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Authors: Gratton, Elena M., McNeil, Darin J., Grozinger, Christina M., and Hines, Heather M.

Source: Environmental Entomology, 52(3) : 491-501

Published By: Entomological Society of America

URL: https://doi.org/10.1093/ee/nvad027

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Environmental Entomology, 52(3), 2023, 491–501
https://doi.org/10.1093/ee/nvad027
Advance Access Publication Date: 3 May 2023
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Local habitat type influences bumble bee pathogen loads and bee species distributions

Elena M. Gratton[1], Darin J. McNeil Jr[2], Christina M. Grozinger[1], Heather M. Hines[1,3,*]

[1]Department of Entomology, Center for Pollinator Research, Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA, USA, [2]Department of Forestry and Natural Resources, University of Kentucky, Lexington, KY, USA, [3]Department of Biology, Pennsylvania State University, University Park, PA, USA *Corresponding author, mail: hmh19@psu.edu

Subject Editor: Juliana Rangel

Received on 23 October 2022; revised on 26 January 2023; accepted on 30 March 2023

Bumble bees (Hymenoptera: Apidae, *Bombus* Latreille) perform important ecological services in both managed and natural ecosystems. Anthropogenically induced change has altered floral resources, climate, and insecticide exposure, factors that 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. We 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 4 leading pathogens in the common eastern bumble bee, *Bombus impatiens* Cresson. 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.

Key words: bumble bee, *Bombus*, pollinator, habitat, pathogen

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. 2007). Many bumble bee species are in decline, with climate change, habitat loss/degradation, and pathogens implicated as leading explanatory stressors (Cameron and 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 and Packer 2008, Williams et al. 2009, Jackson et al. 2022). 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 broods and adults (reviewed in Liczner and 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-Struttmann 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.

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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 highly virulent (Otti and Schmid-Hempel 2007, Rutrecht and Brown 2008) and has been implicated as a leading driver of bumble bee population declines (Cameron et al. 2011). Deformed wing virus (DWV; Picornavirales: Iflavidridae: *Iflavidrus*) 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*: Linnaeus, Hymenoptera: Apidae), DWV does not typically reach high infection levels in wild bumble bee populations (Alger et al. 2019, Ezray 2019, McNeil et al. 2020). Black queen cell virus (BQCV; Picornavirales: Dicistroviridae: *Triatovirus*), in contrast, can be very prevalent, reaching infection frequencies greater than 50% in some wild bumble bees communities (Alger et al. 2019, Ezray 2019, McNeil et al. 2020), although usually not with as high titers as honey bees (Alger et al. 2019, Ezray 2019). 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 (Durrer and Schmid-Hempel 1994, Alger et al. 2019, Burnham et al. 2021). Given the varying epidemiology of these pathogens, we 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 (Brunner et al. 2014, Branchiccela et al. 2019, Figueroa et al. 2021). Certain types of weather (e.g., precipitation) can lead to high prevalence of disease infection in bumble bee populations (Gardner et al. 1977, Gisder et al. 2010, Chen et al. 2012, Neidel et al. 2017, Palmer-Young et al. 2018, 2019, McNeil et al. 2020). Furthermore, toxins introduced into the environment by humans (e.g., fungicides [McArt et al. 2017] and insecticides [Andrew et al. 2017]) can play a large role in reducing immune performance of wild bumble bees (Andrew et al. 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; Piot et al. 2022). Additionally, studies have found that declining and thus rare bumble bee species (e.g., the federally endangered *B. affinis* Cresson) harbor higher pathogen loads than more widespread common species, suggesting that these species might be more susceptible (Cameron et al. 2011, Cordes et al. 2012, Cameron and Sadd 2020). 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 disproportionally 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* increased 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*) 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 nonforested valley landscapes.

Here, we 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. We collected wild bumble bees from forested ridges, agricultural valleys, and ecotonal habitats within the replicate-rich hill and valley system in central Pennsylvania (Fig. 1). We examine how habitat as well as other landscape variables influence loads of 4 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 within the same bee species (*B. 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 yr ago (Clark 2001). These ridges include a mix of deciduous- and mixed coniferous forests and are higher elevation (200–660 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, we chose sites across 3 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 (Fig. 1a).

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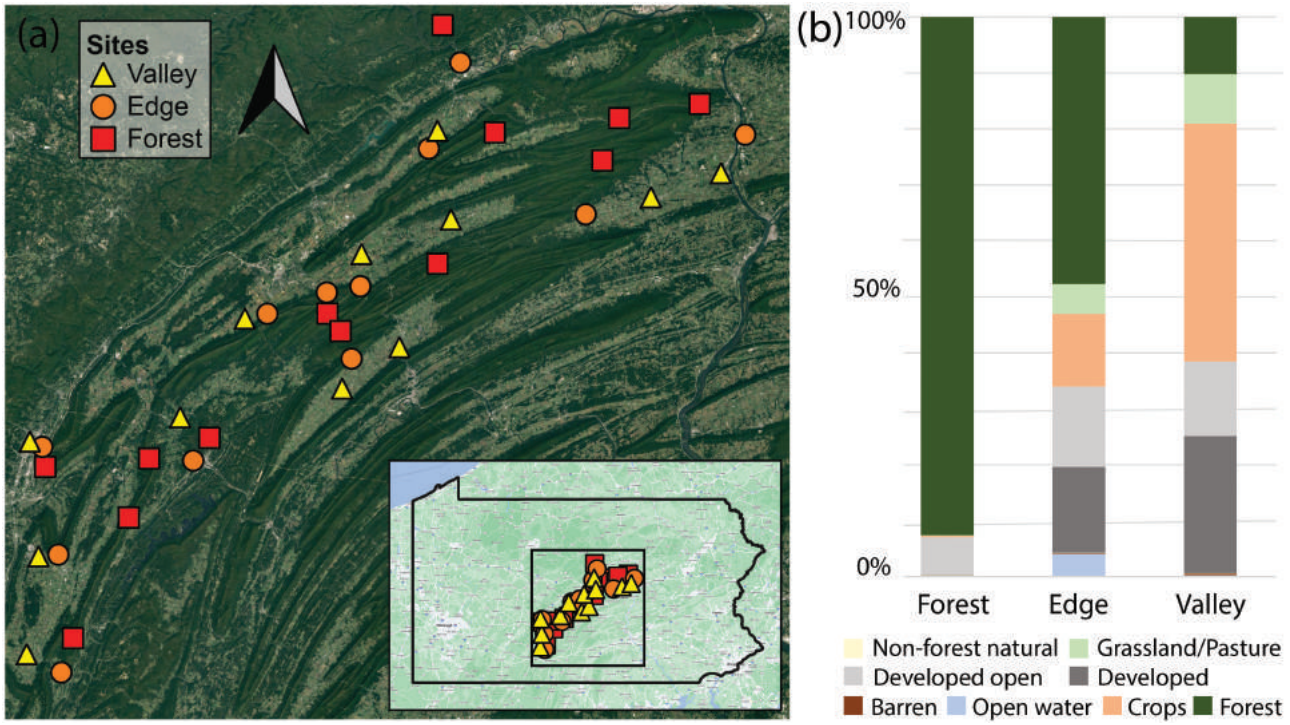

Fig. 1. a) Map of sites across central Pennsylvania and b) landscape cover categories for each habitat.

Using a fairly random walk across each site, we 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 we obtained less than 24 due to a shortage of bumble bees [2 sites at 20, 2 sites at 23]) and we recorded the time it took to collect at each site. For each bee captured, we recorded its species identity, caste, and species of flower upon which it was captured. Bee species were visually identified *in situ* although we retained individuals that could not be identified in the field. We 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 bees, *B. impatiens* is the most common species at all sites, thus providing enough individuals to compare between sites. We 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 to 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, we performed quantitative PCR (qPCR) for 4 pathogens: DWV, BQCV, *Vairimorpha bombi*, and *Crithidia bombi*, along with a control gene *Elongation factor-1α (EF-1α)*. These screens were performed on pooled abdomens of 3 *B. impatiens* workers per sample and we analyzed 1–3 pools per site, depending on how many *B. impatiens* we 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 we performed qPCR on these pooled extracts. RNA was extracted using the Zymo Direct-zol RNA extraction kit and protocols. For the lysis step, 3 bee abdomens were homogenized in 1500 ul TRIzol in a 2 ml vial with 3 metal beads using an Omni Bead Ruptor for two 35-s 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 4 diluted 96 well plates that were kept in a −20 °C freezer until use in quantitative PCR within 5 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 3 nontemplate-controls. Plates were run on an Applied Biosystems 7900 instrument (Thermo Fisher Scientific) using standard conditions, annealing at 60 °C. Amplifications were performed using the following primers: black queen cell virus (BQCV) 5′-TTTAGAGCGAATTCGGAAACA-3′ and 5′-GGCGTACCGATAAAGATGGA-3′ (vanEngelsdorp et al. 2009, McNeil et al. 2020), deformed wing virus (DWV) (A & B) 5′-GTTTGTATGAGGTTATACTTCAAGGAG-3′ and 5′-GCCATGCAATCCTTCAGTACCAGC-3′ (DWV/VDV-1 (80004-8030),F; DWV/VDV-1(8143-8120),R; Ryabov et al. 2014), *Vairimorpha bombi* 5′-GGCCCATGCATGTTTTTGA AGATTATTAT-3′ and 5′-CTACACTTTAACGTAGTTATCT GCGG-3′ (BOMBICAR; (Plischuk et al. 2009, McNeil et al. 2020), *Crithidia bombi* 5′-GGCCACCCACGGGAATAA-3′ and 5′-CAAAGCTTTCGCGTGAAGAAA-3′ (Ezray 2019), *EF-1α* 5′-CCGACAAGGCTCTTCGTTTA-3′ and 5′-ATGCCTG GCTTCAGAATACC-3′ (Tian et al. 2019, McNeil et al. 2020). Each gene was run on a separate plate. Each set of primers has been used

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previously and found to double linearly as expected with cycles, thus a standard curve was not used.

All C_T values above 35 as well as those with no amplification were considered "no detection" and given a value of 35. We used the standard conversion for normalization with 2 delta-delta Ct as was done in McNeil et al. (2020) to obtain log2 fold differences from no detection (0). If a sample did not amplify, it was assigned a value of 0 regardless of *EF-1α* normalization.

DNA Barcoding for Specimen Identification

Due to the difficulty in correctly separating the mimetic (Williams et al. 2014, Ezray et al. 2019) bumble bee species *B. sandersoni* (Franklin), *B. perplexus* (Cresson), and *B. vagans* (Smith) via morphological means (Milam et al. 2020), we 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 (Omega Bio-tek) and associated protocols with 100 µl of elution buffer. Following extraction, each sample was amplified using a standard PCR procedure using a 15 µl reaction with 1 µl DNA, 7.5 µl Taq Mastermix, 0.3 µl 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 30 s; 94 °C for 30 s, 48 °C for 30 s, 72 °C for 40 s, repeated 38 times; 72 °C for 5 min, 4 °C (hold). Following successful amplification, each sample was purified using a 5.6 µl Exosap reaction (Applied Biosystems) and sequenced using Sanger sequencing at the Penn State Genomics Core Facility (University Park, PA). We edited the resulting chromatograms and constructed a neighbor-joining phylogeny in Geneious 8.1.9 (https://www.geneious.com) 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*, Cresson. Although there may be *B. mixtus*, for this study we lump these all into *B. sandersoni* until further work is done on the group. Specimens were pinned and stored as vouchers. We cross-validated our DNA barcode identification with wing morphometric analysis (unpublished data), which also separated the 3 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, we compared the total number of observations of *B. sandersoni*, *B. vagans*, and *B. perplexus* we obtained with records from Pennsylvania on iNaturalist (all records, not only research grade level observations, [all dates available; 2009–13 May 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 3 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 reliability identified, as bees that are uncertain may also not be assigned to species.

Species Abundance and Composition

We 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, we assessed bees-per-minute collected for *B. impatiens*, *B. bimaculatus* (Cresson), and combined *B. perplexus* + *B. sandersoni* + *B. vagans* (referred to hereafter as *B. sandersoni* complex); while all 3 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, we examined the correlation of each species with individual landscape variables outlined below, using the proportion of collected individuals per site. For this, we analyzed only the bee species that occurred at 4 or more sites [excludes *B. terricola* (Kirby), *B. flavidus* (Eversmann), and *B. ternaries* (Say)]. To compare the diversity of bumble bee species between sites, we calculated Shannon diversity index (Shannon 1948) on all of the bees captured at that site.

Landscape and Climate Variables

We 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 (30 m resolution; Homer et al. 2012) using the program R (R Core Team 2022). Specifically, we 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 nonforest open (sum of percent shrubland, percent woody wetlands, percent herbaceous wetlands, and percent grassland/pasture), and percent crop (sum of all agricultural categories). "Nonforest 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. We extracted elevation for each site using ArcGIS Pro's elevation tool using a 30 m DEM (USGS 1999, ESRI 2021).

We 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 and 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. We modified the nesting index to only include the resources for ground nesting bees, which was the most representative option for bumble bees. We 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 and Tscharntke 2000). Finally, we quantified the following weather and climatic factors:

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April, May, and June precipitation, precipitation from the 3 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, we examined how landcover types were distributed across our 3 major habitat types and performed a principal component analysis to determine the extent to which these major variables differ in these 3 habitats, including forest, nonforest 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/min) we used a Kruskal-Wallis test (Ostertagová et al. 2014; $\alpha = 0.05$) 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 comparisons (Jafari and Ansari-Pour 2019). We likewise compared pathogen loads among habitat types (forest/valley/edge) using the same procedure. Finally, we 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 3 major habitat types (valley, edge, and forest) were distinct with respect to land cover and elevation (Fig. 1b, Supplemental Fig. 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 a similar elevation distribution and $\bar{x}$ that was lower (valley = 300.7 m, edge = 327.61 m; Supplementary Fig. 1) than the forest sites (490.27 m; Supplementary Fig. 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; Supplementary Fig. 2). For example, % forest, spring and summer floral index, nesting index, and to a lesser extent elevation and precipitation (Supplementary Figs. 3 and 4) 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 (Supplementary Figs. 3and 4) 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 is, thus, correlated in latitude and longitude) and varies 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 (Supplementary Fig. 5). 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 (Fig. 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*-adj = 0.003), but edge was not different from either habitat type. Similarly, there was a significant effect of habitat type for DWV (Fig. 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 2 viruses, we observed no effect of habitat type of the parasite *V. bombi* levels (Fig. 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 (Supplementary Fig. 5), thus limiting power for inference of landscape influence. *C. bombi* was commonly detected but showed considerable variance in our samples (Supplementary Fig. 5). For *C. bombi*, levels were the inverse trend to that found in viruses, with an overall highly significant effect of habitat (Fig. 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 (Fig. 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 (Fig. 2b) and most positively correlated with nonforested open (grassland/pasture) areas ($r = 0.24$) and developed areas ($r = 0.35$). *V. bombi* had no landcover correlations above $|r| = 0.19$ (nonforest open). *C. bombi* was most and positively correlated with nest ($r = 0.50$) and spring floral availability ($r = 0.47$) (Fig. 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 (Fig. 3a; $\chi^2 = 28.70$, df = 2, $P < 0.001$). There was no difference in *B. impatiens* capture rate between habitat types (Fig. 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 (Fig. 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; Fig. 3b). The relative patterns of species composition are fairly consistent among sites and are not driven heavily by any one site (Supplementary Fig. 6), 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

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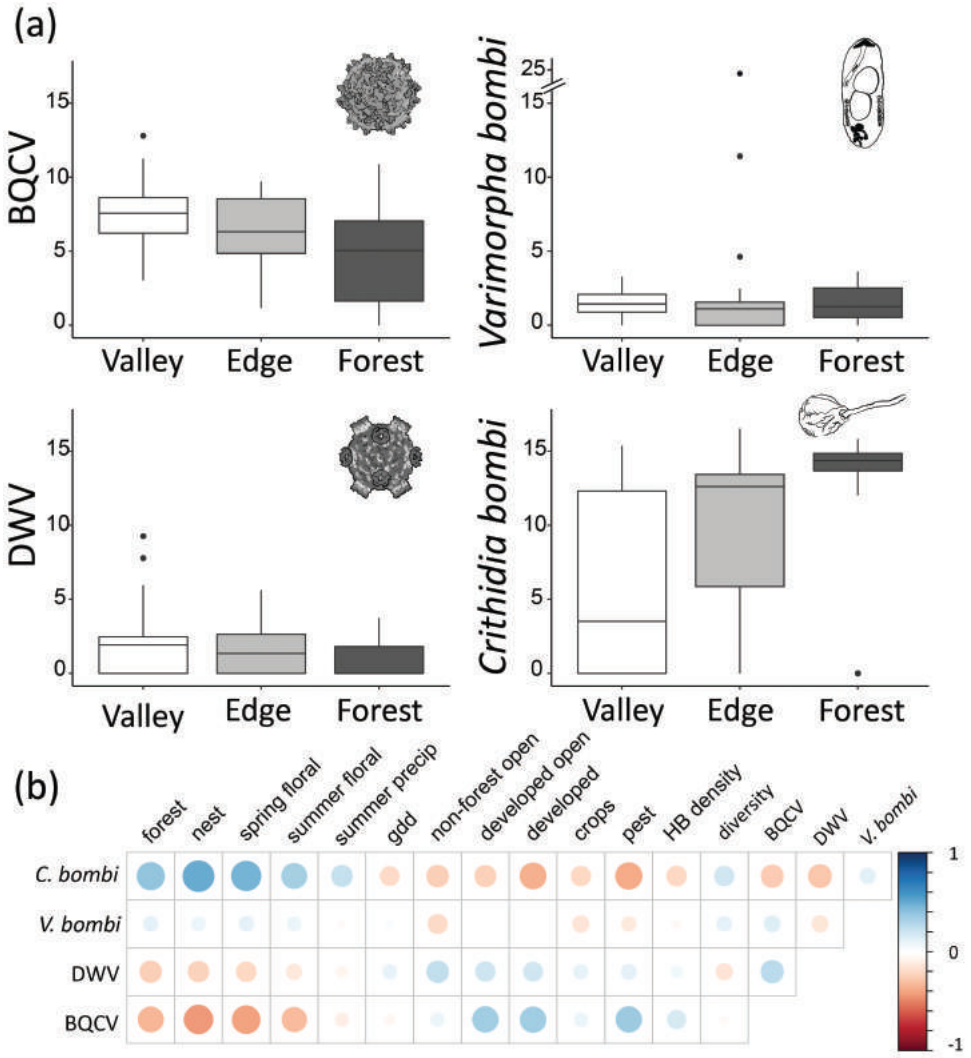

Fig. 2. Impact of landscape features on pathogen loads. a) Pathogen loads across the valley to forest gradient. b) Correlation matrix of pathogen loads with landscape variables, bumble bee diversity, and species composition.

is proportionally less abundant in natural and forested areas and in areas with more floral resources (Fig. 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 (Fig. 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 (Fig. 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 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 3 species for Pennsylvania on iNaturalist and GBIF. While *B. sandersoni* comprised 53% of individuals observed among these 3 species in our data, iNaturalist and GBIF had no to very few (3%) occurrence records for *B. sandersoni*, respectively (Fig. 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* (Fig. 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 (Fig. 3b). *B. vagans* occupies just 1% of bees collected at valley sites, but 6% of both edge and forest sites (Fig. 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; Supplementary Fig. 6). *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 (Fig. 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.

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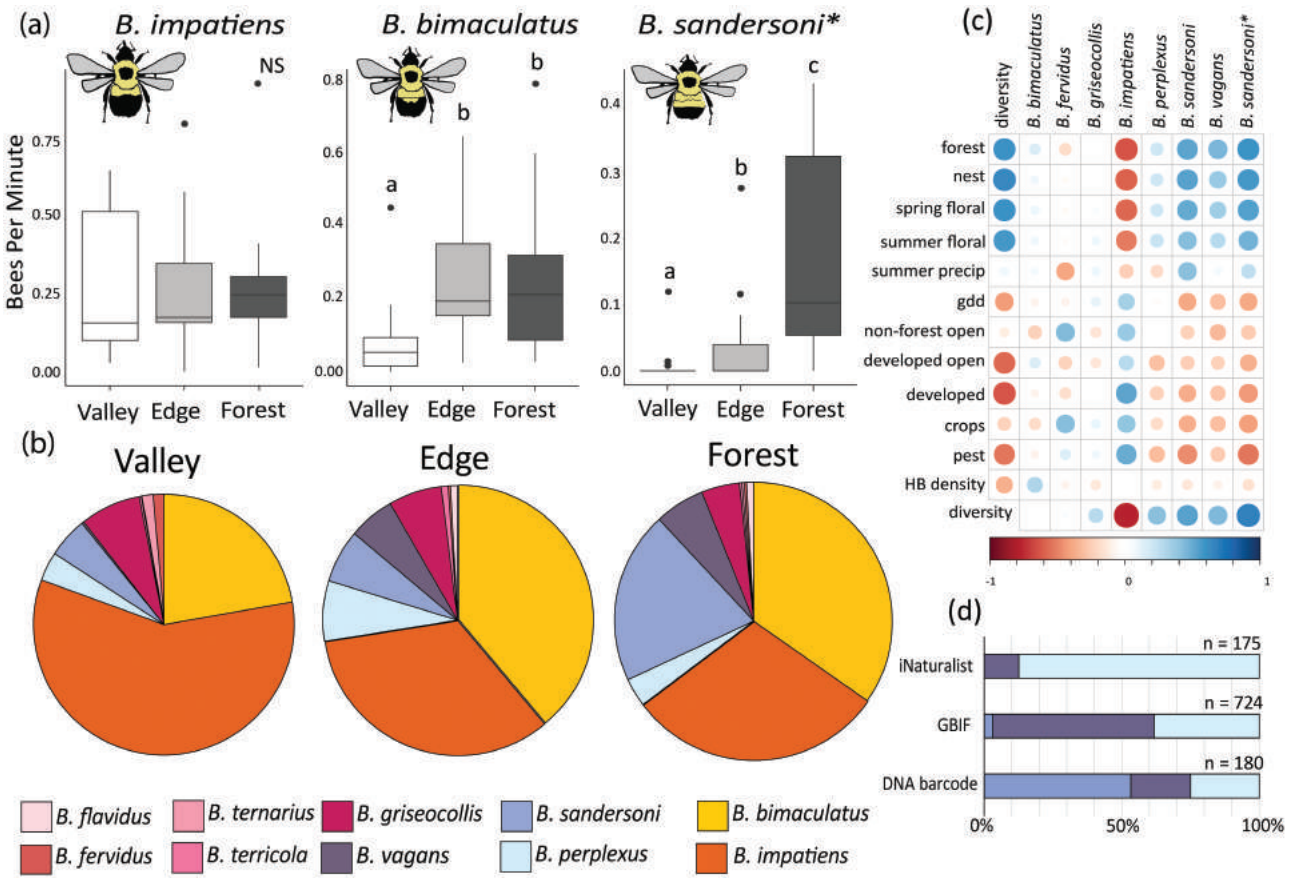

Fig. 3. Distribution of species by local habitat. a) Catch rate (bees per minute) of *B. impatiens, B. bimaculatus*, and *B. sandersoni* across the forest to valley gradient. b) Percent captured of each species in each habitat type. Colors correspond to those in the legend at the bottom of the figure and color groups/bee images match the 3 major groups in (a). c) Correlation of *B. impatiens, B. bimaculatus, B. sandersoni, B. vagans, B. perplexus, B. sandersoni* complex, and Shannon Diversity Index with landscape and weather variables. d) Comparison of observations in Pennsylvania of *B. sandersoni*, *B. vagans*, and *B. perplexus* in iNaturalist and GBIF data sets and our DNA barcoded samples.

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*, *B. terricola*, and *B. ternarius*. *B. fervidus*, a bee known to nest in grassy substrates, was most positively associated with cropland (r = 0.43) and nonforested open areas (grasslands/pasture; r = 0.43).

Diversity-Related Patterns

Bombus 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 (Fig. 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) (Supplementary Fig. 4). 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). (Supplementary Fig. 4)

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, we 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 autocorrelated 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 we 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

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BQCV and DWV). As conservationists in Pennsylvania (PPHTF 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 Koh et al. 2016, McNeil et al. 2020, Gillespie et al. 2022) as well as intermediate/local scales (Mola et al. 2021, Conflitti et al. 2022).

We found levels of 2 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 nonforested 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 (Brunner et al. 2014, Branchiccela et al. 2019, 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.; Figs 2–3; McNeil et al. 2020), there may be synergistic impacts to valley bees that we were 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. 2019, 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 multiyear field data of Manlik et al. (2022), that 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 we did (2018–2019 vs. 2020) and, although loads in that study were similarly small, they were higher than we observed. Given that both studies used identical field and laboratory protocols, we 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 *Vairimorpha 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 and Roulston 2014), thus future work should examine the relative impacts of different *Vairimorpha* on these bees as well.

Conversely, *Crithidia bombi* was more prevalent in bees from forest habitats and showed a general decline in more developed landscapes (Fig. 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 (Koh et al. 2016, McNeil et al. 2020, though see Bosmans et al. 2018). 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 (Figueroa 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 (Figueroa 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 we 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 (Fig. 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 3 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 (Fig. 3d). iNaturalist data mostly just recognize *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 2 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 2 species in Pennsylvania (Fig. 3d). Milam et al. (2020) obtained barcodes of these 3 bee species sampled from across their eastern US range and also identified *B. sandersoni* more often than the other 2 species in the Northeastern United States, 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 United States (e.g., Cameron et al. 2011, Koch et

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al. 2015; Jacbonson et al. 2018), *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 (Koh et al. 2016, Conflitti et al. 2022). 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 (Ratti and Colla 2010, Looney et al. 2019). Indeed, specimen data have shown that *B. impatiens* represents an increasing proportion of bumble bee communities in the eastern Nearctic over the last 50 yrs (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 indicate 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 (Biesmeijer et al. 2006, Albrecht et al. 2012, Brosi and 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 susceptibilities to pathogens (Cordes et al. 2012, Malfi and Roulston 2014) 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* (~2× higher) and *B. bimaculatus* (~3× 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 and Roulston 2014), thus spread of this pathogen could be highly community dependent and better inferred in more vulnerable species. Future studies that monitor pathogen levels in bumble bee species beyond this model species and that compare 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 on conservation aims (Schweitzer et al. 2012, PPHTF 2017).

Acknowledgments

We would like to thank Andy Deans and Codey Mathis for their assistance in the field. We would like to thank Gabriela Quinlan for assistance in obtaining data on geospatial variables and with the statistical analysis. We are also grateful to Allyson Ray for assistance with qPCR. We recognize and are grateful for the considerable efforts of taxonomic experts to identify and database species that populate GBIF and iNaturalist. We are grateful to Jack Hill and the Pennsylvania Department of Conservation and Natural Resources (PA-DCNR) for access to state parks for bumble bee sampling (permit #2019-75). This work was supported by a grant to HMH and CMG from the Foundation for Food and Agricultural Research (FFAR #549032), and by the USDA National Institute of Food and Agriculture and Hatch Appropriations under Project #PEN04716 and Accession #1020527.

Data Availability

Data and code used in this manuscripts are available through Dryad: https://doi.org/10.5061/dryad.j0zpc86k5.

Author Contributions

Elena Gratton (Data curation-Lead, Formal analysis-Lead, Investigation-Equal, Methodology-Equal, Visualization-Lead, Writing – original draft-Lead, Writing – review & editing-Equal), Darin McNeil (Conceptualization-Equal, Data curation-Supporting, Investigation-Equal, Methodology-Equal, Writing – review & editing-Supporting), Christina Grozinger (Conceptualization-Equal, Funding acquisition-Equal, Methodology-Equal, Project administration-Supporting, Writing – review & editing-Equal), Heather Hines (Conceptualization-Equal, Funding acquisition-Equal, Methodology-Equal, Project administration-Lead, Writing – review & editing-Equal)

Supplementary Material

Supplementary material is available at *Environmental Entomology* online.

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