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VARIATION IN POLLINATOR POTENTIAL TO CARRY A BLUEBERRY FUNGAL PATHOGEN AND ASSESSMENT OF TRANSFER EFFICIENCY IN TWO MANAGED BEE SPECIES

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Abstract—Plant diseases are ubiquitous in agricultural systems and are major sources of economic loss. *Vaccinium corymbosum,* or highbush blueberry, is an economically important crop affected by an insect-vectored, fungal pathogen, *Monilinia vaccinii-corymbosi,* or mummy berry disease. Highbush blueberry yield is maximized through outcrossed pollination; however, the pathogen is vectored by pollinators. We used field collections and molecular techniques to identify floral visitors to highbush blueberry and quantify levels of pathogen spores carried by each visiting species. We also conducted a cage trial using single flower visits to determine differences in vectoring efficiency between two managed pollinators, *Apis mellifera* and *Bombus impatiens*. We found that bees, flies, and wasps were all common visitors, and that all bee species and several fly and wasp species carried the pathogen. Of the bee species, *A. mellifera* most often tested positive for the pathogen, while *Dolichovespula maculata* (Bald-faced Hornet) tested positive most among wasps and *Mallota posticata* among flies. Considering only individuals that tested positive, mummyberry levels per individual were highest in *D. maculata* and *Andrena* bees, and relatively low in flies. In cage trials, we found no differences between *A. mellifera* and *B. impatiens* in pathogen load or transfer efficiency, suggesting that these managed species are equally capable of vectoring mummyberry during a single visit to a blighted stem and then a flower. This research demonstrates the variety of floral visitors that carry mummyberry and that two common commercial pollinator species have similar potential to vector mummyberry to blueberry flowers during a single visit.

Keywords: Apis mellifera, blueberry, Bombus impatiens, floral pathogen, Monilinia vaccinii-corymbosi, Vaccinium corymbosum

INTRODUCTION

Over one third of the world's agricultural crops rely on insect-mediated pollination services to reproduce (Klein et al. 2007). A diverse assemblage of pollinators can increase yield as a result of flower visitation (Garibaldi et al. 2013). To increase the efficiency of pollination services, growers in North America often supplement the natural pollinator community with commercially available pollinators, *Apis mellifera* (European honeybee) or *Bombus impatiens* (common eastern bumblebee). Whether present naturally or supplemented by growers, bee pollination is vital to the economic prosperity and stability of agricultural systems (Delaplane & Mayer 2000).

Despite the benefits provided by insect pollinators to many food crops, pollinators and other insect floral visitors may also transmit plant pathogens that reduce both fitness and yield (Dobson & Crawley 1994). At least 26 plant pathogens are vectored by pollinators that infect plant floral reproductive tissue (Roy 1994; McArt et al. 2014). For example, *Microbotryum violaceum,* or anther smut, is a common fungal pathogen vectored by insect pollinators that infect the plants in the family Caryophyllaceae (Jennersten 1988; Shykoff & Bucheli 1995). Additionally, *Erwinia amylovora*, or fire blight, is a bacterial pathogen carried by *A. mellifera* and other pollinators that infects apples, pears, and other crops in the Rosaceae, with domestic losses and control costs exceeding $100 million annually (Norelli et al. 2003). Describing variation in how floral visitors contribute to vectoring pathogens as well as pollinating crops may help to understand tradeoffs between balancing effective pollination services with disease management.

Highbush blueberry is an agriculturally important crop in the United States, with over 588 million pounds of berries produced from the 37,554 hectares dedicated to cultivated blueberry production (Ross et al. 2017). *Monilinia vaccinii-corymbosi* (MVC), or mummy berry disease, is an insect-vectored fungal pathogen that is the most damaging pathogen of highbush blueberry (*Vaccinium corymbosum*), with some infections reducing yield up to 80% and causing severe economic losses (Stretch et al. 2001). MVC employs a two-stage infection process (Batra 1983). Primary infection by mummy-berry ascospores creates 'pseudoflowers' in new blueberry shoots, inducing the production of a sugar-rich solution, while causing blighted shoots to reflect UV light (Batra & Batra 1985) and exude volatile organic compounds that mimic floral scent (McArt et al. 2016). In addition to distribution via wind and rain, pollinators and other insects visit the pseudoflowers and vector conidia, asexual fungal spores, to flowers (Ngugi et al. 2002). These spores mimic pollen grains by germinating on the stigma, and hyphal growth

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extends down the stylar canal from the conidium to the ovary, causing secondary infection (Ngugi & Scherm 2004). Infected flowers develop inedible berries composed of hard, hyphal masses that drop, overwinter, and produce ascospores that begin a new cycle of infection (Batra 1983).

Vaccinium corymbosum is visited by a variety of insect pollinators, including bee species in the Andrenidae, Halictidae, Megachilidae, and Apidae families (Scott et al. 2016) that have the potential to vector MVC. Although mummy berry can be inhibited with repeated fungicide application, the cost of such applications and consumer demand for 'no spray' orchards, coupled with mounting environmental concerns over the use of fungicides (Wightwick et al. 2010) make understanding which pollinators are most likely to vector the pathogen both relevant and economically desirable. Early work in this system established that floral infection is primarily vectored by insects that first visit blighted leaf tissue (Batra 1983), but until recently little was known about the specific insect taxa involved in transmission. Recent observational work combined with nested PCR analysis determined the presence of fungal spores on insect bodies and identified five Hymenopteran and nine Dipteran families as MVC carriers and potential vectors (McArt et al. 2016). Using camera traps to record visits to both flowers and blights, McArt et al. (2016) determined that although bees and flies often visited both blighted leaf tissue and flowers, bees were more likely to visit flowers than flies, and flies were more likely to visit blights than bees. Despite these behavioural differences, bees were more likely to be carriers of fungal spores than flies (McArt et al. 2016). The authors suggest that the discrepancy could be explained by differences in morphology between bees and flies, with the latter lacking branched hairs that are effective at collecting pollen and potentially conidia, or behavioural differences in contact and interaction with floral reproductive structures.

Behaviour and morphology can vary widely among insects, and insect pollinators differ in their pollination efficiency, both in the amount of pollen that can be picked up and in what is transferred from their bodies onto flowers (Primack & Silander 1975; Herrera 1987; Olsen 1997). Since mummy berry conidia mimic pollen grains (Ngugi & Scherm 2004), pollinators may also differ in their effectiveness as vectors for the pathogen. This variation can be caused by differences among pollinator taxa in cuticular structure, body fit to flower structure, or behaviour, such as pollen grooming or collecting nectar vs. pollen (Delaplane & Mayer 2000; Adler & Irwin 2006). Although previous work assessed the presence of fungal conidia on different insect pollinator taxa, we do not know the amount of conidia carried by these taxa, or how this relates to pollinator ability to transfer conidia to new host material. Depending on an insect's body shape, hairs, and way of interacting with flowers, presence or even quantity of conidia carried may not reflect the amount transferred to floral tissues. Therefore, we do not know whether insect pollinators differ in rates of transfer of mummy berry conidia.

The goals of this study were to assess (1) how much MVC is carried on insect taxa visiting *V. corymbosum* flowers and (2) the transfer efficiency of two commercially available pollinator species. We collected blueberry-visiting insects from a no-spray orchard infected with mummy berry. We then identified insect taxa to genus or species using cytochrome c oxidase I (COI) sequencing, and using a targeted sequencing approach combined with a competition assay, we estimated the amount of MVC carried on the sampled insects. Finally, we performed a cage trial to assess comparative transfer efficiency of mummyberry conidia by *Apis mellifera* and *Bombus impatiens*.

MATERIALS AND METHODS

Insect field collection

Insect visitors of highbush blueberry flowers were collected at Quonquont Orchard in Whately, Massachusetts, USA (42.444° N -72.639° W) on May 22, May23 and June 4, 2014, periods of peak bloom. Collection took place on weekdays from 1000 to 1600 hours in weather conditions ranging from full sun to light rain. We did not net specimens to avoid rubbing off conidia due to contact with the net. Instead, we captured insects visiting flowers in snap-cap vials (one insect sample per vial) upon emergence from corollas. Specimen vials were immediately placed in dry ice for transport back to the lab. Two hundred and thirty-two samples were maintained in a -20°C freezer until processing for molecular analysis; final sample sizes for each species are presented in Tab. 1.

Sequencing analysis

Field-collected insects were sent to Floodlight Genomics LLC (Knoxville, TN) in snap-cap vials set in dry ice for processing to determine insect genus and species based on cytochrome c oxidase I (COI) sequencing and to measure the amount of MVC using a targeted-sequencing approach. Insects were weighed to determine wet mass prior to DNA extraction.

DNA extraction

Insects were assigned provisional identifications to order or family based on visual inspections (not removed from plastic containers). Provisional identifications are not reported and only served to confirm that molecular identification was reasonable for easily classified insects (e.g., *Bombus*). Unwashed insects were placed whole into 2 ml or 5 ml tubes containing three to five 3 mm glass balls and freeze-dried for 24 hours. A mixer mill (Retsch GmbH, Germany) was used to disrupt and powder the freeze-dried material prior to genomic DNA extraction.

Genomic DNA was extracted using the MagJET Genomic DNA Extraction Kit (Thermo Fisher Scientific) according to the manufacturer specifications, including lysis with a digestion buffer and Proteinase K followed by magnetic bead separation of genomic DNA from cellular debris, proteins and RNA.

Cytochrome c oxidase I amplifications and sequencing

A multiplex mixture of 11 primers (Tab. 2; see Elbrecht and Leese 2017) with varying degrees of degeneracy were used to amplify the genomic DNA using a Hi-Plex approach (Nguyen-Dumont et al. 2013). The resulting amplicons

TABLE 1. Comprehensive list of species identified by BLAST search with sample sizes and mean counts of MVC ITS sequences indicating pathogen load. Families are listed alphabetically within order, and species are ordered from highest raw ITS count to lowest within family.

	Species	*n*	Family	Common name	Mean ITS
Hymenoptera	*Andrena vicina*	54	Andrenidae	Neighborly Miner Bee	7,423,087
	Andrena carolina	9	Andrenidae	Blueberry bee	140,521.5
	Andrena clarkella	1	Andrenidae	Blueberry bee	51,706
	Apis mellifera	54	Apidae	European Honey Bee	2,136,683
	Bombus bimaculatus	16	Apidae	Two-spotted Bumble Bee	1,947,150
	Bombus impatiens	56	Apidae	Common Eastern Bumble Bee	1,777,119
	Apis florea	1	Apidae	Dwarf Honey Bee	262,579
	Bombus hypnorum	1	Apidae	Tree Bumblebee	101,649
	Bombus perplexus	2	Apidae	Confusing Bumblebee	31,908
	Xylocopa virginica	1	Apidae	Eastern Carpenter Bee	0
	Augochlorella sp.	1	Halictidae	Sweat Bee	0
	Tryphon seminiger	1	Ichneumonidae	Parasitoid Wasp	0
	Pristiphora cincta	1	Tenthredinidae	Saw Fly	70,457
	Empria maculata	1	Tenthredinidae	Saw Fly	0
	Dolichovespula maculata	6	Vespidae	Bald-faced Hornet	10,018,580
	Vespula vidua	14	Vespidae	Widow Yellowjacket	631,404
	Euodynerus foraminatus	1	Vespidae	Potter Wasp	302,476
	Dolichovespula adulterina	1	Vespidae	Parasitic Wasp	258,704
	Dolichovespula arenaria	2	Vespidae	Arial Yellowjacket	102,962
	Vespula sp.	4	Vespidae	Widow Yellowjacket	16,735
	Polistes fuscatus	1	Vespidae	Northern Paper Wasp	0
Diptera	*Delia antiqua*	1	Anthomyiidae	Onion Fly	0
	Pollenia labialis	1	Calliphoridae	Cluster fly	0
	Pollenia pediculate	1	Calliphoridae	Cluster fly	0
	Pollenia rudis	3	Calliphoridae	Cluster fly	0
	Conops rondanii	1	Conopidae	Fly (Bee Parasite)	1,196,821
	Dolichopodidae sp.	3	Dolichopodidae	Long-legged Fly	45,433
	Desmometopa sordida	1	Milichiidae	Freeloader Fly	36,934
	Anthomyiinae sp.	1	Muscidae	House Fly	0
	Coenosia tigrine	1	Muscidae	House Fly	0
	Chrysopilus sp.	3	Rhagionidae	Snipe Fly	0
	Chrysopilus proximus	4	Rhagionidae	Snipe Fly	0
	Blaesoxipha sp.	2	Sarcophagidae	Flesh Fly	0
	Leptocera erythrocera	1	Sphaeroceridae	Lesser Dung Fly	88,580
	Syrphinae sp.	1	Syrphidae	Hover Fly	1,670,285
	Mallota posticata	6	Syrphidae	Hover Fly	549,321
	Eristalis dimidiata	1	Syrphidae	Hover Fly	0
	Parhelophilus sp.	2	Syrphidae	Hover Fly	0
	Platycheirus hyperboreus	1	Syrphidae	Hover Fly	0
	Chrysops carbonarius	11	Tabanidae	Deer Fly	0
	Chrysops dawsoni	1	Tabanidae	Deer Fly	0
	Epalpus signifer	6	Tachinidae	Bristly Fly	316,521
	Klugia marginata	1	Tachinidae	Bristle Fly	122,649
	Gonia ornata	1	Tachinidae	Bristle Fly	0
Coleoptera	*Bibioninae* sp.	1	Cantharidae	Soldier Beetle	83,505
	Pterolophia formosana	1	Cerambycidae	Longhorn Beetle	0

TABLE 2. Cytochrome c Oxidase I (COI) primer sets targeting the Folmer region for DNA metabarcoding of insects.

Primer Name*	Primer Sequence	Amplicon Size	Citation
Uni-MinibarR1	GAAAATCATAATGAAGGCATGAGC	127	Meusnieretal 2008
Uni-MinibarF1	TCCACTAATCACAARGATATTGGTAC	127	Meusnieretal 2008
ZBJ-ArtF1c	AGATATTGGAACWTTATATTTTATTTTTGG	157	Zealeetal 2010
ZBJ-ArtR2c	WACTAATCAATTWCCAAATCCTCC	157	Zealeetal 2010
LepF1	ATTCAACCAATCATAAAGATATTGG	127	Hebertetal 2004
EPT-long-univR	AARAAAATYATAAYAAAIGCGTGIAIIGT	127	Hajibabaeietal 2011
MLepF1-Rev	CGTGGAAAWGCTATATCWGGTG	218	Brandon-Mongetal 2015
BF1	ACWGGWTGRACWGTNTAYCC	217	herein
BR1	ARYATDGTRATDGCHCCDGC	217	herein
L499	ATTAATATACGATCAACAGGAAT	178	VanHoudtetal 2010
H2123d	TAWACTTCWGGRTGWCCAAARAATCA	178	VanHoudtetal 2010

*Primers described in doi: 10.3389/fenvs.2017.00011 (2017)

ranged in size from 127 to 218bp and were sequenced on an Illumina HiSeq X device running a 2x150 paired-end configuration according to manufacturer directions (NovoGene, USA). The resulting raw sequences were processed using CLC Genomics Workbench version 9.5.3 (Qiagen, USA) to merge the paired reads and to conduct *de novo* assemblies using the default settings of CLC. The resulting contigs were BLAST searched (blastn, using non-redundant database) in CLC batch mode at the NCBI using default settings. Contigs receiving 10 or more hits were examined further to assign putative genus and species.

Estimation of *M. vaccinii-corymbosi* using a sequencing approach

Primers amplifying a 93bp portion of the *M. vaccinii-corymbosi* internal transcribed spacer (ITS) region (Forward primer: AAG GGC AGA ACC TCT CCA CCC TT; Reverse primer: AGG GTT AGG TCA TTG GCG GG) were tested on genomic DNA extracted from insects kept in axenic conditions that were entirely free of MVC and insects that had MVC spores applied to them by physically dusting insect bodies with conidia collected from blighted blueberry tissues. The primers amplified a properly sized amplicon from the dusted insects and there was no amplification for MVC-free insects. To determine the amount of fungal ITS carried on wild insects, a competition-assay was devised. The assay included the above primers and a mock-ITS target which had the central bases replaced with a 28bp string of ATCG(x7). The exact *M. vaccinii-corymbosi* amplicon sequence was:

AAGGGCAGAACCTCTCCACCCTTTGTGTATTA TT**ACTTTGTTGCTTTGGCGGGCCGCCTCCGG**GC CTCGCGTGCCCGCCAATGACCTAACCCT

The mock-ITS sequence, added to each PCR amplification, was (replaced bases in bold):

AAGGGCAGAACCTCTCCACCCTTTGTGTATTA TTA**ATCGATCGATCGATCGATCGATCGATCG**GCC TCGCGTGCCCGCCAATGACCTAACCCT

A dilution series for the mock-ITS was tested to estimate the amount of mock template suitable for use as an amplification control and to determine the relative amount of exact *M. vaccinii-corymbosi* sequences in the insect samples. Amplification products were prepared for sequencing on an Illumina HiSeq X device running a 2 x 150 paired-end configuration using the KAPA Hyper-Prep PCR-free kit according to the manufacturer directions (KAPA Biosystems, Wilmington, Massachusetts, USA) and quantified using the KAPA qPCR quantification kit (KAPA, USA). The resulting sequences were then mapped to the above exact and mock ITS sequences, requiring 99% similarity across 99% of the sequence, and the number of exact sequences was divided by the number of mock sequences to provide an estimate of total exact sequences within an insect sample. From these estimates we can compare relative amounts of ITS sequences between insect species.

Cage Trials

From May 29 through June 2, 2017, cage trials were conducted daily to determine the number of fungal conidia deposited per visit on blueberry floral stigmatic surfaces by honey and bumble bees. One nucleus colony of *A. mellifera* was purchased on April 13, 2017 (New England Apiaries, Westfield Massachusetts USA) and transported to Wilbraham, Massachusetts USA (42.136N, -72.434W) to an outdoor, south-facing Langstroth hive attached to a 1.22 x 1.22 x 2.44 m fiberglass insect screen enclosure (Phifer Inc., Tuscaloosa, Alabama, USA). All exits from the hive except those leading to the screen enclosure were secured, ensuring that bees could not forage outside. Two *B. impatiens* research colonies (with queen but no drones) were obtained from Biobest USA, Inc. (Leamington, Ontario, Canada) on May 10, 2017 and placed within a separate screen enclosure (one hive at each end) identical to that for *A. mellifera*. Both *A. mellifera* and *B. impatiens* were fed *ad libitum* sucrose and water solution (1:1) from inverted jar feeders and BeePro FD200 Pollen Substitute (Mann Lake Ltd., Hackensack, Minnesota, USA) on an open platform within the enclosure.

To provide a source of conidia in cage trials, blighted, conidia-producing stems were collected from Quonquont Farm in Whately, Massachusetts, USA (42.444N, -72.639W) on a weekly basis from May 9 to 30, 2017. Blights were left on stems, which were set in Floralife cut-flower solution (15.63 ml/l concentration; Floralife North America, Waterboro, South Carolina USA) prior to use. To assess transfer of conidia to flowers, clippings with unopened floral clusters were taken from the same orchard on the same dates that blighted tissues were collected. These clippings were also provided Floralife solution and kept separate from blighted tissues in an enclosed area to prevent contamination from wild pollinator visitation. Only newly opened 'virgin' flowers were used in trials.

Artificial arrays of blighted tissue and virgin flowers were created by inserting blights and flowers into a 30 cm x 15 cm x 5 cm foam block. Blocks were placed next to sucrose feeders so they could be easily located by foraging bees. Three clippings with blight (one blighted patch per clipping) and three clippings with virgin flowers (1-3 flowers per clipping) were used in each trial. To ensure there was no difference in blight sizes used in each trial, the length and width of blighted tissue on each blight was measured and were not significantly different between honeybee and bumblebee trials ($F_{1,36} = 2.36$, $P = 0.1332$).

To begin each honeybee trial, the gate from the hive to the enclosure was closed, leaving only a small number of foraging bees in the enclosure. The array was observed until a honey bee visited blighted tissue, and bees were not allowed to contact a flower until after visiting blights. If bees were investigating a flower before contacting blight, they were pushed away manually. For blighted tissue, we define 'visit' as an insect fully landing on a blight with cessation of wing movement. The visit time was recorded from moment of landing and cessation of wing movement to departure. Once the visiting bee moved to a flower, we recorded the time spent within the corolla in contact with reproductive parts. The bee was removed from the enclosure after a single visit, and the clipping with visited flowers was removed and returned to a weatherproof screen enclosure in Floralife solution. Stigmas of visited flowers were harvested after three days and fixed in 90% ethanol until subsequent fluorescence microscopy (as in Lehman et al. 2007) to determine how many fungal spores were deposited on the stigma. Clippings of both blighted tissue and floral tissue were discarded after the first visit. We ultimately collected 20 samples where honeybees foraged on blights and then flowers, in which we could measure conidial deposition.

Honeybees were far more apt to visit blighted tissues than bumblebees; *B. impatiens* had to be coaxed to forage on blighted tissue. Individual *B. impatiens* were chilled to 4°C for 20 minutes and then placed and allowed to waken on blighted tissues amongst a floral array similar to that used in honeybee trials; once bumblebees warmed they were more likely to forage. The time spent foraging on blights was measured starting from the first sustained, consistent movement of the bee abdomen lasting longer than one second on blights and ending when the bee left the tissue. Bumblebee visits to flowers were measured using the same honeybee protocols described above, and we collected 24 samples where bumblebees moved from blights to flower and we could measure conidial deposition.

To quantify conidia deposited on floral reproductive surfaces, stigmas were examined using fluorescence microscopy (Lehman et al. 2007). Stigmas were removed from EtOH solution and rinsed twice in sterile dH_2O. Stigmas were then cleared and fixed for 2 hours at 60°C in 0.3% trichloroacetic acid dissolved in a 3:1 vol/vol solution of 95% EtOH and chloroform. Stigmas were again rinsed twice with sterile dHOH and softened in 8 M sodium hydroxide for 20 min at 60°C. Stigmas were then stained in 0.1% methyl blue in 0.1 M K_3PO_4 (pH 12) and again rinsed twice in dH2O. Styles were bisected longitudinally on a glass microscope slide and viewed using a Chroma 31000 filter set (Chroma Technology Corp., Bellows Falls, VT) excitation filter (300 – 400 nm, barrier filter 400 nm, emitter filter 410-500 nm).

Statistical analysis

For field-collected insects on blueberry, we analysed two components of the potential to transmit MVC. First, we analysed the likelihood of insect species carrying MVC using a Chi squared test of independence, with species as the predictor and presence or absence of ITS sequence counts as the response. Then to assess differences in potential to transmit for insects that were carrying MVC, we used generalized linear models with negative binomial distributions (to adjust for overdispersion) and log link functions (glm.nb in the MASS package) to compare the pathogen load, defined as the number of MVC ITS sequences found on insect bodies, between species or functional groups, only including insects in which we detected the presence of MVC ITS sequences. 'Functional groups' included flies, social bees, and solitary bees as categories. Number of copies of MVC (both raw counts and values adjusted by insect bodyweight (ITS count/fresh body weight in g)) were compared with species or functional groups as fixed effects in separate models; there were no other factors in the models. Species with fewer than six samples (Tab. 3) were dropped from this analysis. To compare means of ITS sequence counts, we used Tukey's post hoc comparison in the MultComp package for R (R Development Core Team 2018).

For the cage trials, foraging time on blights and flowers was analysed using ANOVA with species as a fixed factor. To determine whether *A. mellifera* or *B. impatiens* differed in deposition of conidia per floral visit, a generalized linear model with a Poisson distribution was used with 'species' as a predictor and number of conidia deposited as a response. GLMs and associated multiple comparisons were analysed in R (version 3.5.1, R Foundation for Statistical Computing), and all other statistical analyses were carried out using JMP Pro 13.2.1 (SAS Institute Inc., Cary, NC, 1989-2007).

RESULTS

We identified 47 species of insects spanning 21 families visiting *Vaccinium corymbosum* flowers, 25 of which carried MVC on or in their bodies (Tab. 1). Of the 232 specimens

TABLE 3. Sample sizes and percentages of taxa testing positive for MVC. Species are listed alphabetically. Only species with sample sizes of 5 or greater were included.

	Percent with MVC	Individuals with MVC	Individuals without MVC	Sample Size
Bees				
Andrena carolina	44.4	4	5	9
Andrena vicina	44.2	23	29	52
Apis mellifera	76.9	40	12	52
Bombus bimaculatus	40	6	9	15
Bombus impatiens	55.6	30	24	54
Flies				
Chrysops carbonarius	0	0	10	10
Epalpus signifer	83.3	5	1	6
Mallota posticata	100	6	0	6
Wasps				
Dolichovespula maculata	83.3	5	1	6
Vespula vidua	64.3	9	5	14

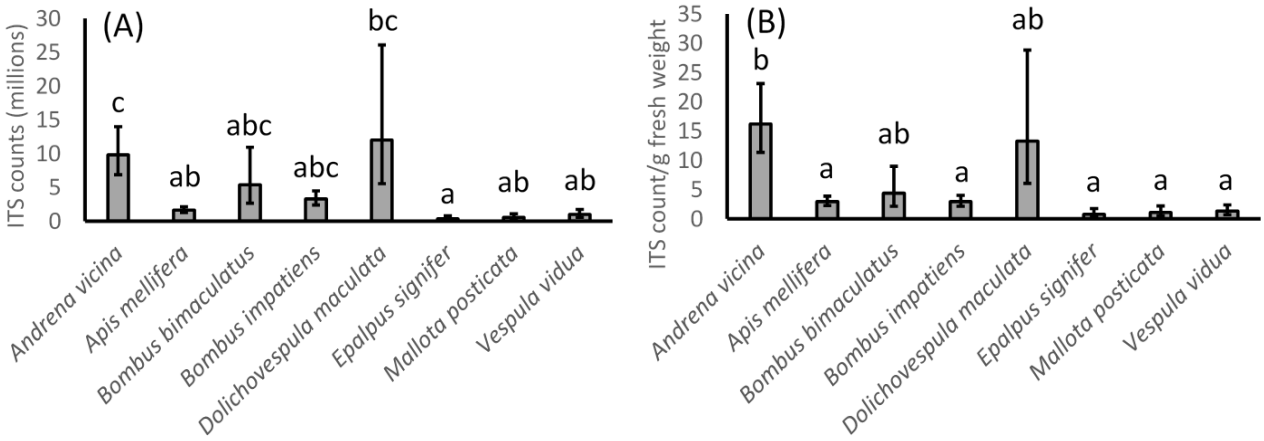

FIGURE 1. Species comparison of MVC ITS regions for (A) counts in millions and (B) counts in millions adjusted by body size (g fresh weight). Samples without MVC and species with fewer than five samples were not included in this analysis. Species with different letters are statistically different as determined by Tukey's post hoc comparisons. Means and error bars are back-transformed model estimates; error bars are exponentiated +/- one standard error of the mean.

collected, 164 were comprised almost equally of *B. impatiens, Andrena vicina* and *A. mellifera*. We found that species was a significant predictor of the presence of MVC ($\chi^2 = 37.157$, df = 9, $P < 0.0001$). Of all bee species, *A. mellifera* was most likely to carry MVC (76.9% positive), while *D. maculata* (83.3% positive) was highest among the wasps and *Mallota posticata* (100% positive) highest among the flies (Tab. 3). Of insects that carried MVC, comparison between generalized linear models with and without species as a fixed factor revealed that both raw counts of ITS sequences and those adjusted by insect body weight varied by species ($\chi^2 = 32.34$, df = 7, $P < 0.0001$ and $\chi^2 = 32.28$, df = 7, $P < 0.0001$, respectively). Additionally, results from Tukey's post hoc analyses show significant differences in both ITS and adjusted ITS means between species, with *A. vicina* carrying significantly more raw ITS sequences than *A. mellifera,* and more than both *A. mellifera* and *B. impatiens* when adjusted for body weight (Fig. 1). Comparisons of generalized linear models with and without functional group as a fixed factor revealed that both raw counts of ITS sequences and those adjusted by body weight varied by functional group ($\chi^2 = 19.30$, df = 2, $P < 0.0001$ and $\chi^2 = 24.74$, df = 2, $P < 0.0001$, respectively). Tukey's post hoc tests showed differences in ITS and adjusted ITS counts between functional groups (Fig. 2) with solitary species carrying the most pathogen load in both raw counts and analyses adjusted by bodyweight, and solitary species carrying more than flies in raw counts.

In our cage trials, *A. mellifera* and *B. impatien*s did not differ in conidia deposition ($\chi^2 = 0.01385$, df = 1, $P =$

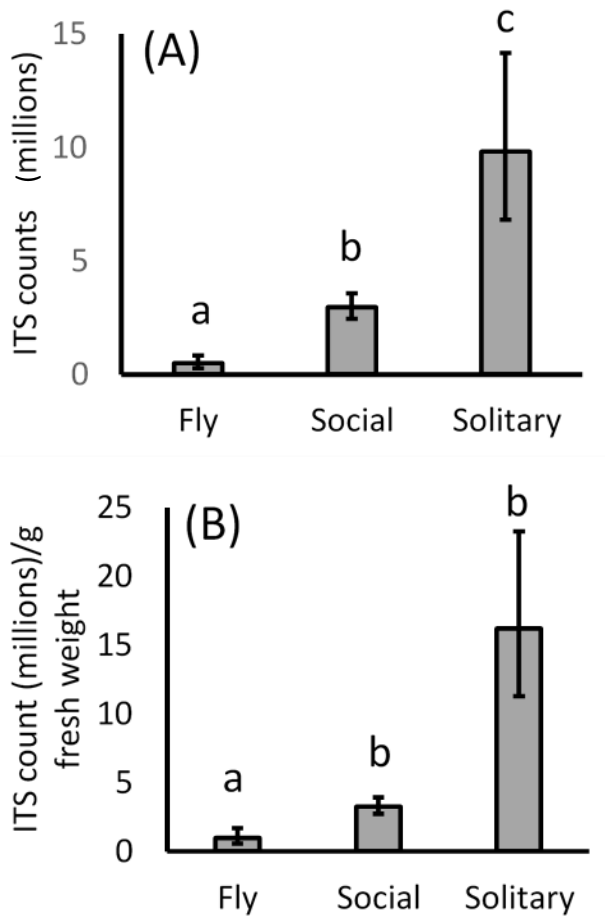

FIGURE 2. Functional group comparison of MVC ITS regions for (A) counts in millions and (B) counts in millions adjusted by body size (g fresh weight); 'social' and 'solitary' refer to bee species. Groups with different letters are statistically different as determined by Tukey's post hoc comparisons. Means and error bars are back-transformed model estimates; error bars are exponentiated +/- one standard error of the mean.

0.9063; Fig. 3). *A. mellifera* and *B. impatiens* also did not differ in time spent foraging per flower ($F_{1, 46} = 0.1022$, $P = 0.7507$). Although *A. mellifera* spent 35.5% more time on blighted tissues than *B. impatiens*, this difference was not significant ($F_{1, 51} = 2.3577$, $P = 0.1310$).

DISCUSSION

Pollination is needed to maximize yield in highbush blueberry (Ehlenfeldt 2001), and a wide diversity of species visit *V. corymbosum* flowers (MacKenzie & Eickwort 1996; Tuell et al. 2009). Generally, bees are considered to be the primary pollinators (West & McCutcheon 2009; Isaacs & Kirk 2010). Our study found 11 bee species from three families visiting blueberry at a Massachusetts site, and other work investigating *V. corymbosum* in Oregon recorded 30 bee species spanning 5 families (Rao et al. 2009). Because bees are considered the most important pollinators, little attention has been paid to non-bee visitors. We also found non-bee hymenopteran visitors such as Ichneumonids and seven species of Vespids, including common and aerial yellowjackets and Bald-faced Hornets (Tab. 1). The contribution of these species to blueberry pollination is unclear since these taxa may be nectar robbers, thieves, or searching for prey species instead of pollinating. We also identified 13 species of flies (Tab. 1); fly species are often overlooked as contributors to pollination services in both natural and agricultural systems (Larson et al. 2001; Ssymank et al. 2008). The decline of several bee species, including bumblebees and honeybees (Cameron et al. 2011; Smith et al. 2013) has prompted concerns over the effects on managed crop systems, including blueberry yield (Gibbs et al. 2016). Diverse assemblages of native pollinators may be able to provide 'biological insurance' that protects against the loss of key pollinator taxa (Winfree et al. 2007). Although not all of the insects found in our study are considered key pollinators, the broad community of insect visitors may indicate that *V. corymbosum* crops will be resilient to decline of particular pollinator species.

A wide variety of floral visitors were potential vectors of MVC. We found that 25 of the 46 floral visitor species tested positive for MVC (Tab. 1), and of those species with six or more representatives, 9 out of 10 species tested positive (Tab. 3). The prevalence of MVC in our study is congruent with prior work that found 18 of 28 families and 5 of 6 orders that tested positive for the presence of MVC DNA (McArt et al. 2016). Overall, solitary bees carried more MVC than social bees, and social bees carried more than flies when comparing raw ITS counts (Fig. 2A). When comparing counts adjusted for body size, social and solitary bees did not differ significantly from one another but did carry more than flies (Fig. 2B). Below we address in turn specific patterns for bees, flies, and other insects.

Of bees in our study, *A. mellifera* tested positive for MVC most often, with over 76% of samples returning positive results, but the solitary bee *Andrena vicina* carried the most MVC when it was present. *A. mellifera* are widely used as supplemental pollinators due to their commercial availability, large colony size, and high pollination efficacy (Delaplane & Mayer 2000). However, because MVC conidia mimic pollen grains in their mode of delivery (Ngugi & Scherm 2004), *A. mellifera* may also be an efficient mummy berry vector. Thus, growers considering using honeybees as supplemental pollinators may want to consider their potential to also vector MVC. In our comparison of ITS regions adjusted for insect body size, we found that *A. vicina* had the highest average MVC ITS count when MVC was present. *Andrena vicina* is a solitary bee and common *V. corymbosum* forager and pollinator (Scott et al. 2016). In addition, one *A. vicina* sample had the highest MVC count of any insect sampled, with over 198 million ITS copies, almost three times as much as the next highest sample. In some cases, solitary bees can be more effective pollinators than *A. mellifera* (Vicens 2009). Additionally, many solitary bee species lack corbiculae, or pollen baskets, on their hind legs, instead carrying pollen on brushes of hairs (scopa) on their ventral abdomen or legs (Chambers 1946). While conidia mimic pollen grains in rough form and function, they are smaller than blueberry pollen and thus may be more easily transferred on scopa. This morphological difference may explain why MVC occurred in large quantities on *A. vicina* when it was present.

Congruent with prior work (McArt et al. 2016), we found that flies are less likely to carry MVC than bees, and when

they do they carry MVC, they carry less. That said, we also found a great disparity in the presence of MVC between fly species (Tab. 3). For example, none of the ten specimens of *Chrysops carbonarius* (deer fly) tested positive for MVC. However, in *Mallota posticata*, a bee mimicking fly, all 6 samples tested positive for MVC. Behavioural differences are unlikely to explain this disparity in MVC presence since *M. posticata* and *C. carbonarius* are both floral visitors (Maier & Waldbauer 1979; Karolyi et al. 2014), but morphological differences may play a role. Bee mimics have an abundance of body hairs compared to deer flies like *Chrysops* and other Tabanids, perhaps making the transfer of conidia more likely. On a broader scale, the wide variety of behaviours exhibited by Dipteran taxa found in our study may help to explain the variation in amounts of MVC. For example, Sarcophagidae species often feed on nectar (Rathman et al. 1990) and may be attracted to blights due to pseudoflower mimicry of floral volatiles, leaving shortly after discovering no nectar rewards. However, flies in the Sphaeroceridae are often larval microbial grazers on decaying plant material or fungi, and thus may visit blights at less frequent intervals and with different behaviours than other Dipteran taxa. Recent camera trap work has shown that flies were more likely to visit blighted blueberry tissues than bees, although bees were more likely to carry MVC than flies (McArt et al. 2016). This suggests that both blight-visiting behaviour and morphological features may work in tandem to maximize vectoring potential. Given the vast diversity of Dipteran species, more study relating fly behaviour to MVC transmission is needed.

Surprisingly, some of the highest MVC loads were carried by insects other than bees and flies. In our comparison of raw counts of MVC ITS regions on insects testing positive, *D. maculata*, or the Bald-faced hornet, carried the highest average MVC load of all insects sampled (Fig. 1, Tab. 1). Prior work using PCR to determine MVC presence on or in insect bodies found that the presence of MVC in Vespids was relatively low when compared to other taxa (McArt et al. 2016), which contrasts with our results in which 5 of 6 samples tested positive for MVC (Tab. 3). This suggests that while incidence of Vespids carrying MVC may vary, when MVC is present it may be carried in large quantities. Although *D. maculata* is primarily a predator upon insects including other Vespids, it often also acts as pollinator while searching for nectar (Jacobs 2015), and is commonly found foraging on *V. corymbosum* flowers (McArt et al. 2016). However, field observations during 2014 and 2016 suggest that large Vespid species may visit *V. corymbosum* flowers too infrequently to be a major vector of MVC, having comprised only 7% of all visitor observations (Boyer, unpublished data).

In cage trials we found that *A. mellifera* and *B. impatiens* did not differ in their ability to transfer conidia to blueberry stigmas after visiting blighted tissue, although we note that overall deposition was low (Fig. 3). Although *A. mellifera* spent 35.5% more time on blighted tissues than *B. impatiens*, this difference was not significant. Differences in vectoring success between *A. mellifera* and *B. impatiens* may be complex, and while single visit deposition is a good first step to understanding pathogen transmission efficacies between these taxa, it may not reflect deposition in the field due to differences in bee behaviour. Bees of either species may visit

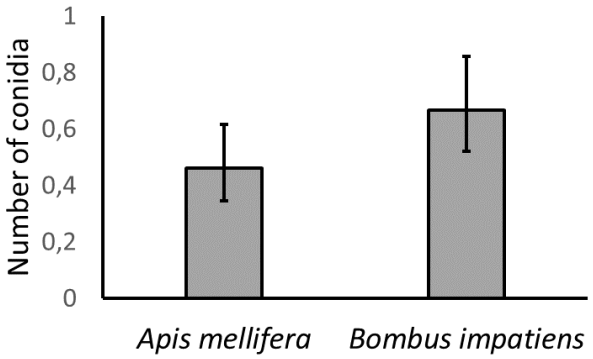

FIGURE 3. Mean conidia on stigmas visited by *A. mellifera* and *B. impatiens* in field cage trials. Bee species do not significantly differ in conidia deposited per single visit (see Results). Means and error bars are back-transformed model estimates; error bars are exponentiated +/- one standard error of the mean.

multiple blights before any given flower, or *vice versa*, and the preference of *A. mellifera* to forage on blights may explain why *A. mellifera* was most likely to carry MVC in our field samples. Conidia deposition may rely on the cumulative effect of multiple visits, in which case single visits may not be enough to determine differences between species. Higher proportions of *A. mellifera* visitation are associated with greater incidence of fruit infection in field observations (Boyer, unpublished data). Thus, while our single-visit experiment demonstrated that conidial deposition per visit does not differ between these species, more comprehensive behavioural observations of visits to blights and flowers in the field may be necessary to understand transmission dynamics.

Encouraging native pollinators may help to decrease reliance on managed bees (Rogers et al. 2014), but managed colonies of *A. mellifera* and *B. impatiens* are often used by growers (Delaplane & Mayer 2000). *Apis mellifera* was more likely to carry MVC than *B. impatiens* and had a non-significant tendency to forage for longer on blighted tissue than *B. impatiens*. However, we found no significant difference in raw or adjusted ITS counts (Fig. 1), nor in number of conidia deposited by *A. mellifera* and *B. impatiens* (Fig. 3). In addition to considering potential to vector MVC, pollination efficiency is of course a key characteristic when deciding whether and which species to use for supplemental pollination. *Bombus impatiens* are more effective extracting blueberry pollen due to their sonication behaviour, as blueberries have poricidal anthers adapted to buzz-pollinators (Delaplane & Mayer 2000). *A. mellifera* often have difficulty legitimately pollinating blueberry flowers due to corolla structure, orientation, and a lack of buzz-pollination behaviour (Delaplane & Mayer 2000). Despite the lack of sonication behaviour, honeybees may incidentally release small amounts of pollen while retrieving nectar from blueberry flowers (Javorek et al. 2002). Although the amount pollen released is small compared to buzz-pollination on a per-visit basis, differences in colony size between the two species may increase overall honeybee pollination effectiveness. Bumblebee colonies have hundreds of individuals, while a single honeybee colony often has tens of thousands. A recent study investigating efficacy of highbush blueberry pollinators found that while per-visit efficiency of *A. mellifera* was low, they

were also the most abundant pollinators observed in the field (Rogers et al. 2013), but this was often dependent on the presence of managed hives. While we speculate that *A. mellifera* may be more likely to vector MVC than *B. impatiens* as well as being a less effective pollinator, further data are needed to make responsible recommendations for whether and which supplemental pollinators to use to maximize yield and minimize disease transmission.

Blueberry is an important economic crop in the United States whose pollinator community has been previously described (MacKenzie & Eickwort 1996; Ross et al. 2017). Our study adds to this knowledge by providing molecular identification of the floral visitor community to the species level. Additionally, our study is the first to assess relative quantities of MVC carried on insect bodies, as well as to evaluate differences in pathogen transmission between two commonly supplemented pollinator species. All of the bee species and many of the fly species we collected tested positive for MVC, and of those that carry the pathogen, *D. maculata* carried the most in terms of raw counts, but *A. vicina* carried the most when adjusted for body size. Although honeybees were more likely to carry MVC than bumblebees we found no significant difference between the amount of MVC carried by bumblebees and honeybees, nor was there a significant difference in per visit deposition. In total, our findings increase our understanding of the potential for both wild and managed pollinator species to contribute to the vectoring of a highly damaging blueberry pathogen.

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