate change, and pathogens, but the impact of each of these factors depends on the sensitivity of the species. By assessing changes between historical and current *Bombus* communities at a landscape scale, the habitat needs of species can be assessed and management recommendations can be made. Using bumble bees collected from 37 sites in 2021 and 31 sites in 2022 across North Carolina, correlations between *Bombus* spp. and landscape factors were assessed. Community composition was also compared to historical data collected over the last 120 years across the whole state. The data support bumble bees utilizing ecoregions depending on habitat needs. Additionally, higher quality sites, or sites with more dense and diverse floral resources, have higher *Bombus* species richness, but not significantly higher overall diversity. Low quality sites had low species richness and were dominated by *B. impatiens*. Comparing percentages of *B. impatiens* over the last 120 years revealed they have steadily increased over time from 30% of collected bees between 1900-1930 to 60% of collected bees in our study, and in turn several fewer common species are no longer observed. Additionally, *B. impatiens* is positively associated with warmer temperature and negatively associated with natural cover, highlighting the disturbance resilient nature of this bee. Improving floral availability and understanding specific species needs are key elements to maintaining sensitive bumble bee populations, however, given the correlations between *B. impatiens* and warm temperatures, mitigating climate change will likely be critical to maintain bumble bee diversity.



## Introduction

There are over 250 species of bumble bees worldwide, which provide ecosystem services through pollination and serve as models for co-evolution and mimicry (Cameron et al., 2007; Fisher et al., 2022; Jordan et al., 2021; Miller-Struttman et al., 2015; Ranta & Lundberg, 1980; Tian et al., 2019). Many of these species are facing declines due to climate change, habitat loss, pathogens, and invasive species (Cameron & Sadd, 2020; Goulson et al., 2008). In North America alone at least half of species are exhibiting declines (Cameron et al., 2011; Colla et al., 2012). The different biotic and abiotic factors contributing to these declines can interact in complex ways, and have different effects on the different bumble bee species (Cameron & Sadd, 2020). Understanding the relative importance of each of these stressors on individual species allows us to critically assess what is driving *Bombus* declines, and to determine how best to support struggling species (Cameron & Sadd, 2020).

Habitat loss and climate change are two of the major drivers of *Bombus* declines, however some species are more susceptible to declines than others. Loss of habitat can lead to reduced food resources and more competition with conspecifics or invasive species (Cameron & Sadd, 2020; Goulson et al., 2008). Broadly, increasing landscapes resources tends to increase overall bumble bee diversity (Hines & Hendrix, 2005; Nicholls & Altieri, 2013). However, while bumble bees are typically generalist pollinators and can forage on a diverse array of flowering plants, many species still have specific habitat and climate needs (Colla, 2016; Liczner & Colla, 2020). While a broader diet breadth appears to buffer species against the loss of floral resources (Bartomeus et al., 2013; Colla & Packer, 2008; Wood et al., 2019), specialization can contribute to potential declines as a species has fewer options for food, habitat, or climatic range, and thus is especially susceptible to changes of those factors (Goulson et al., 2008) (Goulson et al., 2008). For example, in North America, due to climate change, many bumble bee species are no longer able to subsist in the warmer parts of their geographic range, however there is no evidence

that these species are moving farther north thus their ranges are only contracting (Jackson et al., 2022; Kerr et al., 2015; Sirois-Delisle, 2017; Soroye et al., 2020).

To better understand the impacts of habitats on distribution and resilience in bumble bee species, I describe the current and historic state of *Bombus* communities in North Carolina. For this I compare shifts in community composition by comparing recent field collections against historical records and using landscape analyses to understand needs of individual species and how they respond to landscape quality. In eastern North American, regional studies (North East [Jacobson et al., 2018; Richardson et al., 2019], Midwest [Grixti et al., 2009; Hemberger et al., 2021]) have compared historic to recent records to find declines of more rare, socially parasitic (*B. citrinus*, *B. bohemicus*), and three recently declining species (*B. terricola*, *B. affinis*, *B. pensylvanicus*). Bumble bee distributions in the southeast United States has previously not have been assessed at the regional scale, although it has been included in broader nationwide analyses (Cameron et al., 2011; Colla et al., 2012; Guzman et al., 2021). North Carolina is an excellent region to examine influences on changing bumble bee communities, as it has three major ecoregions with significantly different landscapes and climates (NC Department of Natural and Cultural Resources, 2020) and is home to 15 native species of bumble bees (Levenson & Youngsteadt, 2019) that represent most east coast fauna (Strange & Tripodi, 2019). Furthermore, there are ample historic records available given that bee expert Theodore Mitchell (Mitchell, 1960, 1962) actively conducted surveys in the state in the earliest twentieth century. I compare this historic data to new surveys across 37 total sites spanning the ecoregions of North Carolina. These data demonstrate that bumble bee species diversity has significantly declined across North Carolina in the last 60 years and *Bombus impatiens* has become an increasingly dominant species. Moreover, landscape analyses reveal that habitat quality is a factor driving declines in diversity.

## Methods

I collected bumble bees at 37 sites across North Carolina from June 22<sup>nd</sup> to July 17<sup>th</sup>, 2021 and resampled 31 of the same sites June 7<sup>th</sup> to July 7<sup>th</sup>, 2022. These time periods represent a time in bumble bee phenology that includes all species near their peak abundance, typically in worker phases, although some species were producing males. Sites were initially selected to be distributed evenly across the state and represent a variety of habitats and habitat qualities (Figure 11). At each site, I conducted an unlimited-length visual encounter survey for all *Bombus* spp.. Surveys were conducted in low wind speed, above 15.5°C and below 36°C. During each survey, I examined all available flower species for foraging bumble bees, until I captured 20 *Bombus* spp.. Each visual encounter survey consisted of one to two highly trained field researchers searching each site, making a conscious effort to avoid biasing sampling on a single floral species or patch within each habitat. The majority of the *Bombus* spp. collected were identified in the field and released, and those unable to be identified in the field were stored on dry ice and identified in the lab using either morphology or DNA (see Chapter One methods DNA barcoding for specimen identification). Additionally, I recorded the start- and end times at each site also using a stopwatch to keep track of time spent searching in 2022. Bumble bee diversity at a site was calculated using Shannon-diversity index using the vegan package in R (Oksanen et al., 2022). The percent of each species at a site was calculated by dividing the number of each species caught by the total number of bees collected at the site.

In 2021 site quality was assigned using a qualitative score based on a rough field estimate of floral diversity, abundance, and density. In 2022, floral availability at each site was quantitatively assessed using measures of floral species richness, floral abundance, and floral density. During the unlimited-length survey or immediately following the unlimited length survey, one observer would record all species of blooming flowers and estimate the number of flowers of each species within two

meters of the survey transect. During this time the observer responsible for the survey would use a GPS tracking app (Strava, Inc., 2022) to record where the observer surveyed. If the floral survey was done after the bumble bee survey the observer would retrace their steps using the GPS app. The stop watches were stopped while the observer was counting and recording the floral data. The GPS routes were imported into ArcGis pro (ESRI, 2021), where they were buffered by 2 meters and calculated the total floral survey area. The floral density was calculated by dividing the abundance by the total area surveyed. This was done for both the total number of flowers at a site and for each individual species in bloom. Floral diversity was calculated using the Shannon diversity index in the package vegan in R (Oksanen et al., 2022).

In addition, at each site a qualitative overall floral quality score was assigned immediately after the surveys, using the same scale as in 2021. This was based on species richness, abundance, and qualitative density of flowers each separately categorized with a score of one through four. Qualitative scores of density and diversity were calculated after the fact. For floral species richness, a score of four including high numbers of 15 or above blooming floral species, three was 10-20 blooming floral species with moderate number of all blooming flowers, two was 5-15 blooming floral resources dominated by few species, and one was 1-10 blooming floral resources dominated by one or two species. For floral abundance, four was extremely abundant (too many flowers to easily survey them all), three was abundant (almost too many flowers to easily survey them all), two was less abundant (some flowers, and easy to survey all), and one was extremely low abundance (very easy to survey all flowers, need to resurvey some flowers). For floral density, four was extremely high density of blooming floral resources across the entire site, three was large patches of high-density floral resources, two was medium-small patches of medium density floral resources, one was few – small patches of low-density floral resources. The scores of each category were averaged for each site (rounding up above 0.5 and down below 0.5)

and this score is referred to as “habitat quality”; one is low quality, two is medium-low quality, three is medium-high quality, and four is high quality. This score qualitatively describes each site and is referred to as “habitat quality” and provided a coarse habitat quality level for each site to use when comparing pathogen loads across groups, while also being used during our field work to avoid over sampling sites of higher or lower quality.

From the floral data I calculated several other floral interaction variables to help illuminate some of the specific relationships at a site. The number of floral species foraged is a summation of all flower species on which bees were collected on at a site. The percent of floral species foraged at a site was calculated by dividing the number of floral species foraged by the total species richness. Percent clover was calculated by dividing the number of clovers at a site by the total floral abundance. This was assessed given that many bees in more disturbed areas are found in places with mostly just lawn clovers available for forage.

### *Landscape Data*

Landscape characteristics were extracted from various landcover data layers to compare bumble bee diversity and occupancy to the broader landscape. GIS methods and landscape variables followed methods outlined in Chapter Two (methods: landscape data; tables: Table two).

### *Historical data*

Historical data was obtained to compare bumble bee communities over time in North Carolina. Records from the North Carolina State Insect Museum (1321 records) and GBIF (representing mostly other collections’ material, n=335, extracted in 2012) were pared down to represent one record of

each species from each collection event to avoid redundant records. In general, most sites were represented with a single specimen and there were thus few redundancies. As such, these records are used as presence data and relative occurrence, not as an absolute representation of the communities at the time.

### *Analysis*

Correlations between landcover factors and percent *Bombus* species at each site were compared with a Pearson's correlation matrix. Bumble bee diversity index for 2021 and 2022 were compared across ecoregion and habitat quality using ANOVA ( $\alpha = 0.05$ ). Bumble bee diversity index for 2021 and 2022, and proportion *B. impatiens* in the surveyed population were each modeled using mixed-effects models using the lme4 packaging in R (using gaussian link function; Bates et al., 2017). Ecoregion was used as a random effect to account for inherent regional differences in bumble bee communities. Candidate models consisted of single variables, single quadratic variables, and all two non-correlated variables ( $r^2 < |0.7|$ ). AIC model selection was used to determine which model best described our data (Akaike, 1973, 1998; Mazerolle, 2020). I included a null model for model comparison purposes. Any models within two delta AIC were considered equivalent. A variable was considered non-significant if the 95% confidence interval (CI) included zero or if the null model was within 2 delta AIC (Akaike, 1998).

## Results and Discussion

Bumble bee community diversity varied depending on region, habitat quality, and over time. Overall community richness and evenness was not different between ecoregions; however, the community composition did differ (Figure 12a). These differences reflect the habitat requirements of the species that occur in these areas. For example, I found *B. fraternus* and *B. pensylvanicus* in the Piedmont and Coastal Plains which is consistent with their preferences for grassland habitats (Colla, 2016; Novotny et al., 2021; Tripodi & Szalanski, 2015). *B. perplexus*, *B. vagans*, and *B. sandersoni* were found in the Appalachians, reflective of their preferences for forested landscapes (Chapter One; Colla, 2016).

These habitat needs are reflected in the correlations I see between percent of a given *Bombus* species at a site and landscape factors (Figure 13). For example, *B. perplexus*, *B. vagans*, and *B. sandersoni* were consistent between both years and all three species were positively correlated with elevation (*B. perplexus* 2021:  $r = 0.26$ ; 2022:  $r = 0.38$ ; *B. vagans*, 2021:  $r = 0.58$ ; 2022  $r = 0.25$ ; *B. sandersoni*, 2021:  $r = 0.72$ ; 2022:  $r = 0.83$ ) and negatively correlated with growing degree days (*B. perplexus*, 2021:  $r = -0.27$ ; 2022:  $r = -0.40$ ; *B. vagans*, 2021:  $r = -0.54$ ; 2022:  $r = -0.27$ ; *B. sandersoni*, 2021:  $r = -0.61$ ; 2022:  $r = -0.70$ ). Additionally, all three species were positively correlated with percent forest, nesting, spring floral resources, and precipitation; and negatively correlated with development, agriculture, and insecticide. Again, this coincides with the habitat requirements for these species as they are most likely found around forests (Colla et al., 2012; Colla, 2016; Giles & Ascher, 2006).

*B. bimaculatus* was most variable between years, however this could be due to phenology. In 2021, *B. bimaculatus* was positively correlated with spring floral availability ( $r = 0.29$ ), elevation ( $r = 0.23$ ), and nesting resources ( $r = 0.18$ ) and negatively correlated with development ( $r = -0.26$ ), developed open space ( $r = -0.23$ ), and growing degree days ( $r = -0.15$ ). In 2022, *B. bimaculatus* was positively

correlated with latitude ( $r=0.45$ ), elevation ( $r =0.36$ ), forest ( $r =0.29$ ), and nesting ( $r =0.28$ ). Due to the slight difference in survey times between 2021 and 2022 (surveys were conducted earlier in 2022), it is possible that these differences were due to phenology: in 2021 there were 31 *B. bimaculatus* observed and in 2022 there were 79. *B. bimaculatus* are a generally stable species found ubiquitously and often in mixed habitats (Cameron et al., 2011; Colla et al., 2012; Colla, 2016; Novotny et al., 2021), for example in Chapter One *B. bimaculatus* was found across all habitat types but were most common in the edge habitats.

There were few *B. fervidus* caught in both 2021 and 2022, (2021:  $n = 5$ ; 2022  $n=2$ ), at two sites in 2021 and one site in 2022. The inconsistency in the correlations reflect these low numbers. For example, in 2021 *B. fervidus* was negatively correlated with development ( $r = -0.11$ ) and in 2022 it was positively correlated with development ( $r = 0.15$ ). Two of the sites were community parks and one site was a state park; all sites were in the Appalachians. *B. fervidus* is listed as vulnerable on the IUCN red list, can be found in forests or open habitats, and the most well documented threat against this species is habitat loss (Colla et al., 2012; Colla, 2016; Novotny et al., 2021).

*B. fraternus* was slightly more consistent between years (2021:  $n = 28$ ; 2022:  $n = 32$ ). Both years *B. fraternus* was negatively correlated with forest (2021:  $r = -0.34$ ; 2022:  $r = -0.52$ ), and positively correlated with longitude (2021:  $r = 0.46$ ; 2022:  $r =0.61$ ). Additionally in 2022, *B. fraternus* was positively correlated with floral density ( $r = 0.66$ ). My data suggests that the most likely place to find *B. fraternus* are the open, florally dense, the eastern parts of the state. This is consistent with previous recognition of this bee as a grasslands and prairie bee (Tripodi & Szalanski, 2015).

*B. griseocollis* was also consistent between years (2020:  $n = 63$ ; 2022:  $n = 89$ ). Both years *B. griseocollis* was positively correlated with longitude (2021: 0.44; 2022: 0.25), percent natural cover (2021:  $r = 0.69$ ; 2022:  $r = 0.30$ ), and growing degree days (2021:  $r = 0.31$ ; 2022:  $r = 0.38$ ). Both years *B.*



*griseocollis* was negatively correlated with latitude (2021:  $r = -0.28$ ; 2022:  $r = -0.24$ ), forest (2021:  $r = -0.37$ ; 2022:  $r = -0.53$ ), and precipitation (2021:  $r = -0.31$ , 2022:  $r = -0.35$ ). This suggests the *B. griseocollis*, thought to be a mostly stable species found largely in mixed habitats (Cameron et al., 2011; Colla, 2016; Novotny et al., 2021), would be found in the southeastern part of the state in dry hot open natural areas.

In 2021 and 2022 I found 0.03% of bees (2021:  $n = 30$ ; 2022:  $n = 8$ ) were *B. pensylvanicus*. This species was negatively correlated with development (2021:  $r = -0.17$ ; 2022:  $r = -0.19$ ), mean elevation (2021:  $r = -0.19$ ; 2022:  $r = -0.19$ ), and the number of flowers foraged (2021:  $r = -0.21$ ; 2022:  $r = -0.14$ ). In both years they were positively correlated with longitude (2021:  $r = 0.13$ ; 2022:  $r = 0.28$ ) and natural habitats (2021:  $r = 0.19$ ; 2022:  $r = 0.31$ ). In 2021 *B. pensylvanicus* was positively correlated with agriculture ( $r = 0.23$ ) and in 2022 positively correlated with floral diversity ( $r = 0.38$ ). *B. pensylvanicus* has seen significant declines in parts of its range (Grixti et al., 2009). My data suggests *B. pensylvanicus* prefers open areas in the central part of the state and abundant floral resources which is consistent with the known range and needs for this species (Colla, 2016; Colla & Packer, 2008; Novotny et al., 2021). Clear understanding of these needs is critical as *B. pensylvanicus* has seen significant declines and is currently under review for federal listing as endangered (Grixti et al., 2009; MacPhail et al., 2019; Tyler et al., 2021).

There were three species only collected in 2021: *B. auricomus*, *B. citrinus*, and *B. flavidus*. *B. citrinus* and *B. flavidus* are generally uncommon species as they are both socially parasitic bumble bees (Colla & Packer, 2008), and the different sampling periods could also mean having missed the peak phenological window in 2022. Additionally, each of these species were only collected at one site each, thus their correlations reflect the site they were found at. There was only one *B. auricomus* collected at a high elevation, forested site: at Mt. Mitchell state park, the tallest mountain in the Appalachian

Mountain range. In some areas *B. auricomus* is in decline (Wood et al., 2019) while in other areas, populations may be stable or increasing (Colla, 2016; Tripodi & Szalanski, 2015). This species is typically found in open habitats (Colla, 2016) which is contradictory to the bee I collected at Mt. Mitchell who was on forest-edge. Additionally, *B. auricomus* has different phenology depending on region (Tripodi & Szalanski, 2015) and thus our survey windows may have missed peak abundance in North Carolina. There was one *B. flavidus* collected, she was collected on a mountain top; suggesting that these bees can prefer cool, high elevation, forested sites. Additionally, these needs mirror *B. flavidus*' host, *B. perplexus* (Colla, 2016); at the site where the *B. flavidus* was collected, *B. perplexus* represented 25% of the bees collected. There were four *B. citrinus* collected and they were all collected at a state park in the Appalachians thus are all positively correlated with forest. *B. citrinus* are nest parasites often parasitizing *B. bimaculatus*, *B. vagans*, or *B. impatiens* (Colla, 2016), and but over all this species appears to be in decline potentially due to diet specialization (Colla & Packer, 2008). At the site where *B. citrinus* was collected, *B. impatiens* represented 80% of the bees collected at that site in 2021.

Conversely, *B. impatiens* was the most abundant bee found across all sites in both 2021 and 2022 (2021: n = 540; 2022: n = 353). In 2021, *B. impatiens* is positively correlated with developed open space ( $r = 0.27$ ), development ( $r = 0.24$ ), and growing degree days ( $r = 0.11$ ) and negatively correlated with natural cover ( $r = -.34$ ) and spring floral resources ( $r = -0.22$ ). In 2022, *B. impatiens* is positively correlated with growing degree days ( $r = 0.47$ ), percent clover ( $r = 0.24$ ) and developed open space ( $r = 0.12$ ). *B. impatiens* is negatively correlated with elevation ( $r = -0.46$ ), precipitation ( $r = -0.17$ ), and nesting resources ( $r = -0.15$ ). *B. impatiens* has a broad diet breadth as well as a long flight season, which could both explain their persistence in developed areas, which has also been observed in other studies (Giles & Ascher, 2006; Novotny et al., 2021; Tripodi & Szalanski, 2015; Wood et al., 2019).

Habitat quality differs not only in community composition but also in richness and evenness (Figure 12a). In my study, habitat quality was scored according to floral density, diversity, and richness. The low-quality sites are overall dominated by *B. impatiens*, and I only found four species total in any of these sites. This supports repeated claims that *B. impatiens* is a disturbance resilient bee, making it an excellent candidate for invasion (Ratti & Colla, 2010), commercialization, as well as persistence in rapidly changing landscapes (Colla, 2016). In contrast, the highest quality sites are less than 50% occupied by *B. impatiens* and have the highest diversity of other *Bombus* species. This suggests that floral availability (as defined in this study using richness, density, and abundance), plays a role in the presence and absence of less resilient *Bombus* spp.. Floral availability is key to maintaining native pollinator populations (Goulson et al., 2008), but this score does not necessarily consider other disturbances on the landscape such as pesticides or development which could play a role in species presence (Goulson et al., 2015). However, when comparing habitat quality using overall Shannon-diversity of each site and ANOVA, the diversity indices are not statistically different from each other (Figure 14). Additionally, there were no significant models for 2022 or 2021 *Bombus* Shannon diversity index.

Comparing community composition across North Carolina from the 1900s to 2022, there is an overall increase in proportion of *B. impatiens* and a loss of several *Bombus* species (Figure 12b), which could be explained by *B. impatiens* resiliency to disturbance at warmer temperatures. This is consistent with other findings arguing that *B. impatiens* is increasing across the east coast (Cameron et al., 2011; Novotny et al., 2021; Ratti & Colla, 2010; Tripodi & Szalanski, 2015). Additionally, the top model describing proportion of *B. impatiens* in a population was Growing Degree Days (Figure 15; Appendix Table 1). There are several competing models describing proportion of *B. impatiens* at a site: elevation and percent natural cover both have a negative effect on proportion of *B. impatiens* at a site. Elevation

and temperature are highly correlated and thus are never in the same model. However, the strong negative effect of natural cover and positive effect of temperature again suggests that *B. impatiens* is particularly resilient to increases in temperature and land use changes even over time. As I used percent *B. impatiens* within a population as the response variable, this suggests that other species are less tolerant of high temperatures and disturbance. Additionally, this is consistent with overall climate changes in North Carolina. According to the North Carolina Climate Science Report average temperatures have increased by 0.56°C since 1895, which, while less than the global average of 1°C, mostly happened between 2008-2018 (Kunkel et al., 2020). 2019 is still the hottest year on record (Kunkel et al., 2020). Despite sampling 1,349 bees across all sites in 2021 and 2022, compared to 1,160 historic samples, I found no representatives of *B. affinis*, *B. frigidus*, *B. terricola*, and *B. variabilis*. Some of these species were historically rare and appear to be very rare or perhaps extirpated from the region today.

## Conclusions

Overall, these studies provide valuable information about the habitat needs of ten bumble bee species in the Southeastern United States. Clearly, some species (*B. pensylvanicus* and *B. fraternus*) prefer grasslands while others (*B. perplexus*, *B. vagans*, *B. sandersoni*) prefer forested habitats. Additionally, some species prefer high elevation versus low elevation conditions, likely reflecting preferences in temperature conditions. Additionally, within a particular habitat type, floral availability at a site can contribute to increases in overall species diversity and evenness. Thus, improving floral availability at the local scale can influence the broader *Bombus* population. Additionally, where other species are not occurring, whether because of floral resources, temperature, or time, *B. impatiens* is a

larger percentage of *Bombus* populations. This does not mean that overall populations of this species are increasing as our data cannot make claims about abundance. However, it does mean that there is a loss of *Bombus* biodiversity which likely weakens ecosystem function (Oliver et al., 2015). Indeed, when comparison data collected in 1900 versus 2021 and 2022, the relative proportion of *Bombus impatiens* found in North Carolina increased from 30% to more than 60%, and the total number of *Bombus* species identified decreased from 16 to ten. While improving floral availability overall may support biodiversity, the *B. impatiens* relationship with growing degree days is especially concerning. It suggests that in North Carolina, climate change mitigation could make a large impact on the ability for other *Bombus* species to persist.

### Figures

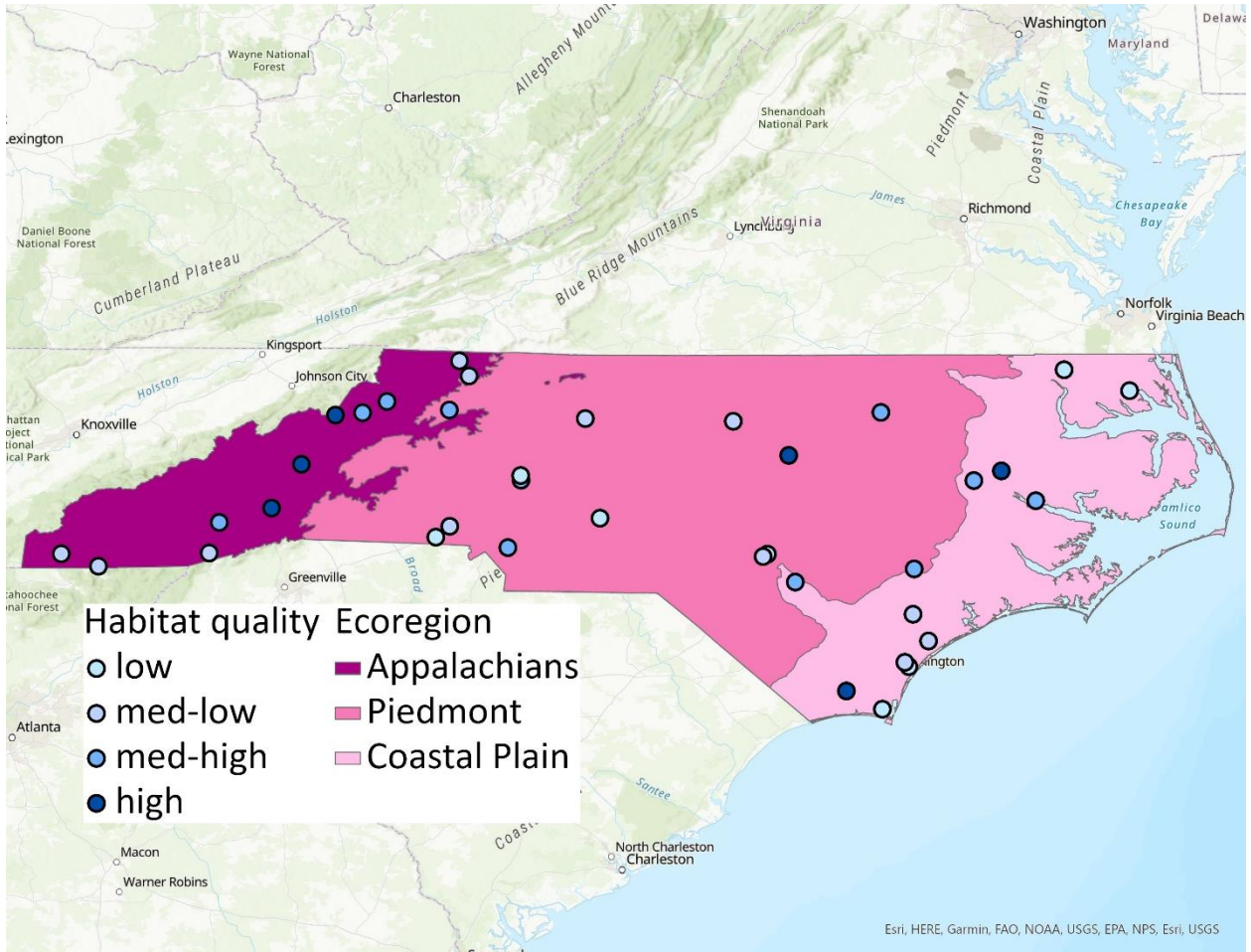


Figure 11: Map of surveyed sites across North Carolina colored by habitat quality.

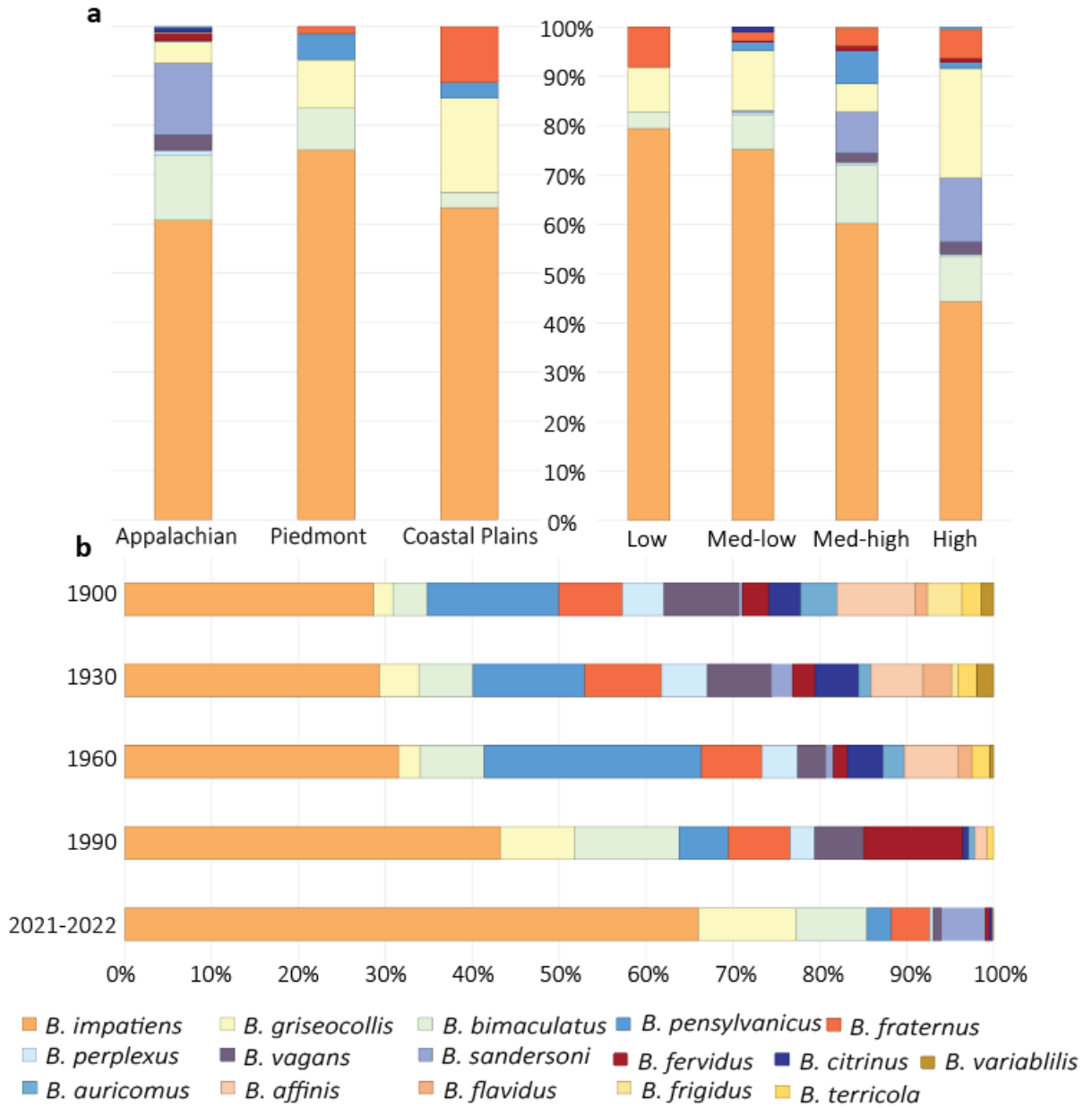


Figure 12: a) All bees at sites in each ecoregion and all bees at sites for each habitat quality for both 2021 and 2022. b) Bumble bees collected across North Carolina from 1900 to through 2022.

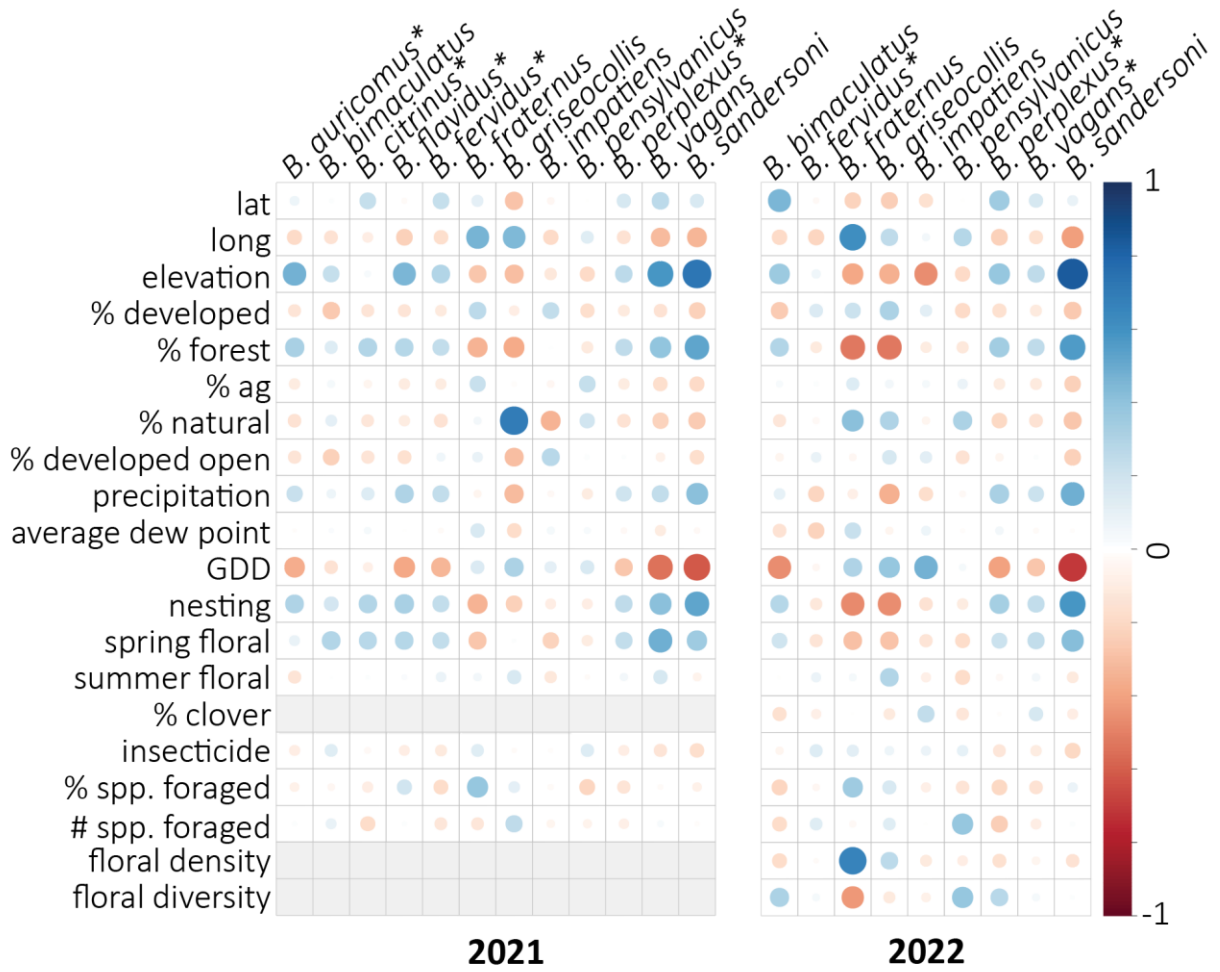


Figure 13: Correlation matrix describing correlations between percent of species at site and various landscape factors in 2021 and 2022. Asterisks indicates five or fewer bees collected.



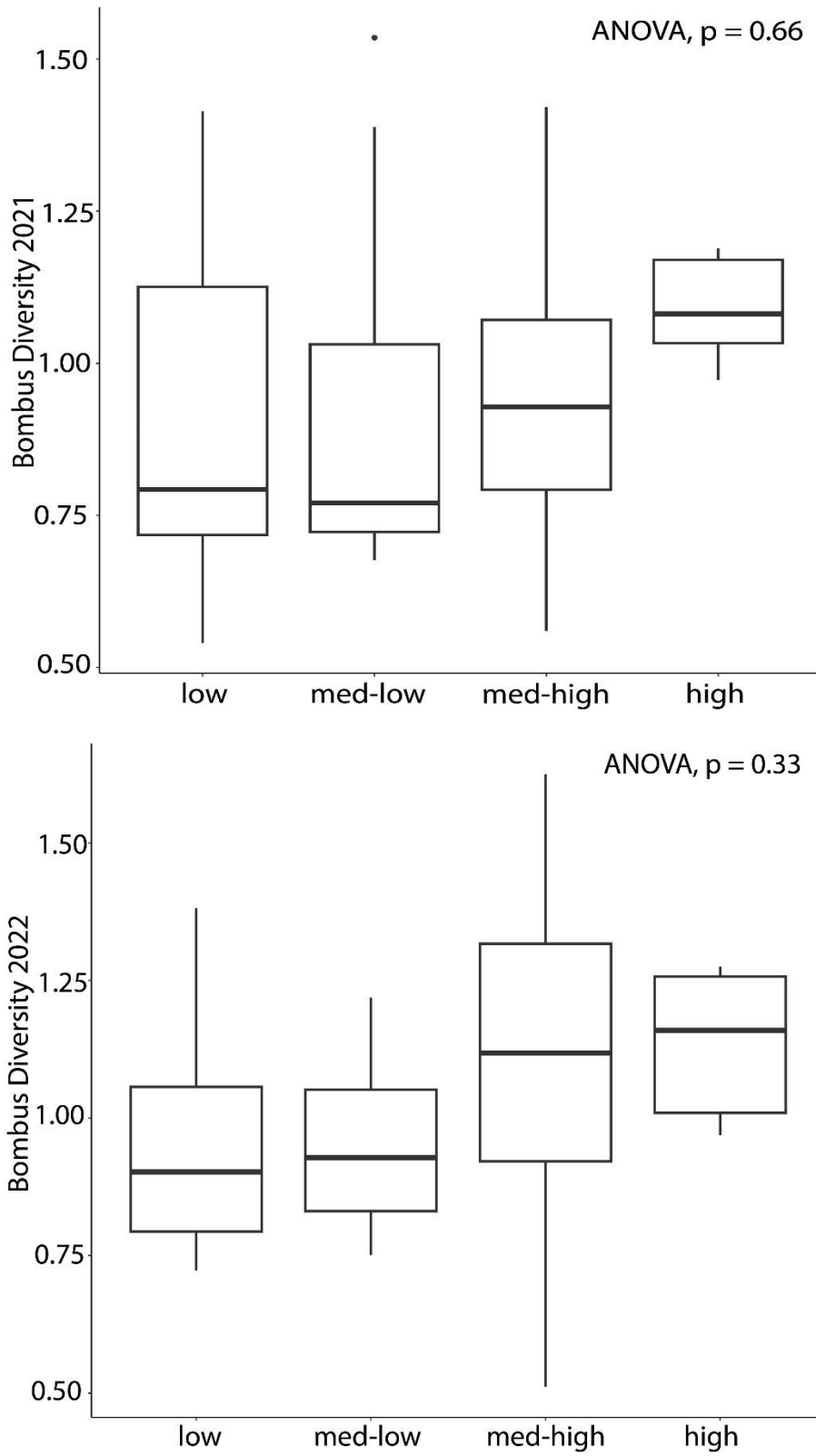


Figure 14: Comparison of Shannon diversity index compared across habitat quality.

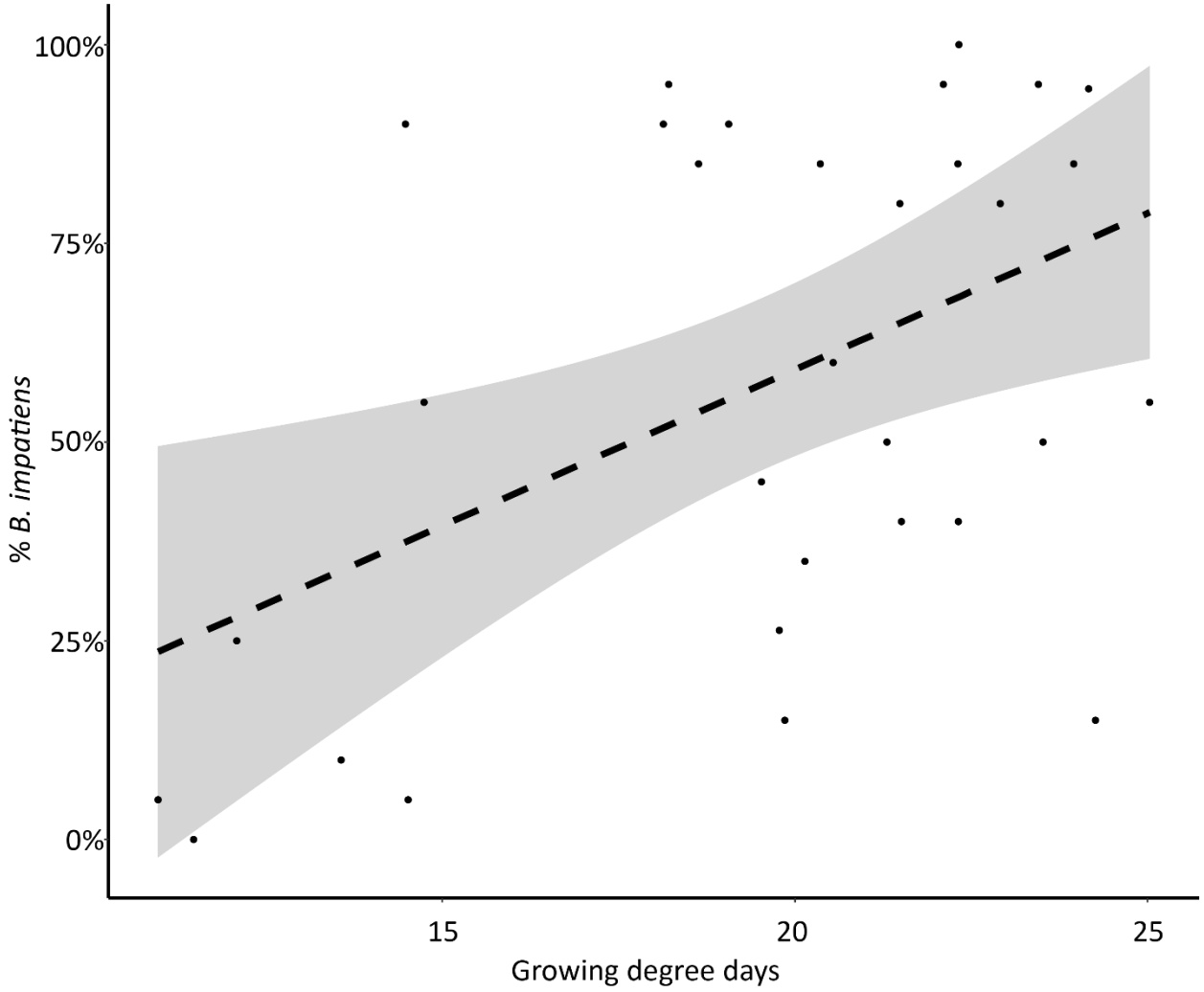


Figure 15: Growing degree days was the top model predicting percent *B. impatiens* at a site.

## Tables

Table 5: AIC model selection table for *B. impatiens*. All competitive models with a  $\Delta AIC$  of less than 2 when compared to the model with the lowest AIC are shown. Significant models are indicated with an asterisk.

Model	$\Delta AIC$	ModelLik	w	variable	beta	lower CI	upper CI
gdd *	0.00	1.00	0.08	gdd	0.50	0.16	0.89
elevation*	0.13	0.94	0.08	elevation	-0.57	-0.96	-0.14
natural + gdd*	0.62	0.73	0.06	natural	-0.35	-0.69	-0.02
gdd				0.64	0.31	0.99	
elevation + natural*	0.95	0.62	0.05	elevation	-0.64	-0.99	-0.30
natural				-0.37	-0.71	-0.02	
gdd + honey bee	1.05	0.59	0.05	gdd	0.51	0.20	0.82
honey bees				0.48	-0.12	1.09	
elevation ^2	1.47	0.48	0.04	elevation^2	-0.33	-0.66	0.00
longitude + gdd	1.48	0.48	0.04	long	-0.33	-0.70	0.04
gdd				0.67	0.30	1.04	
gdd + % clover	1.52	0.47	0.04	gdd	0.53	0.20	0.89
% clover				0.28	-0.02	0.59	
gdd ^2	1.73	0.42	0.03	gdd^2	-0.29	-0.61	0.03
forest + gdd	1.87	0.39	0.03	forest	0.31	-0.08	0.70
gdd				0.67	0.28	1.06	

## Conclusion

Supporting healthy bumble bee populations requires a broad understanding of the complex interactions between communities, landscapes, and pathogens. This work aims to provide context and data to conserve biodiverse *Bombus* communities, to reduce pathogen prevalence, and mitigate the effects of pathogens. Using the washboard landscape of central Pennsylvania and the varied habitats in North Carolina, I quantified the relative roles of landscape types, resource quality, and climate on bumble bee pathogens and communities. As bumble bees are critical pollinators, this work is key to understanding how best to support declining populations. These data, along with data from the literature review, enables a consolidated perspective on large-scale patterns in bumble bee pathogens across North America. This work highlights the gaps in the knowledge about bumble bee pathogens and how they are influenced by human activity. Ultimately this work should be used to make management decisions regarding bumble bee and broader pollinator health.

In chapter one I found distinct partitioning in pathogens and bumble bee species by habitats. The valleys were dominated by *Bombus impatiens* while the forests had a higher proportion of *B. sandersoni*, *B. vagans*, and *B. perplexus*. Additionally, Black Queen Cell Virus (BQCV) loads were highest in the developed valley sites than in more natural forests, a result that matched a previous study on bumble bee pathogens across Pennsylvania. This supported the hypothesis that disturbed and lower quality habitat fosters sicker bees. However, while I did not find major differences in Deformed Wing Virus (DWV) and *Vairimorpha* levels between our sites, I did find higher *Crithidia bombi* loads in the forest sites. While this was contrary to what I initially expected, this could be due to the condition-dependent virulence that other studies have observed with *C. bombi*: high quality habitats foster more *C. bombi* because the bees are less stressed and thus can survive higher loads. It may also be due to differing communities in these habitats and the different levels of these pathogens that they harbor, as different

bumble bee species are known to vary in their sensitivity to *Crithidia* infections. Given the local context of this study, I was able to demonstrate that both bees and pathogens partition at a local scale.

Understanding the differences between habitats provide a baseline for any future conservation efforts.

In chapter two I demonstrated patterns of varying strength and consistency in pathogen prevalence and load when comparing across landscapes in North Carolina, Pennsylvania, and other studies in North America. Across North Carolina, I detected very low prevalence of DWV and no *Vairimorpha bombi* in the bumble bee samples, which was consistent across many studies. However, I found BQCV loads were positively correlated with a summer floral availability index, which, while initially opposite expectations, was consistent with the varied effects of floral availability found in the literature. Some measures of floral availability, for example floral density, are associated with decreased BQCV prevalence, while other measures of floral availability, for example floral richness, are associated with increased prevalence. *C. bombi* levels were not reliably described by any landscape or site variables, however, throughout the literature and to some degree in both Pennsylvania and North Carolina, *C. bombi* is influenced by the extended pollinator community. Most consistently in our study and others, honey bees and habitat disturbance, resulting from development and agriculture, are associated with increased pathogen loads. In our study there is evidence that honey bee infection levels are reduced in areas with high floral availability and natural habitat, suggesting that landscape management practices can reduce managed honey bee pathogen loads and thereby mitigate spillover to wild bumble bees.

In Chapter Three I described key habitat preferences of *Bombus* species in North Carolina and highlighted loss of diversity over time. *Bombus* communities differed across ecoregions based on the habitat needs, which were also consistent with the correlations seen across the landscape. For example, *B. pensylvanicus* was found mostly in the Coastal Plains and Piedmont and was correlated with

agriculture, which reflects its preference for grasslands. Additionally, species richness was higher in habitats of higher quality suggesting that floral availability can increase *Bombus* species richness. In more disturbed, lower quality habitats, there is a significantly higher proportion of *B. impatiens*. *B. impatiens* is considered a disturbance resilient bee, which was further supported with the modeling results which showed a negative correlation with natural cover and a positive correlation with growing degree days. This coincides with a significant increase in proportion of *B. impatiens* over the last 120 years in North Carolina and the loss of several species, suggesting that *B. impatiens* is particularly resilient to the changes occurring in the state, while other *Bombus* species are disappearing. This suggests that while floral availability can improve species richness, addressing climate change could be critical to maintaining overall species diversity.

These studies combined revealed two understudied aspects of wild bee pathogen management: the importance of community composition and the importance of scale. Our results indicate that *Vairimorpha* and *Crithidia* infections vary across large spatial scales and can be influenced by historical land use and climate. This explains the significant variation between regions in not only Pennsylvania and North Carolina, but the rest of the continent as well. However, BQCV, DWV, and *Crithidia* all seem to have some relationships with habitats and landscape factors at smaller scales. This could be associated with differences in bee communities between habitats. Bee community explains significant variation in our data as well as the broader literature: as not all bumble bee species are equally susceptible to pathogen transmission, they differentially influence the pathogens within their community. Additionally, we may be able to see potential influences of the broader pollinator community, for example honey bees, again can vary at a smaller scale.

From this thesis I have several recommendations for future pathogen-landscape work and conservation measures. First, I recommend focusing studies on those pathogens that are best suited for

the question at hand. For example, in a classical disease ecology framework, *C. bombi* levels are highly influenced by the structure and community and could serve as an excellent model for examining disease transmission questions like the relationship between mechanical and viral vectors. However, as *C. bombi*, and other pathogens like DWV, are not serious immediate threats, they may not require interventions to control and are less of a conservation concern. However, it should be noted that such pathogens may still have a negative effect on health and fitness even if not highly prevalent or lethal. Focus on more lethal pathogens and less understood pathogens, such as *Vairimorpha bombi* and potentially *Vairimorpha apis*, would likely serve as a better step to reducing major threats to bumble bee communities in North America. Second, understanding of the interactions between bumble bees and their disease vectors, including honey bees, would also help in not only understanding transmission of pathogens, but also determining methods for mitigating spillover. In the case of BQCV, from my studies and others it is clear that there is spillover from managed honey bees to wild bees, but my studies also indicate that landscape conditions can reduce levels in managed bees: thus, strategies to better manage landscapes to reduce viral loads in honey bees may help mitigate spillover effects. Third, given that *B. impatiens* and other stable *Bombus spp.* carry low loads of *V. bombi* across multiple studies and shows no clear patterns of declines in relation to pathogens, using more susceptible *Bombus spp.*, such as *B. pensylvanicus*, to study *V. bombi* levels would lead to a better understanding of the dynamics of the pathogen. Furthermore, to make inferences on transmission we need more understanding of how species in communities differ in their susceptibility to pathogens. Lastly, given the few large-scale multi-region landscape pathogen studies, a meta-analysis combining all smaller scale landscape pathogen projects with the large-scale pathogen projects would be beneficial to understanding continental-scale pathogen patterns that could ultimately lead to better conservation recommendations, while simultaneously, state-by-state research can provide a strong base to address specific regional needs.

In terms of land management recommendations, we are learning that landscapes are complex and thus can influence pathogen loads in unexpected ways that do not always generate strong single-factor effects. In general, however, we see that the presence of managed honey bees increases loads in bumble bees, that more developed open areas are associated with higher viral loads, and that pathogens can vary by habitat type in their transmissibility likely due to which members of the community support and transmit infections. The easiest factor to manage is quality of floral resources for bees. My data shows that higher quality resources can sometimes facilitate transmission, but they can support higher bee diversity (e.g., Figure 12a), and healthier bees that then harbor lower pathogen levels (e.g., honey bee pathogen loads). Land management for increased floral availability and reduced disturbance from agriculture or development, i.e., protected natural areas, should thus support healthy pollinator populations overall.

Bumble bees are a significant contributor to native ecosystems and agricultural production. While there are many causes for bumble bee population declines, and pathogens interact with many of these factors, and thus serve as an important target for conservation and as an indicator of other stressors. This research focused on providing context to patterns of pathogen prevalence found in bumble bees across North America, ultimately highlighting the future needs for research and conservation if we are to maintain and expand healthy and diverse bumble bee communities.



**Appendix: Assessing Factors that Explain the Persistence of Crickets and Katydid within Intensive Agricultural Landscapes: The Relative Roles of Pesticide Exposure and Microhabitat Characteristics**

**Authors:**

Elena M. Gratton, Darin J. McNeil Jr., and Christina M. Grozinger

**Abstract:**

Habitat loss and degradation are primary drivers of insect population declines. In the United States, agricultural intensification has resulted in direct habitat loss (for species intolerant of agriculture) as well as habitat degradation due to pesticide applications. Although insecticides have the clear potential to impact invertebrates at small scales, relatively little is known regarding how broad-scale patterns of pesticide use impact insect species occurrence patterns. Here, I examined occurrence patterns for a sensitive group of insects (crickets and katydids; Orthoptera: Ensifera) across several agricultural valleys spanning gradients of pesticide toxicity. Specifically, I conducted 180 aural point count surveys for Ensifera from 7 – 27 September, 2020 in central Pennsylvania. Species occurrence models indicated that taxa differed in their habitat associations in ways consistent with life history characteristics. Of the six species I modeled, three were associated with more ‘suburban’ settings (*i.e.*, high impervious cover, *e.g.*, *Microcentrum rhombifolium*) while the other three showed the opposite pattern, being most likely to occur where habitat was dominated by row-crop fields and pastures (*e.g.*, *Neoconocephalus ensiger*). Only one species, the canopy-dwelling *Pterophylla camellifolia* was clearly impacted by insecticide toxic load. Collectively, our results provide novel insights into how night-singing Orthoptera partition structure within novel habitat types (agricultural valleys) and are impacted by pesticide loads.

**Implications for insect conservation.** Although agricultural landscapes can provide habitat for *Ensifera*, the presence of certain habitat features coupled with insecticide stressors can play a major role in shaping these communities.

## Introduction

As keystone organisms in many ecosystems, insects perform a variety of essential ecological functions like pollination (Gallai et al., 2009; Klein et al., 2007), supporting complex food webs (Sabo & Power, 2002; Sipura, 1999), and decomposition (A’Bear et al., 2013). With that in mind, the drastic population declines reported for many taxa (Dirzo et al., 2014; Martin et al., 2018) are drawing widespread concern among conservation biologists (Wagner, 2020). Indeed, population declines have been reported in a wide variety of insect taxa, from bumble bees (*Bombus* spp.; Cameron et al., 2011; Goulson et al., 2005) to spittlebugs (Hemiptera; Karban & Huntzinger, 2018) and beyond, ultimately including at least 33% of described insect species (Dirzo et al., 2014). The drivers behind these declines are varied, however, some of the most important threats include climate change, increased pesticide use/toxicity, habitat loss/degradation, invasive species, and emerging infectious diseases (Dirzo et al., 2014; Douglas et al., 2022; Forister et al., 2019; Sánchez-Bayo & Wyckhuys, 2019). For example, exotic insects (especially honey bees, *Apis mellifera*) imported into agricultural areas can introduce novel pathogens to naive-native insects (McNeil et al., 2020; Vilcinskas, 2019). Climate change has been documented as a major driver behind the declines of a variety of insect species in the Neotropics (Janzen & Hallwachs, 2019) and beyond (Stange & Ayres, 2010). However, habitat loss/degradation remains one of the most pervasive threats to insect populations across the globe (Didham et al., 2020; Lewis et al., 2020), especially in the form of agricultural intensification (Wagner, 2020).

The conversion of natural/semi-natural ecosystems into intensive agriculture has occurred over many regions across the world (Matson et al., 1997; Potts et al., 2016). The effects of agricultural intensification on insect populations are stark (Raven & Wagner, 2021; Tschardt et al., 2005). In the United States, approximately 46% of land that was once natural/semi-natural cover has now been converted to intensive agriculture (Bigelow & Borchers, 2012). Agricultural intensification poses a threat to insects through a variety of mechanisms. For example, while heterogeneous “mosaic” landscapes with semi-natural components (fencerows, woodlots, etc.) allow the persistence of some beneficial insect populations, landscapes dominated by intensive agriculture largely lack the essential habitat elements necessary for the persistence of some important insect species (Cardoso et al., 2020; Sonoda et al., 2011). Moreover, even when agricultural landscapes contain natural/semi-natural aspects, they may act as population sinks due to the increasingly heightened quantity and toxicity of pesticides applied across many such regions (Douglas et al., 2021; Main et al., 2020; Raven & Wagner, 2021). Still, in other contexts, agriculturally intensive landscapes may provide high quality habitat for certain groups of native insects, highlighting the complexity of this issue (Burkle et al., 2017; Evans et al., 2018).

Although agricultural intensification can impact insect populations via habitat loss, another agent that stresses insect populations are insecticides (Sánchez-Bayo and Wyckhuys 2019). In the United States, the area of agricultural land treated with insecticides has increased dramatically since the 1990s and the types of insecticides being applied are also more toxic (Douglas et al., 2022). In addition to growing toxicity, pesticides are increasingly applied using a multitude of methods spanning from soil treatments to aerosolized sprays (Jeschke et al., 2011), thus impacting the potential for a wide variety of organisms to be affected across disparate substrates. Neonicotinoids, a particularly toxic class of insecticide, have become increasingly popular due to their high water solubility which allows them to be

sprayed on soil and incorporated into plant tissues via root absorption (Douglas et al., 2022; Jeschke et al., 2011). Neonicotinoids can persist in soils anywhere from 90 to 6,391 days (>17 years) depending on the specific compound (Hussain et al., 2016). Additionally, these pesticides can be found beyond agricultural areas within runoff and nontarget plants surrounding an agricultural field (Bonmatin et al., 2015). As with many pesticides, neonicotinoids can have a variety of lethal (Kwon, 2008; Parli et al., 2020; Rundlöf et al., 2015) and sub-lethal effects (Martinou et al., 2014; Neuman-Lee et al., 2013; Parli et al., 2020).

Among the most sensitive insect taxa are Orthopterans (grasshoppers, crickets, and katydids), an insect group that has seen some of the most dramatic declines across the world (Dirzo et al., 2014). Their sensitivity to environmental degradation means that Orthoptera may be useful as indicator species (Fartmann et al., 2012), potentially serving as ‘indicators’ of habitat quality for a broad suite of taxa (Landres et al., 1988). Indeed, a variety of life-history characteristics may cause Orthoptera to be especially vulnerable to the effects of insecticides including that many lay their eggs in the soil or live plant tissues where many pesticides are applied (Borror et al., 1989; Gardiner et al., 2005) and their folivorous diets, thus facilitating direct ingestion of potentially toxic plant tissues (Borror et al., 1989). While some are considered agricultural pests (Curry, 1993), Orthoptera are significant contributors to ecosystems as they are important prey for vertebrates like birds and bats (Rodríguez & Bustamante, 2008; Tryjanowski et al., 2003). Despite the value of Orthopterans as indicator species and members of healthy ecosystems (Fartmann et al 2012, Dirizo 2014, Raven and Wagner 2021), most species have been poorly studied and their responses to environmental stressors like insecticides remain largely anecdotal (Gwynne, 2001; McNeil & Grozinger, 2020; Shapiro, 1998). This may be because they are generally difficult to study, often requiring intensive field sampling with nets or expensive recording equipment to document stridulations (Gardiner et al., 2005; Gibb et al., 2019). However, the

development of new aural survey methods for North American Ensifera (long-horned Orthoptera) can make rapid sampling across habitats and landscapes more efficient (Mankin et al., 2011; McNeil & Grozinger, 2020; Penone et al., 2013).

Given the threats presented to native insects within landscapes dominated by agriculture, understanding the factors that allow sensitive insects like Ensifera to persist therein is important for the long-term conservation of these populations across the eastern U.S. (Raven & Wagner, 2021). A recent study demonstrated that many Ensifera species in the eastern United States are apparently abundant in agricultural landscapes (McNeil & Grozinger, 2020), suggesting that such places may potentially serve as a focus for the conservation of Orthoptera biodiversity (Benton et al., 2002; Norris, 2008). To better understand how insecticide exposure might impact native Orthoptera populations in agriculturally intensive landscapes, I quantified the presence of cricket and katydid species across several agricultural valleys in central Pennsylvania that contained site-specific variation in insecticide toxic load and microhabitat characteristics. More specifically, I developed a study with the following objectives: 1. Assess the impacts of insecticide toxic load on species presence within intensive agricultural landscapes and 2. Quantify the role of microhabitat conditions on species presence within these landscapes. I discuss these results in the context of insect conservation (Orthoptera and beyond) as well as how species life history traits interact with pesticide threats and habitat characteristics.

## **Methods**

### *Study area and site selection*

I focused our sampling to six agricultural valleys within the Ridge-and-Valley region of central Pennsylvania (Appendix Figure 1). Valleys in this area provide the ideal setting for assessing the role of

pesticides and other characteristics on insect biodiversity because the region's rolling ridges individually isolate the low-elevation, agricultural valleys (Cuff, 1989). Moreover, these agricultural valleys exhibit local variation in insecticide toxic load (within- and among valleys), based on the agricultural crops present, as estimated in previous studies (Douglas et al 2020). Each valley was chosen based on its predicted insecticide toxic load-gradient (average oral/contact median lethal dose [LD50] for a model insect, *Apis mellifera*; Douglas et al 2020) within the valley (mean LD50: 0.33 within 100 m, SD: 0.13; Appendix Figure 1; see below for details on how insecticide toxic load was calculated). To select sampling locations within each of the six valleys, I drew the longest straight-line transect possible from one end of the valley to the other. Next, along this transect, I plotted 10 equidistant points that maximized the distance between the nearest neighboring points for a total sample of 60 survey locations. I then selected sites on roads closest to the equidistant-line points that could safely be surveyed by a human observer (i.e., parking lots next to the road or pull-offs; Appendix Figure 1). All sites were at least 1.6km apart from the nearest neighboring point.

### *Point count surveys*

To quantify Ensifera species present at each of the 60 survey locations, I conducted aural point counts as described in McNeil and Grozinger (2020). In short, a single observer stood in each location for three minutes and recorded the presence of each species detected; I also noted whether the closest individual was within/beyond 100m of the survey location. Surveys were conducted from the 7<sup>th</sup> to 27<sup>th</sup> September, between 20:00-23:59, and each site was visited three times over this period. At each point, I recorded the presence of the following crickets: Allard's Ground Cricket (*Allonemobius allardi*, Alexander and Thomas 1959), Striped Ground Cricket (*A. fasciatus*, De Geer 1773), Carolina Ground

Cricket (*Eunemobius carolinus*, Scudder 1877), Fall Field Cricket (*Gryllus pennsylvanicus*, Burmeister 1838), and Jumping Bush Cricket (*Hapithus saltator*, Uhler 1864). Additionally, I recorded detections for the following katydids: Greater Angle-wing (*Microcentrum rhombifolium*, Saussure 1859), Nebraska Conehead (*Neoconocephalus nebrascensis*, Bruner 1981), Round-tipped Conehead (*N. retusus*, Scudder 1878), Sword-bearing Conehead (*N. ensiger*, Harris 1841) and Common True Katydid (*Pterophylla camellifolia*, Fabricius 1775). Immediately prior to conducting each point count, I measured temperature (with a handheld thermometer), percent cloud cover (estimated to the nearest 25%), wind (Beaufort scale), and index of noise (“0” = no noise through “3” = noise severely impacting the ability to hear). To maximize detection probability, I avoided sampling in high winds (wind index 4-5), low temperatures (less than 15°C), and when noise severely impacted ability to hear (noise index 3). Surveying for Ensifera under these conditions promotes high detection probability of nearly 1.0 when coupled with repeated visits as I have here (McNeil and Grozinger 2020).

### *Landscape variables*

Using the raster package (Hijmans et al., 2022) in program R version 3.6.178,79 (R Core Team, 2022), I extracted remote-sensed habitat characteristics from two data sources: the Cropland Data Layer (CDL; Homer et al., 2012) and Chesapeake Bay Watershed land cover database (CBW; Allenby & Phelan, 2012; Appendix Table 1). From the CBW database, I extracted the following variables as percent cover within 100m around each point: percent forest, percent trees over turf, percent canopy (trees over turf plus forest), percent agriculture, and percent turf grass. From the CDL, I extracted percent developed (open, low, and medium, plus all combined), and combined forest plus wetland plus barren plus grassland/pasture into a ‘percent natural’ cover. Finally, from the CDL, I extracted all

individual crops from 2020 as well as all combined (percent row crops; Appendix Table 1). To measure distance to the nearest building I used the Google Maps measure tool to calculate the Euclidean distance between each point and the nearest visible building.

To estimate the insecticide toxic load at each point location, I followed the protocol developed by Douglas et al. (2022) to calculate the agricultural insecticide loading. Specifically, I obtained a 2020 crop reclassification table for the state of Pennsylvania and used it to reclassify the USDA Cropland Data Layer (Homer et al., 2012) with a distance-weighting procedure to more heavily weight insecticide application near our study sites (McNeil et al. 2020). This gave us crop-specific toxic loads applied to year-specific crop plantings across each of our agricultural valleys from which I generated a raster map of insecticide load across our study area (Appendix Figure 1).

### *Statistical analysis*

Using cumulative detections from each site summed over three visits, I calculated naïve occupancy for each species observed at each site. Because cumulative detection probability summed across three visits for these species approaches 1.0, I interpreted cumulative naïve occupancy as ‘occurrence probability’ (McNeil & Grozinger, 2020). I modeled habitat relationships for species detected with naïve occupancy between 0.4 - 0.85 because very common species (naïve ~ 1.0) and rare species (naïve < 0.4) exhibit limited variation for which patterns could be modeled. For this subset of species, I modeled naïve occupancy as our response variable using simple logistic regression in R. Specifically, for each species, I used a set of nine variables that I selected as being the most ecologically relevant for our study: insecticide toxic load (LD50 per hectare), percent row crop, canopy cover (binary; 1/0), percent turf grass, percent agriculture, percent developed, distance to nearest building (m),



percent corn, and percent ‘natural’. Using these nine variables, I specified univariate models for each covariate as well as an intercept-only “null” model against which to compare these univariate models. I assessed the relative value of each model using Akaike’s Information Criterion adjusted for small sample size ( $AIC_c$ ; Akaike, 1998; Burnham & Anderson, 2004). Any models within 2.0  $AIC_c$  ( $\Delta AIC_c$ ) were considered competing, and I considered variables with  $\beta$  parameter 95% confidence intervals including zero to be weak biological effects (K. P. Burnham & Anderson, 2004). Finally, prior to analyses, I created a correlation matrix to check for correlated variables ( $|r| > 0.7$ ; Sokal & Rohlf, 1969).

## Results

From 7-27 September 2020, I conducted 180-point count surveys on 24 nights across our six focal valleys. Sampling locations were largely a mix of row crops (mean cover = 40% at 100m radius, range: 0-85%, SD: 22%) and mowed turf grass (mean = 26%, range: 7-53%, SD: 13%). Sites were also usually not far from buildings (mean = 105m, range: 2-493m, SD: 99m) and primarily characterized by sparse canopy cover (mean = 9%, range: 0-65%, SD: 13%). Insecticide toxic load averaged 0.33  $LD_{50}/ha$  (SD: 0.13  $LD_{50}/ha$ ) but ranged between 0.11 to 0.70  $LD_{50}/ha$ .

During our surveys, I recorded all 10 target species at least once. Several species were too common to model because they were found at every (or nearly every) site, including Fall Field Cricket (naïve occupancy: 1.00), Allard’s Ground Cricket (naïve occupancy: 0.90), Round-tipped Conehead (naïve occupancy: 0.90). In contrast, Nebraska Conehead was too rare to model (naïve occupancy: 0.08). The remaining species, Carolina Ground Cricket, Greater Angle-wing, Jumping Bush Cricket, Striped Ground Cricket, Sword-bearing Conehead, and Common True Katydid all had naïve occupancy rates from 0.40 - 0.85 which allowed for model convergence (Carolina Ground Cricket: 0.80, Greater Angle-

wing: 0.82, Jumping Bush Cricket: 0.70, Striped Ground Cricket: 0.40, Sword-bearing Conehead: 0.48, Common True Katydid: 0.77).

Our models suggested that species occurrence patterns were often driven by aspects of agricultural cover, especially for the three cricket species. For example, percent row crop was within the competing models for Greater Angle-wing (negative;  $\beta = -1.39$ , SE = 0.45), Jumping Bush Cricket (negative;  $\beta = -1.11$ , SE = 0.36), and Striped Ground Cricket (positive;  $\beta = 0.78$ , SE = 0.30; Appendix Table 2, Appendix Figure 2). Similarly, percent agriculture was predictive for Jumping Bush Cricket (negative;  $\beta = -1.27$ , SE = 0.41), Carolina Ground Cricket (positive;  $\beta = 0.64$ , SE = 0.34), and Striped Ground Cricket (positive;  $\beta = 0.74$ , SE = 0.31; Appendix Table 2, Appendix Figure 2).

Other variables that were important predictors of species presence included distance to nearest building, canopy cover, and percent turf grass. More specifically, distance to nearest building predicted presence of Sword-bearing Conehead ( $\beta = 0.80$ , SE = 0.35) and Carolina Ground Cricket ( $\beta = 0.82$ , SE = 0.52; Appendix Table 2, Appendix Figure 2). The presence of canopy cover negatively predicted the presence of the Sword-bearing Conehead ( $\beta = -1.30$ , SE = 0.568; Appendix Table 2, Appendix Figure 2). Percent turf grass predicted the presence of Carolina Ground Cricket ( $\beta = -0.75$ , SE = 0.35) and Striped Ground Cricket ( $\beta = -0.76$ , SE = 0.31).

Insecticide toxic load was the only competing model for Common True Katydid and had a negative influence on the probability of species occurrence ( $\beta = -1.06$ , SE = 0.37; Appendix Table 2, Appendix Figures 2-3).

Overall, when examining occurrence patterns related to percentage of agriculture or percentage of developed land (Appendix Figure 2), three species appear to be “*agriculture associates*” (Carolina

Ground Cricket, Striped Ground Cricket, Sword-bearing Conehead) and “*agriculture avoiders*” (Common True Katydid, Greater Angle-wing, and Jumping Bush Cricket).

## Discussion

Although it seems intuitive that folivorous insects would be strongly impacted by pesticides (Dirzo et al., 2014; Fartmann et al., 2012), I found limited evidence that insecticide toxic load drove variation in the Orthoptera community within agricultural valleys in the Central Appalachians. This may suggest that, for the community I sampled, Orthoptera may be somewhat resistant to the effects of pesticide use on agricultural crops and persist despite occasionally heavy applications. This would be consistent with literature suggesting that Orthoptera are particularly well-suited to resist the effects of pesticides (Brahimi et al., 2020), potentially via behavioral shifts or other means (Kostromytska et al., 2018). Alternatively, there may be tradeoffs between sub-lethal pesticide effects that are outweighed by the presence of other habitat components (e.g., habitat structure) that drives *Ensifera* space use (McNeil & Grozinger, 2020). With that in mind, there are potential alternative explanations for most species’ presence being disconnected from pesticide toxic load. For one, the Douglas et al. (2020) representation of the insecticide toxic load is not necessarily representative of how the Orthoptera I surveyed interact with pesticides because it was optimized for honey bees (Kammerer et al., 2020; McNeil et al., 2020). With that in mind, although the presence of most species appeared to be independent of pesticide load, one species appeared to be strongly impacted: the Common True Katydid.

The stark relationship I observed between Common True Katydid occurrence and insecticide toxic load is somewhat unexpected given the species’ highly arboreal life history (Borror et al., 1989). Still, there are a number of ways these canopy-dwelling insects could be exposed to pesticides applied to

low-strata crops. For example, although the species spends nearly its entire life in the canopy, individuals can be occasionally found on the ground after storms or during dispersal events (Himmelman, 2009) potentially exposing them to ground-level pesticides. Additionally, some insecticides can volatilize and become airborne, thus exposing katydids while they remain in the canopy (Bedos et al., 2002; Brain et al., 2019). Indeed, up to 40% of a sprayed insecticide can drift beyond the field to which they were applied for absorption by non-target plants (such as trees; Maybank et al., 1978). There is evidence that trees can absorb insecticides in detectable levels in their leaves which Common True Katydid use as forage (Botías et al., 2016; Karthikeyan et al., 2004). Chronic pesticide exposure by large-bodied Orthoptera such as the Common True Katydid can impact a variety of aspects of life history and physiology. For example, *Wetas* (*Hemideina crassidens*) in New Zealand that consumed pesticides exhibited significantly reduced activity and defensiveness which could lead to reduced mating and increased predation risk (Parli et al., 2020). Moreover, Common True Katydid lay their eggs directly within the branches of live woody plants (Gwynne, 2001). If these eggs are oviposited within pesticide-laden plant tissues, it seems likely that egg survival would be reduced (Manna et al., 2020). Thus, our study demonstrates that, even where Common True Katydid habitat remains intact, pesticides may render conditions unsuitable for persistence.

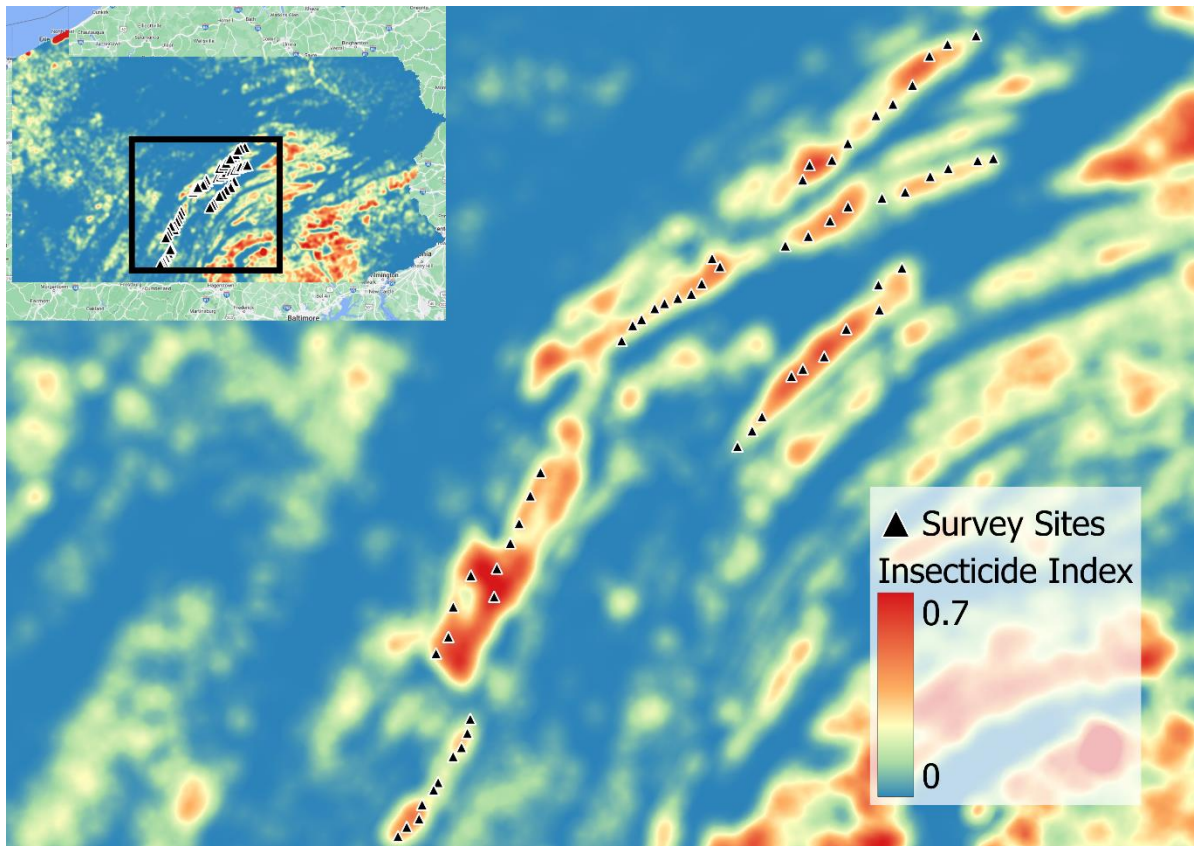
Although pesticide toxic load was not a strong predictor of occurrence for most species, aspects of microhabitat explained occurrence patterns for all three cricket species I modeled and two of the katydids. In particular, within highly agricultural landscapes, I observed two broad patterns among the six species I studied: “*agriculture associates*” (both ground crickets and the Sword-bearing Conehead) and “*agriculture avoiders*” (Common True Katydid, Greater Angle-wing, and Jumping Bush Cricket) with reverse patterns associated with human development for each group (Appendix Figure 2). These patterns are consistent with general knowledge of species’ life history characteristics; for example,

Jumping Bush Crickets in this region are known to prefer urban areas (McNeil & Grozinger, 2020) where low trees and shrubs provide song perches for singing males (Himmelman, 2009; Maier, 2017; O'Brien & O'Brien, 2015). Likewise, Sword-bearing Coneheads sing predominantly from tall grass, a feature common along the margins of agricultural fields and pastures (McNeil et al 2020) but less so in yards/parking lots. Similarly, the Carolina Ground Cricket sings exclusively from the ground and, in many cases, concealed beneath leaf litter and thatch vegetation which may be more abundant along the margins of agricultural fields (Urquhart, 1938). Ultimately, these ecological patterns indicate that habitat use by Ensifera species is non-random in agricultural landscapes and, in most cases, they do so in ways constrained by life history needs.

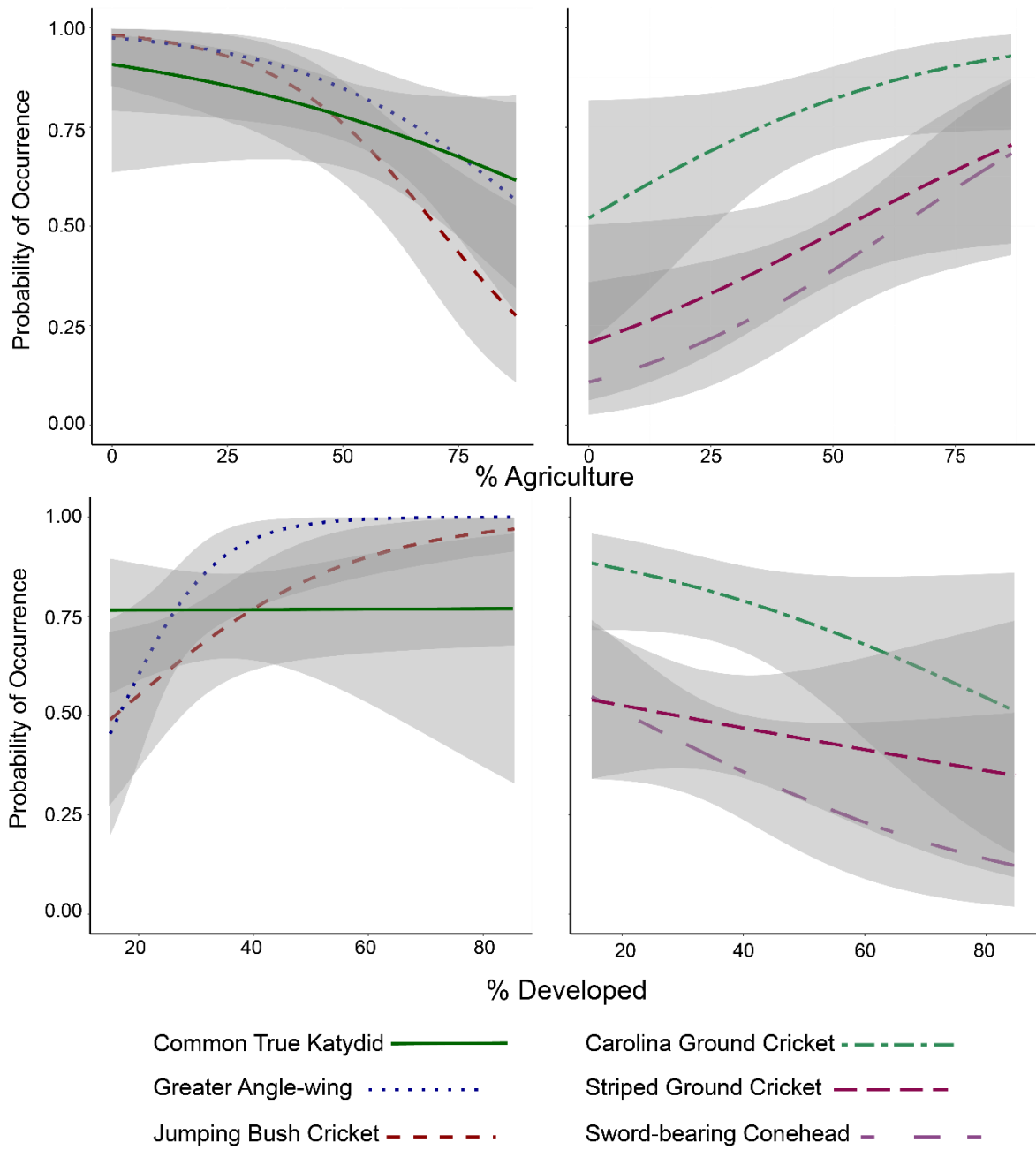
While intensive agriculture is often thought of as a degraded habitat (Norris, 2008), our data demonstrates that such landscapes can provide habitat for a variety of species. In fact, beyond the six species I modeled, three species (Fall Field Cricket, Allard's Ground Cricket, and Round-tipped Conehead) were so common in agricultural habitats that there was insufficient variation in their occurrence patterns to model. In addition to an increased understanding of how crickets and katydids can persist within novel habitats like agriculture, our analyses provide insights into the poorly-known ecological requirements for these species, most of which have only been described anecdotally (Gwynne, 2001; Shapiro, 1998). In North America, there are 431 species of crickets and katydids (*Singing Insects of North America (SINA)*, 2020), I only addressed a small handful of charismatic species. A lack of information regarding the habitat associations for these insects makes the interpretation of ecological data somewhat challenging – whether within agricultural landscapes or natural ones (Bakewell et al., 2020; Sheehan & Miller, 2021). One way to begin improving our understanding of the ecological needs of night-singing Orthoptera would be through the use of a concerted monitoring effort that occurs routinely across a specified focal area (akin to state-level “bird

atlases”; *e.g.*, Wilson et al., 2012), potentially using point count surveys (McNeil & Grozinger, 2020). Alternatively, monitoring efforts using automated recording units (*e.g.*, AudioMoth; Hill et al., 2019) could be an option for long-term monitoring without requiring high sampling effort, though this would require the development of species-specific classifiers (*sensu* Lapp et al., In Press). Such a monitoring effort would help to inform conservation efforts for Ensifera, especially in a context as I observed here for the Common True Katydid where intact habitat may become unsuitable through the addition of toxins. Pairing Ensifera monitoring data with advances in the availability of high-resolution remote-sensed data (*e.g.*, Light Detection and Ranging data [LiDAR]) might provide increased information on microhabitat use with relatively low sampling effort (Atkins et al., 2018). With these ideas in mind, roadside point count surveys of crickets and katydids, as I employed here, provide important insights as to how these ecologically sensitive insects respond to insecticides and other characteristics associated with novel (*i.e.*, agricultural) ecosystems.

## Figures and Tables

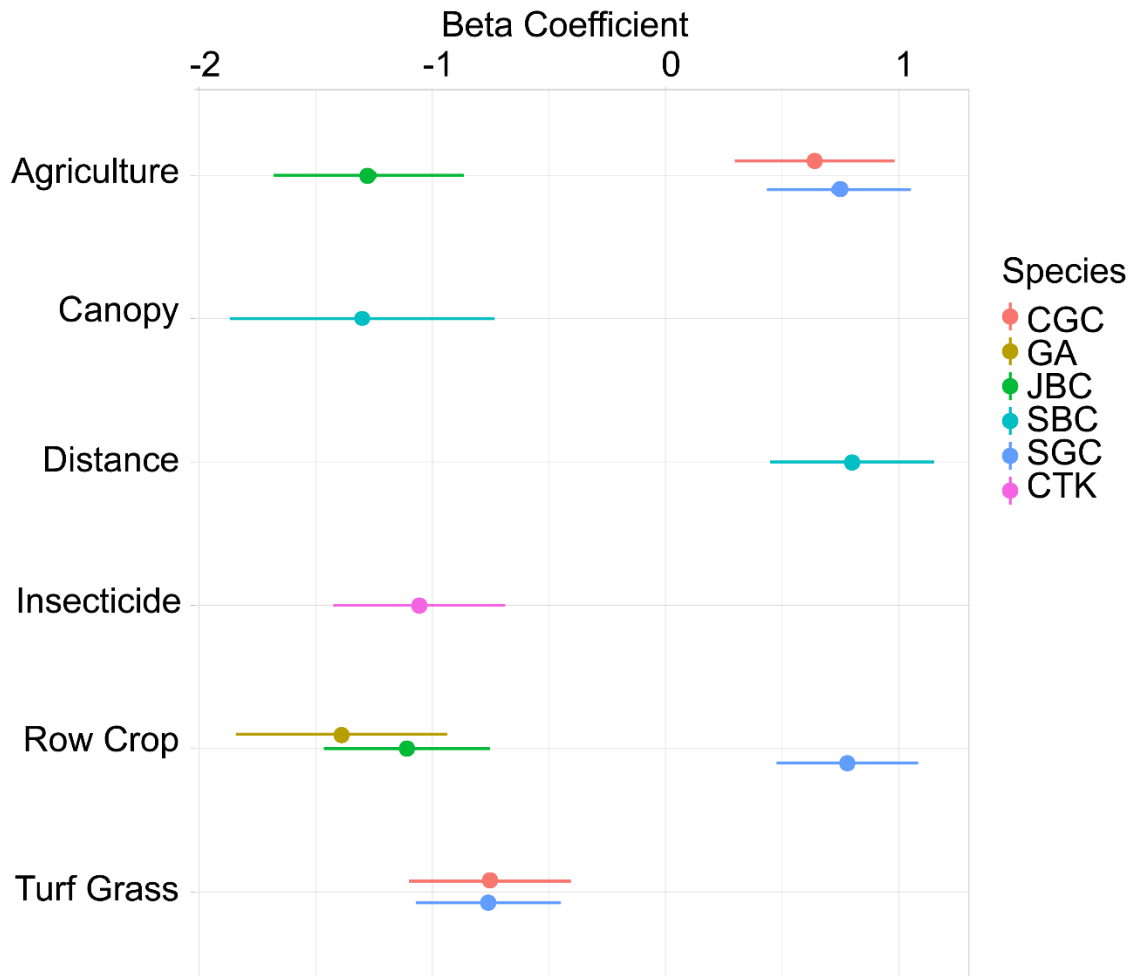


*Appendix Figure 1: A map of our Pennsylvania study area highlighting the region where I focused our study. Survey sites are indicated with black triangles. The color scheme of the map indicates insecticide toxic load (LD50/ha) with cool colors representing low values and increasingly warm colors representing higher LD50 values.*



*Appendix Figure 2: Shown are modeled functional relationships between Ensifera species and percent agriculture cover (100m radius, top row) and percent developed cover (100m radius, bottom row). Each species' ecological pattern estimate is shown with a different color of line and the 95% confidence intervals is shown in gray.*





*Appendix Figure 3: Plotted beta ( $\beta$ ) coefficient values for all biologically meaningful logistic regression models (circles), with 85% confidence intervals around each estimate (lines).*

*Appendix Table 1: All habitat covariates used in our analyses. For each variable, I denote the source (Cropland Data Layer [CDL], Chesapeake Bay Watershed dataset [CBW], etc.), mean value, standard deviation (sd). All variables are presented in the form of percentages except distance-to-nearest-building and insecticide toxic load are expressed in meters and LD50/ha, respectively.*

<b>Variable name</b>	<b>Source</b>	<b>Mean (sd)</b>
% agriculture	CBW	49.8 (22.48)
% canopy	CBW (composite)	8.53 (13.48)
% corn	CDL	23.00 (17.56)
% developed	CDL (composite)	35.50 (16.44)
% natural	CDL (composite)	15.70 (15.52)
% row crop	CDL (composite)	40.00 (22.17)
% turf grass	CBW	26.20 (12.55)
Distance-to-nearest-building	Google Maps	105 (99)
Insecticide toxic load	Douglas et al. 2020	0.34 (0.13)

*Appendix Table 2: The candidate model sets for each species, ranked in descending order of  $\Delta$  Akaike's Information Criterion adjusted for small sample size ( $\Delta$ AICc). Also shown is the number of model parameters ( $k$ ), model weight ( $w$ ) and the  $\beta$  parameters for models with covariates (competing models*

only;  $\Delta AIC_c < 2.0$ ). For each  $\beta$  parameter shown, I also indicate whether the  $\beta$  85% confidence intervals include zero (no asterisk) or do not include zero (asterisk).

Carolina Ground Cricket ( <i>Eunemobius carolinus</i> )				
Model name	k	$\Delta AIC_c$	w	$\beta$
Percent turf grass	2	0.00	0.34	-0.75*
Percent agriculture	2	1.42	0.17	0.64*
Distance-to-nearest-building	2	1.74	0.14	0.82*
Percent developed	2	2.84	0.08	-
Null (intercept only)	1	3.01	0.08	-
Striped Ground Cricket ( <i>Allonemobius fasciatus</i> )				
Percent row crop	2	0.00	0.32	0.78*
Percent turf grass	2	0.67	0.23	-0.76*
Percent agriculture	2	0.89	0.21	0.74*
Percent canopy	2	2.31	0.10	-
Percent developed	2	4.55	0.03	-
Jumping Bush Cricket ( <i>Orocharis saltator</i> )				
Percent agriculture	2	0.00	0.49	-1.27*
Percent row crop	2	1.24	0.26	-1.11*
Percent natural	2	3.26	0.10	-
Percent canopy	2	3.67	0.08	-
Distance-to-nearest-building	2	4.39	0.05	-
Sword-bearing Conehead ( <i>Neoconocephalus ensiger</i> )				

Distance-to-nearest-building	2	0.00	0.45	0.80*
Percent canopy	2	1.26	0.24	-1.30*
Percent agriculture	2	2.41	0.13	-
Null (intercept only)	1	4.69	0.04	-
Percent turf grass	2	4.79	0.04	-
Greater Angle-wing ( <i>Microcentrum rhombifolium</i> )				
Percent row crop	2	0.00	0.66	-1.39*
Percent developed	2	2.03	0.90	-
Percent canopy	2	5.53	0.95	-
Distance-to-nearest-building	2	6.68	0.97	-
Percent agriculture	2	7.60	0.98	-
Common True Katydid ( <i>Pterophylla camellifolia</i> )				
Insecticide load	2	0.00	0.81	-1.06*
Percent canopy	2	4.41	0.09	-
Percent natural	2	6.73	0.03	-
Percent row crop	2	7.01	0.02	-
Null (intercept only)	1	8.10	0.01	-

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