March 2019

The Good, The Bad, and The Ugly: Pollinators as Vectors of Mummy Berry Disease in Highbush Blueberry

Matthew Boyer

THE GOOD, THE BAD, AND THE UGLY: POLLINATORS AS VECTORS OF MUMMY BERRY DISEASE IN HIGHBUSH BLUEBERRY

A Dissertation Presented

by

MATTHEW D. H. BOYER

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2019

Organismic and Evolutionary Biology

and Entomology Graduate Programs

THE GOOD, THE BAD, AND THE UGLY: POLLINATORS AS VECTORS OF MUMMY BERRY DISEASE IN HIGHBUSH BLUEBERRY

A Dissertation Presented

by

MATTHEW D. H. BOYER

Approved as to style and content by:

_____
Lynn Adler, Chair

_____
Anne Averill, Member

_____
Robert Wick, Member

_____
Joan Milam, Member

_____
Paige Warren, Graduate Program Director
Organismic & Evolutionary Biology

_____
Patricia Wadsworth, Director
Interdepartmental Graduate Programs

DEDICATION

To my son, Avery.

ACKNOWLEDGMENTS

I will be forever grateful to my advisor, Dr. Lynn Adler, as I truly did not deserve such a brilliant and supportive mentor.

Thank you to all my friends and family that helped me along the way. To my parents, David Cox and Ann Boyer. To my grandfather, Henry Boyer. To my wife, Caroline Curtis. To my son, Avery, for rolling with all the rough seas.

Thank you to my committee members kind enough to donate their time to help in my success: Rob Wick, Anne Averill, Joan Milam, Martha Hoopes, Ben Normark, Joe Elkinton, Bruce Byers, Paul Sievert, and Jill Miller. And to all the unofficial advisors, especially Scott McArt and John Hulvey.

To the Biology Department staff who have helped navigate me through graduate school: Penny Jaques, Zander Crowley, Sally Ives, Sarah Czerwonka, and Maggie DeGregorio.

To the agencies that have supported me: USDA NIFA, The Lotta M. Crabtree Fellowship, UMass Natural History Collections, The UMass Graduate School and The Graduate Program in Organismic and Evolutionary Biology.

Finally, to all of those I did not mention, but nonetheless played a role in my success, I thank you.

ABSTRACT

THE GOOD, THE BAD, AND THE UGLY: POLLINATORS AS VECTORS OF MUMMY BERRY DISEASE IN HIGHBUSH BLUEBERRY

FEBRUARY, 2019

MATTHEW D. H. BOYER, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Lynn S. Adler

Background: Many plants must balance the need for pollination services with mediating the risk of pollinator-vectored pathogens. *Vaccinium corymbosum*, highbush blueberry, is negatively affected by an insect-vectored, fungal plant pathogen, *Monilinia vaccinii-corymosi* (MVC), the cause of mummy berry disease, in which the asexual spore mimics pollen grains and is transferred from blighted tissue to flowers via pollinators, resulting in inedible, hardened fruits. Highbush blueberry plants require outcrossed pollen for maximum yield and fecundity. Therefore, yield of blueberry plants rely on a balance between adequate pollination service and disease avoidance.

Approach: To explore the relationship between pollinator community and infection we used field observations and infection assessments to determine if differences in floral visitors can help to explain variation in infection between cultivars. To better understand the key vectors involved in transmission of MVC we used molecular quantification techniques to assess pathogen load on insect bodies and used a cage trial to determine how much of the pathogen is deposited by two common pollinators in a single visit. Finally, we used inoculation trials followed by fluorescence microscopy to determine if

plants must balance pathogen inhibition with fertilization success, as well as assessing whether pathogen germination contributes to disease resistance.

Results and Conclusions: When investigating community composition we found that *Apis, Bombus* and *Andrena* visitation varied with cultivar and that there was also a positive relationship between the proportion of floral visits by honeybees to individual plants and the percentage of infected fruits. This is the first study to our knowledge comparing fruit infection with visits by different bee species.

In our investigation of pathogen load on vectors and single-visit transmission success we found that bees, flies, and wasps were all common visitors and that all the bee species and several species of flies and wasps carried the pathogen. We found no differences between *A. mellifera* or *B. impatiens* in pathogen load or transfer efficiency in cages, suggesting that both of these species are equally capable of vectoring MVC during a single visit to a blighted stem and then a flower. Taken together, this research emphasizes the wide variety of floral visitors capable of carrying the MVC pathogen and demonstrates that two common pollinator species have similar potential to vector MVC to blueberry flowers during a single visit.

Finally, we found no tradeoff between pollen and fungal spore germination on floral reproductive parts, suggesting that disease resistance traits mediated by stigma traits may not come at a cost of reduced pollination. We also did not find a relationship between spore germination and published disease resistance. This study adds to our understanding of disease resistance in natural and agricultural systems, which is especially important due to mounting concerns over the use and cost of fungicides,

including negative effects on non-target organisms. Our findings also increase our understanding of the potential for both wild and managed pollinator species to contribute to the vectoring of a highly damaging blueberry pathogen, and plant pathogens in other systems as well.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

POLLINATOR COMMUNITY ASSOCIATED WITH PROPORTION OF *Vaccinium corymbosum* FRUITS INFECTED BY *Monilinia vaccinii-corymbosi* (MUMMY BERRY DISEASE)

Abstract

Plant diseases are a major source of economic loss and reduction in agricultural yield. Many plant diseases are vectored by the same pollinators relied upon on for outcrossing and increased fruit-set. Cultivars of crop plants often have both varying levels of resistance to these pathogens, and differences in floral traits that can influence pollinator preferences. *Monilinia vaccinii-corymbosi*, or mummy berry disease, is a fungal plant pathogen of highbush blueberry (*Vaccinium corymbosum*) in which the asexual spore mimics pollen grains and is transferred from blighted tissue to flowers via pollinators, resulting in inedible, hardened fruits. We used field observations of 14 blueberry cultivars over two years to investigate whether pollinator community varies by cultivar, if bee visitation is associated with fruit infection, and if the proportion or absolute number of floral visits by honeybee, bumblebee, and solitary bees is associated with differences in cultivar resistance. We found that *Apis, Bombus* and *Andrena* visitation varied with cultivar. We also found a positive relationship between the proportion of floral visits by honeybees to individual plants and the percentage of infected fruits. While bees have been known to carry *M. vaccinii-corymbosi*, this is the first study to our knowledge comparing fruit infection with visits by different bee species.

Introduction

Plant diseases cause major loss to food producers worldwide, and hinder the efforts of production agriculture to meet the food demands of an ever-growing human population (Pinstrup-Andersen 2000). Over one third of all agricultural crops are pollinated by animals, mostly insects (Klein et al. 2007). Unfortunately, many devastating plant diseases are vectored by the same insects plants rely on for pollination services (McArt et al. 2014a). Chemical pesticides and fungicides can help to mediate losses from disease in the short term, but both the use of chemical treatments and losses from disease continue to increase (Pinstrup-Andersen 2000), often due to the adaptation of pest organisms (Orke et al. 1994). Economic costs of chemical treatments (Legard et al. 2005), concerns of human health effects (Pearson et al. 2016), and negative effects on non-target organisms (Ladurner et al. 2005; Dijksterhuis et al. 2011) make finding alternative ways to mediate losses from disease a chief concern in production agriculture.

Morphological features of flowers can affect the suite of floral visitors (Bell 1985; Wilson et al. 2004), and cultivars of crop species often vary in their floral displays (Rick et al. 1978; Suso et al. 2007). For example, in highbush blueberry, pollinator visitation can vary with small differences in floral morphological traits between cultivar (Courcelles et al. 2013). Pollinators also vary greatly in their ability to transfer pollen (Adler & Irwin 2006), often due to morphological and behavioral differences (e.g., the presence of branched hairs and sonication, respectively) (Delaplane & Mayer 2000). Therefore, the suite of pollinators visiting individual cultivars may vary in their ability to vector pollen.

For pollinator-vectored diseases, variation in the suite of floral visitors could not only affect pollen transmission, but disease transmission as well. *Vaccinium corymbosum*, or highbush blueberry, is a commercial crop that is impacted by an insect-vectored, fungal pathogen. *Monilinia vaccinii-corymbosi* (MVC), or mummy berry disease, can reduce yield by up to 80% and cause major economic losses (Stretch et al. 2001). MVC produces conidia (secondary spore) that mimics pollen grains morphologically and behaviorally, in that spores adhere to insect bodies upon visitation (Batra 1983). Resistance to MVC infection varies greatly by blueberry cultivar (Ehlenfeldt et al. 2010), presenting an opportunity to examine the relationship between pollinator community composition and cultivar variation in disease resistance to a pollinator-vectored, plant pathogen.

V. corymbosum flowers are visited by a wide variety of insect species, many with the potential to vector MVC. A recent study found insects from 6 orders and 28 families visiting *V. corymbosum* flowers (McArt et al. 2016). Additionally, using a nested PCR analysis the same study found that of those insects collected, 5 orders and 18 families tested positive for the presence of MVC DNA. Social bee species, such as *Bombus impatiens* and *Apis mellifera*, are often used to supplement natural pollination services due to their large numbers of individuals per hive (hundreds to tens of thousands, respectively) and commercial availability. However, many solitary bees in the Andrenid and Halictid families also visit blueberry plants (Moisan-Deserres et al. 2014), and some solitary bees have been shown in certain systems to be more efficient pollinators than *A. mellifera* (Vicens 2009). However, the effects of solitary bees on disease transmission are unknown.

For growers seeking to reduce their dependency on chemical fungicides or those with a 'no-spray' or 'pick-your-own' business model, understanding the impacts of natural and managed pollinators is clearly important. In this study, we investigate the relationship between pollinator communities and MVC disease transmission by asking: 1.) Do key pollinator taxa vary in visitation between cultivars, 2.) How is total bee visitation to flowers associated with fruit infection, and 3.) Is visitation by social vs solitary bees associated with fruit infection?

Methods

Study system

Monilinia vaccinii-corymbosi is an ascomycete fungal plant pathogen infecting *Vaccinium corymbosum* plants across North America (Batra 1983). MVC infection is characterized by two distinct stages beginning with the distribution of windborne spores released from apothecia emerging from the substrate in the spring. This primary infection affects new shoot and stem tissues of *V. corymbosum*, creating blights, or 'pseudoflowers', that attract insect visitors by exuding volatile organic compounds that mimic floral scent (McArt et al. 2016) and by visually mimicking floral UV reflectance (Batra & Batra 1985). These co-opted tissues also produce larger, asexual spores (conidia) that, although sometimes spread by wind and rain, are primarily vectored by flower-visiting insects (Ngugi et al. 2002). Once vectored to receptive *V. corymbosum* stigmas, conidia mimic pollen grains in both their method of delivery and germination on stigmatic surfaces (Ngugi & Scherm 2004). Hyphae then grow, analogous to pollen tubes, extending down the stylar canal to the ovary resulting in secondary infection (Ngugi & Scherm 2004). Fruit infection results in fungus-filled locules, causing the

mature berry to become hard and grey instead of ripening normally. Infected berries, or mummies, then fall to the ground, overwinter, and emerge as ascocarps the following spring, each releasing upwards of 800,000 windborne spores and beginning a new infection cycle (Batra 1983; Batra & Batra 1985).

Field observations

From 29 May to 2 June 2014 and from 13 May to 2 June 2016, 239 highbush blueberry plants representing 14 cultivars (Table 1) at the Cold Spring Orchard in Belchertown MA (42.251143, -72.362321) were surveyed for insect visits. Cultivars were selected to span the breadth of resistance to MVC infection. Three-year-old plants were sourced from Fall Creek Nursery (Lowell, Oregon, USA), were between 0.5 and 1.5 m in height, and were planted randomly in six equidistant rows (3 m between plants, 3 m between rows). Each year, plants were surveyed on clear days, with 5 minutes per plant, from approximately 10 am to 3 pm, for a total of 44 person-hours/year, with each plant observed at least twice each year. We recorded visitor identity each time a new insect visited a plant during the 5 minute observation time, with a 'visit' being defined as each time a new insect landed on and probed a flower, or group of flowers, on that plant. Insect visitors in 2014 were identified to functional group (e.g. bumblebee, honeybee, solitary bee, Vespid, Coleopteran, Hemipteran, and Lepidopteran) according to general morphology, while in 2016 all insects were identified at least to genus and to species when possible. *Andrena vicina* was indistinguishable from *A. carlini* on the wing, as was *A. carolina* and *A. bradleyi*, and were combined for identification purposes in 2016. In 2014, total number of open flowers per plant was recorded once on 30 May during peak flowering to use as a covariate in analyses. All plants were netted to prevent loss from

foraging birds prior to fruit maturation post-bloom, after observations had ceased. Upon maturation in 2014, both healthy and infected fruits were counted, including those that had fallen to the ground. Fruit infection was measured only in 2014 due to the host farm's application of fungicide to control MVC in 2016.

Statistical analysis

To determine whether number of visits per 5 minute observation by *Andrena*, *Bombus*, and *Apis* species (the three most common genera to visit plants) differed between cultivar in 2014, we used generalized linear models with a Poisson distribution and log link function, cultivar as a fixed factor and number of flowers per plant as a fixed covariate. We then analyzed differences between cultivar using Tukey's HSD pairwise comparisons in the MultComp package for R (R Development Core Team 2018). To determine if the proportion of visits by *Andrena*, *Bombus*, and *Apis* species (i.e. the number of visits by each genus per observation divided by the number of all visitors per observation) differed between cultivar in 2014, we used generalized linear models with binomial distributions and logit link functions, cultivar as a fixed factor and number of flowers per plant as a covariate. We conducted the same analyses for the 2016 data, but included date as a random covariate because observations spanned a longer rate of dates, and *Andrena* at the species scale. *Apis mellifera* visits were too infrequent to analyze in 2016.

To determine whether the proportion of bee visitation (i.e. number of combined *Andrena*, *Apis*, *Bombus,* Halictid and Megachilid visits per observation divided by total visits per observation) is associated with the percentage of infected fruits for each plant, including all cultivars, linear regression analyses were used for the 2014 data.

Regressions included number of flowers per plant as a covariate. Linear regressions were also used to determine the relationship between the proportion of *Andrena*, *Apis*, and *Bombus* visits separately and the percentage of infected fruits. To determine whether cultivars varied in the proportion of infected fruits for 2014 only, arcsine transformed proportions of infected fruits to healthy fruits were analyzed using an ANOVA with cultivar as a fixed factor and a Tukey's HSD to determine differences between cultivars. GLMs assessing bee visitation differences between cultivars and associated multiple comparisons were analyzed 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 recorded insect visitors from four orders: Hymenoptera, Diptera, Coleoptera, and Lepidoptera. However, Hymenoptera represented the majority of visits, including species in Andrenidae, Apidae, Halictidae, Megachilidae, and Vespidae. *Bombus impatiens*, *Andrena* spp., and *A. mellifera* were the most common taxa in 2014, in that order, while *Andrena* spp., *B. impatiens*, were the most common in 2016.

Results from generalized linear models applied to absolute insect counts from 2014 revealed insect visits by *Andrena, Apis* and *Bombus* species varied significantly with cultivar (Table 1-2). Number of flowers per cultivar was also a significant predictor of bee visitation (Table 1-2). Results from Tukey's HSD multiple comparisons showed significant differences between cultivars in *Bombus* (Figure 1-1) and *Andrena* (Figure 1-2) visits in 2014, while there were no differences in *Apis* visits (Figure 1-3). Generalized linear models for absolute insect counts from 2016 revealed insect visits by all *Andrena*

species, *A. vicina/carlini*, and *Bombus* varied by cultivar (Table 1-3). However, results from Tukey's HSD multiple comparisons showed no significant differences between individual cultivars. There was no significant difference between models with and without date as a random factor, and thus date was dropped from the model. In any year, only absolute counts of insects were significantly associated with cultivar while percentage of visitation was not.

There was a significant, positive relationship between the percentage of infected fruits and both proportion of overall bee visits (Figure 1-4. $r^2 = 0.06$, $n = 239$, $p < 0.0013$) and proportion of *A. mellifera* visits (Figure 1-5. $r^2 = 0.265$, $n = 239$, $p = 0.0001$). However, there was no significant relationship between fruit infection and proportion of visits by any other bee taxa, including Bombus (Figure 1-6; $r^2 = 0.188$, $n = 239$, $p = 0.6046$), and solitary bees (Figure 1-7; $r^2 = 0.19$, $n = 239$, $p = 0.6761$). Cultivars varied significantly in proportion of infected fruit (Figure 1-8; $F_{13, 233} = 28.1975$, $p < 0.0001$). Jubilee had the highest proportion of infected fruit, with over 45% of total fruits infected by MVC, followed by Blueray with 23%, and Earliblue and Toro each at approximately 15%, while Bluejay, Reka, and Southmoon had the lowest with under 5% infection.

Discussion

Insect visitors recorded in this study were consistent with those in prior work assessing *V. corymbossum* pollinator communities. We recorded visitations by four orders: Hymenoptera, Diptera, Lepidoptera, and Coleoptera. By far, Hymenoptera were the most common and were comprised of the families Andrenidae, Apidae, Halictidae, and Vespidae. One study using molecular identifications of insects netted in blueberry plantings recorded three additional Hymenopteran families: Megachilidae, Cephidae, and

Tenthredinidae (McArt et al. 2016). Another study characterizing *V. corymbosum* bee visitation in central New York state found predominantly *A. mellifera, B. impatiens*, and *Andrena* spp. (MacKenzie & Eickwort 1996). Interestingly, that study found differences in the dominant species from site to site. Although all observations in our study were conducted at a single site, we saw a two-fold reduction in the abundance of *A. mellifera* from 2014 to 2016. Yearly variation in pollinator communities is not uncommon. For example, one study examining pollinator efficiency in *Asclepias tuberosa* noted a ten-fold increase in *A. mellifera* as well as a 50% reduction in *B. impatiens* abundance over a two-year survey (Fishbein & Venable 1996). Other studies on *V. ashei* have noted fluctuation in temporal abundance of *A. mellifera*, likely resulting from shifts in foraging preference between competing flowering species (Cane & Payne 1993). Changes in the abundance of one pollinator species can affect others (Brosi & Briggs 2013); for example, one study found that in the presence of honeybees, bumblebee diet became more limited or shifted to different plant species (Forup & Memmott 2005). This indicates that although key pollinators may be consistent across blueberry plantings, the dominant pollinator species may change spatially and temporally, possibly impacting pollination services and disease transmission.

There was a significant effect of cultivar on insect visitation, indicating that pollinators may prefer floral traits that vary between highbush blueberry cultivars. Cultivar had a significant effect on *Apis, Andrena*, and *Bombus* visits in 2014 (Table 1-2), and on *Andrena* and *Bombus* visits in 2016 (Table 1-3). Additionally, cultivar had a significant effect on *A. vicina/carolina* visits, but not on *A. carlini/bradleyi,* in 2016 (Table 1-3). Insect pollinators are often attracted to flowers by shapes, colors, and scent

(Leonard et al. 2011), and these traits can vary between cultivars of agricultural plants. Highbush blueberry cultivars often vary in flower morphology (Arrington & Wasko DeVetter 2018), and a recent study found differences in bumblebee and honeybee visits to plants between four *V. corymbosum* cultivars based on five floral dimensions (Courcelles et al. 2013). That study concluded that *A. mellifera* and *B. impatiens* visits may be a function of corolla opening and depth. Only one cultivar, Bluecrop, was common between that study and ours, as our cultivars were selected based on disease resistance and not differences in floral size or shape. Bluecrop was least preferred by honeybees and bumblebees in Courcelles et al. and was among those least preferred by both *Andrena* (Figure 1-1) and *Bombus* (Figure 1-2) in our study in 2014. Bluecrop flowers have a smaller throat diameter that may make access for larger bees difficult, but easier for the smaller *Andrena* species. Since Bluecrop was not preferred by small or large bees (*Andrena* and *Bombus*, respectively), this suggests that floral dimensions alone are not the trait to which these genera are attracted, but instead that a suite of traits may work in combination. A recent study has shown that volatile organic compounds vary between blueberry cultivars, and that blueberry pollinators were attracted to traps baited with compounds mimicking floral VOCs (Rodriguez-Saona et al. 2009), providing another source of variation that may explain differences in pollinator visits between cultivars.

We found a significant, positive relationship between bee visits and percentage of infected fruits (Figure 1-4). A recent study using a nested PCR analysis to detect the presence of MVC on insects netted in the field showed that not only do bees carry MVC (5 of 7 Hymenopteran families sampled), but that many Dipteran families do as well (7 of

9 families), and so all are potential vectors (McArt et al. 2016). Additionally, that study used camera traps to film insect visitors and found that bees accounted for 75-80% of total visits, with flies at 14- 23%. Flies were more likely to visit blighted tissues than bees, and MVC conidia were more likely to be present on bees than flies, raising the question of what role flies play in overall disease transmission. The rarity of observing flies on floral tissues, coupled with our result that bee abundance is significantly associated with fruit infection, demonstrates that bees are likely a primary vector of MVC to *V. corymbosum* flowers.

A wide variety of bee species have been recorded visiting highbush blueberry, but generally *Apis*, *Bombus*, and solitary bees are considered the primary pollinators (Rao et al. 2009; West & McCutcheon 2009; Isaacs & Kirk 2010). In examining how visitation by these individual groups affects MVC infection, we found a significant, positive relationship between the proportion of honeybee visitations and the percent of infected fruits (Figure 1-5), and no significant relationship between the proportion of bumblebee (Figure 1-8) and solitary bee visitations (Figure 7). In a cage trial evaluating MVC transmission between the two species, *A. mellifera* readily visited pseudoflowers while *B. impatiens* did not (Boyer unpublished data), and thus may carry more MVC conidia when visiting flowers. Additionally, honeybees only accounted for 5% of total bee visits overall, yet had the strongest relationship with fruit infection (Figure 1-5), suggesting that they are the most efficient vector of MVC of the species we tested. In some studies the most abundant pollinator is the most important to pollen transfer (Olsen 1997), but in other systems, common floral visitors do not contribute equally to vectoring of pollen grains by either carrying low levels of pollen loads on their bodies (Watts et al. 2011) or

having low transfer efficiency of pollen to flowers (Javorek et al. 2002). Pollinators vary greatly in their pollination efficiency (Adler & Irwin 2006), or the number of pollen grains picked up and transferred to receptive stigmas (Primack & Silander 1975; Herrera 1987). Since fungal conidia mimic pollen grains chemically and morphologically (Ngugi & Scherm 2004), as well as in delivery method (Batra 1983), it is likely that pollinator species vary in MVC transmission as well. While honeybees are expected to be a vector of MVC (Batra & Batra 1985), this is the first study to our knowledge to compare visits by different pollinator species with subsequent fruit infection.

The relationship between honeybees and fruit infection may be important for growers wishing to supplement natural pollination services. *Vaccinium corymbosum* yield is often increased by outcrossed pollination (Delaplane & Mayer 2000; Ehlenfeldt 2001a), and many farms supplement pollination services with commercially available colonies of *A. mellifera* or *B. impatiens*. While honeybee colonies are perennial, workers are numerous, and are able to pollinate a wide variety of crops, they may also be more likely vectors of MVC. Although bumblebee colonies are short-lived with fewer workers, bumblebees demonstrated no significant relationship between visitation and fruit infection, work in a wider variety of weather conditions, and are able to sonicate blueberry flowers to release greater amounts of pollen per visit (Delaplane & Mayer 2000). Farms with a history of MVC infection may consider supplementation with bumblebees, while farms opting for *A. mellifera* may consider a targeted biocontrol strategy to curb losses to MVC. Efforts to use bees as vectors of biocontrol agents to control MVC in *Vaccinium virgatum* have been successful. For example, *Bacillus subtilis* dispensers mounted to *A. mellifera* hives reduced the incidence of MVC in

Vaccinium virgatum (Dedej et al. 2004), and may be effective in *V. corymbosum* as well.

Cultivars in our field plot differed significantly in levels of infection (Figure 1-8). Jubilee and Blueray had the highest proportion of infected fruit, followed closely by Earliblue and Toro (Figure 1-8). This is consistent with prior work assessing cultivar resistance to MVC (Batra 1983), with proportion of infected fruit corresponding to resistance rankings developed over a multiyear field study (Ehlenfeldt et al. 2010). Additionally, Blueray and Jubilee had the highest numbers of both *Andrena* and *Bombus* visits (Figures 1-1 & 1-2), but there was no significant relationship between visits by these taxa and infected fruits. These cultivars may exhibit traits that make them generally desirable to many pollinator species and thus receive more conidia, or other biological mechanisms, such as conidia and hyphal inhibition in the stigma and stylar canal (Lehman et al. 2007), may help to explain variation in resistance to secondary infection. While long surmised that bees were the likely primary vectors of MVC, this is the first study, to our knowledge, linking a particular clade of bees to the proportion of infected fruit. Honeybees service a wide variety of crops and are often considered top pollinators (Delaplane & Mayer 2000). Even in systems such as blueberry with poricidal anthers that require buzz pollination, incidental contact of anthers by honeybees, coupled with abundant visits, makes them effective pollinators of blueberry. However, their effectiveness as pollinators may extend to vectoring MVC as well. In this study, we provide evidence that cultivars vary in both their proportion of infected fruit as well as pollinator visits. Further, we provide supporting evidence that bees are the primary vectors of MVC. In particular, honeybees are linked to higher proportion of infected fruit

and have no preference for specific cultivars in our study. These findings may help to guide growers in their decisions pertaining to the use of supplemental pollinator species.

Acknowledgments

I would like to thank Cold Spring Orchard and Shaun McIntyre for the upkeep of our blueberry plot. Thanks to Rebecca Warren and Graham Snyder for their help with data collection. This work was made possible by funding from USDA NIFA award 2016-67011-24722, the Lotta M. Crabtree Fellowship, and the Organismic and Evolutionary Graduate Program at the University of Massachusetts Amherst.

Table 1-1. Cultivars in observational plot. n = number of plants of that cultivar.

Cultivar	***N***
Bluecrop	19
Bluegold	19
Bluejay	18
Blueray	18
Chippewa	18
Darrow	18
Earliblue	18
Hardyblue	18
Jubilee	16
Patriot	15
Polaris	18
Reka	18
Southmoon	10
Toro	16
Total	**239**

Table 1-2. Summary of GLM results testing the effect of cultivar and number of flowers per plant on *Andrena, Bombus*, and *Apis* visitation in 2014. Significance is considered at p=<0.05 (bold type).

Factor	***Andrena***			***Bombus***			***Apis***		
	χ^2	df	*p*	χ^2	df	*p*	χ^2	df	*p*
Cultivar	58.9590	13	<0.0001	59.5460	13	<0.0001	43.8410	13	<0.0001
Flowers	19.3500	1	<0.0001	41.0490	1	<0.0001	28.7990	1	<0.0001

Table 1-3. Summary of GLM results testing the effect of cultivar on *Andrena, A.vicina/carlini,* and *Bombus impatiens* visits in 2016. Significance is considered at p=<0.05 (bold type).

Factor	**Cultivar**		
	χ^2	df	*p*
All Andrena	28.8020	13	**0.0070**
Bombus	23.7790	13	**0.0332**
A. vicina/carlini	22.4090	13	**0.0493**
A. carolina/bradleyi	20.7550	13	0.0779

Figure 1-1. Mean number of *Bombus* visits per observation by cultivar. Error bars are +/- one standard error of the mean. Cultivars not sharing letters are significantly different, as determined by Tukey's HSD comparison.

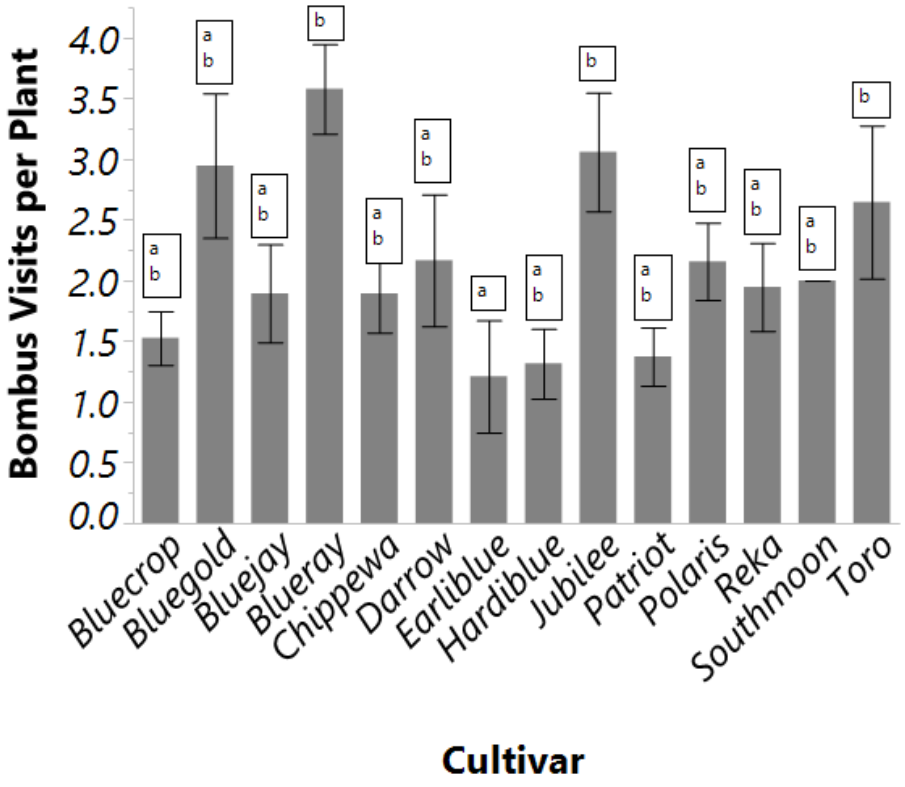


Figure 1-2. Mean number of *Andrena* visits per observation by cultivar. Error bars are +/- one standard error of the mean. Cultivars not sharing letters are significantly different as determined by Tukey's HSD comparison.

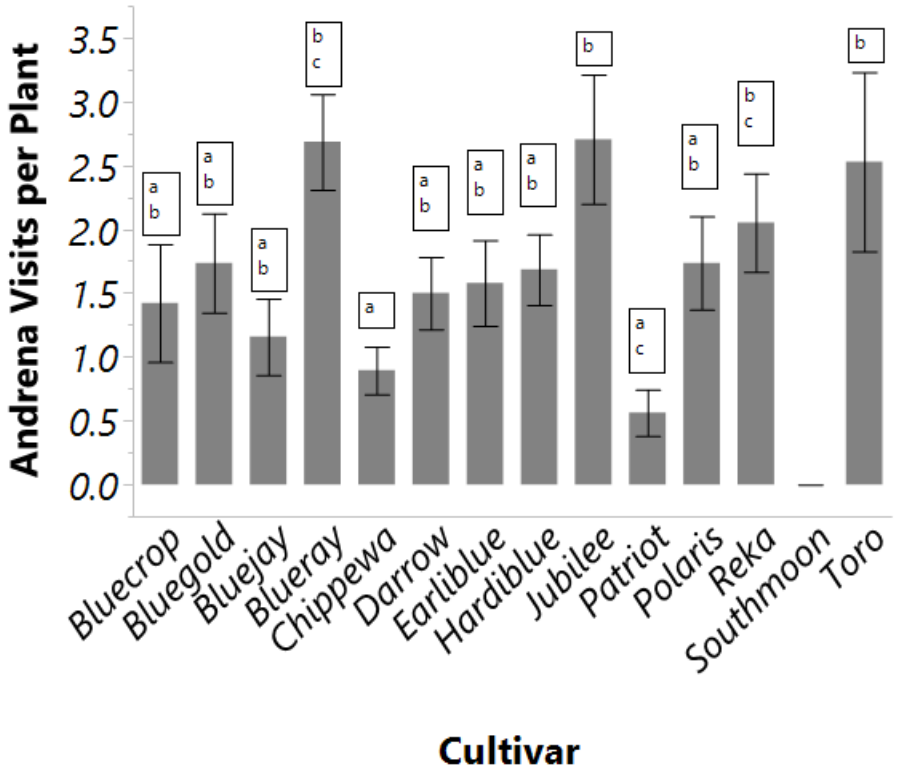


Figure 1-3. Mean number of *Apis* visits per observation by cultivar. Error bars are +/- one standard error of the mean. No significant differences were found between cultivars.

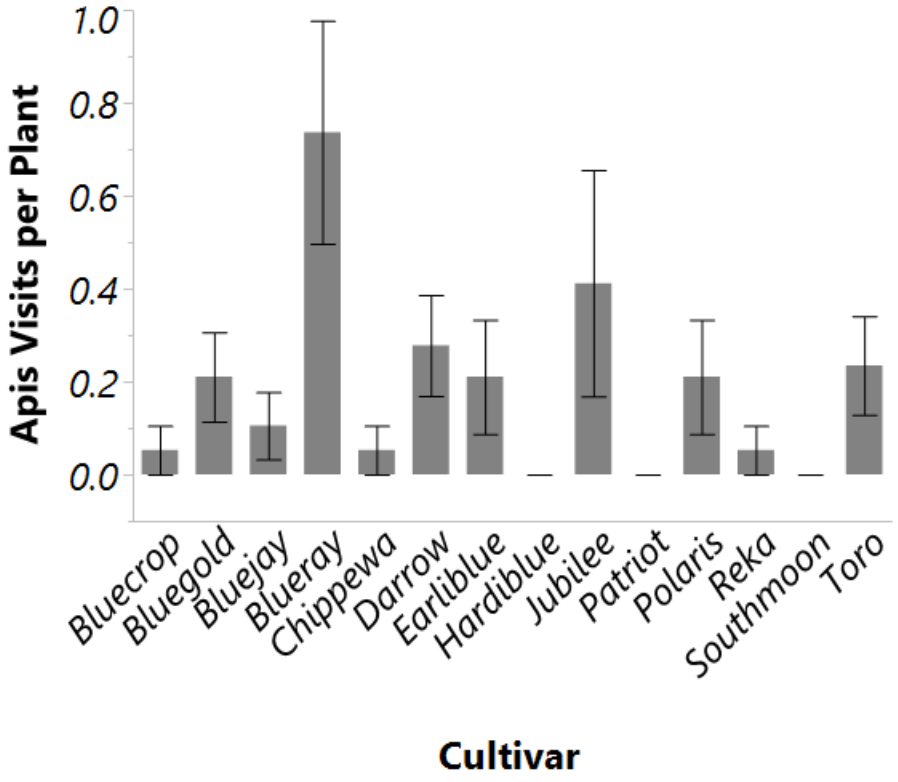


Figure 1-4. Results from linear regression illustrating the relationship between the proportion of visits by bees (all *Andrena*, *Apis*, and *Bombus* divided by total number of visits by all taxa) and the percentage of infected fruits. $r^2 = 0.06$, n = 239, $p < 0.0013$.

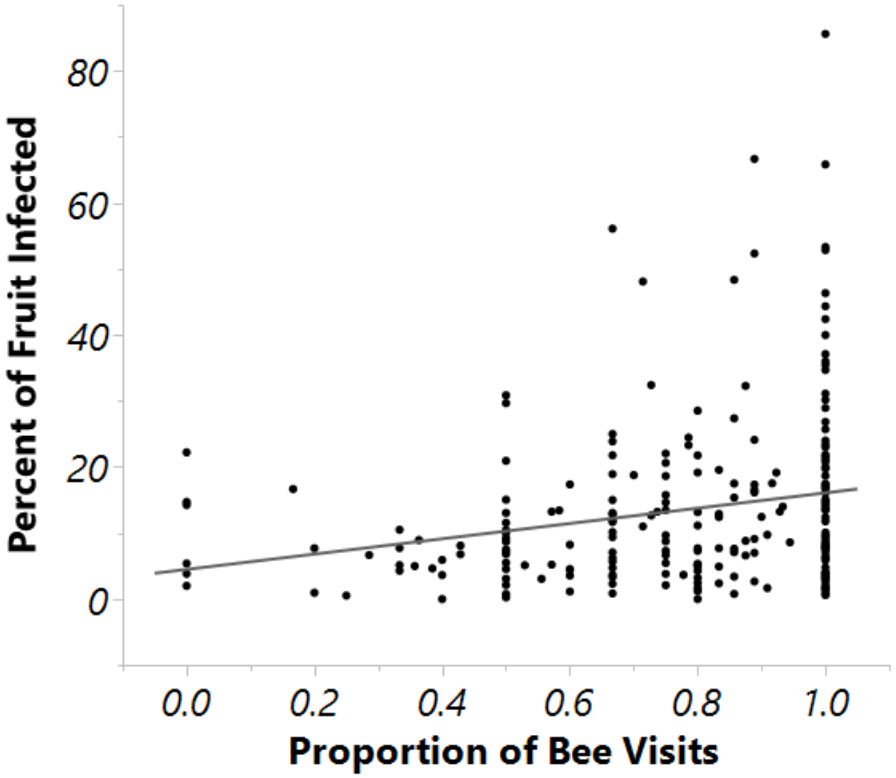


Figure 1-5. Results from linear regression illustrating the relationship between the proportion of visits by honeybees and the percent of infected fruits. $r^2 = 0.265$, n = 239, p = 0.0001

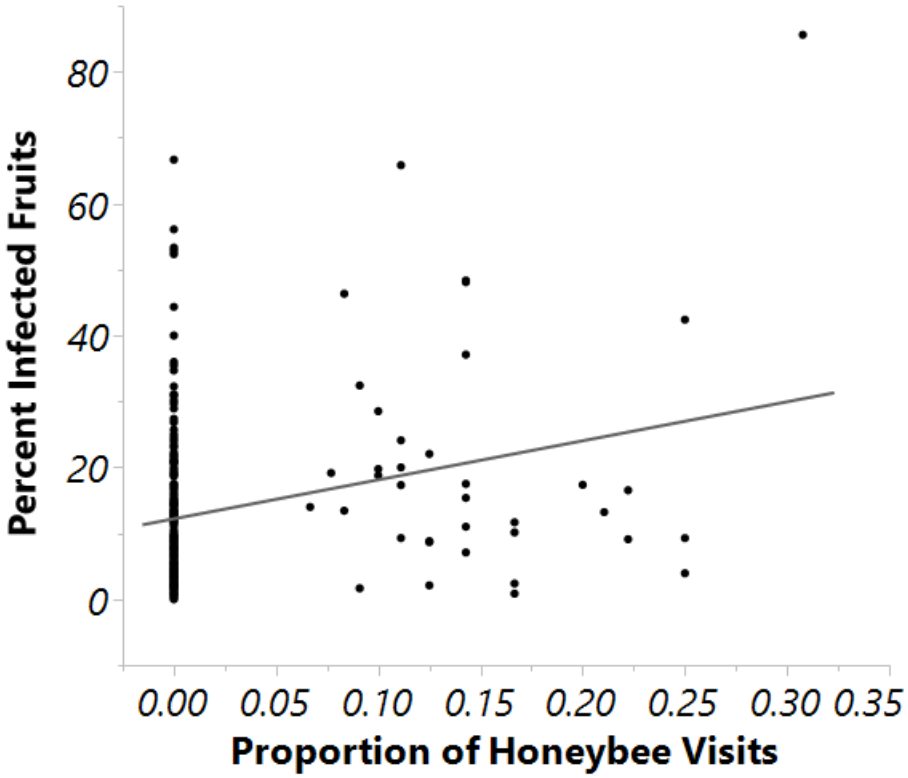


Figure 1-6. Results from linear regression illustrating the relationship between the proportion of visits by bumblebees and the percent of infected fruits. $r^2 = 0.188$, n = 239, p = 0.6046

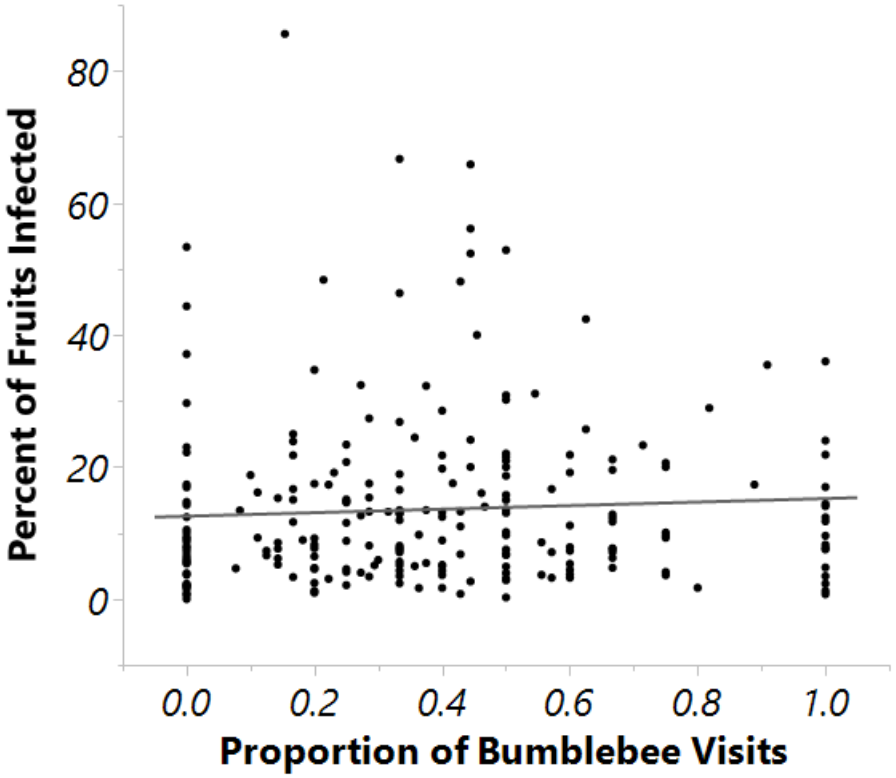


Figure 1-7. Results from linear regression illustrating the relationship between the proportion of visitations by *Andrena* and the percent of infected fruits. $r^2 = 0.19$, n = 239, p=0.6761,

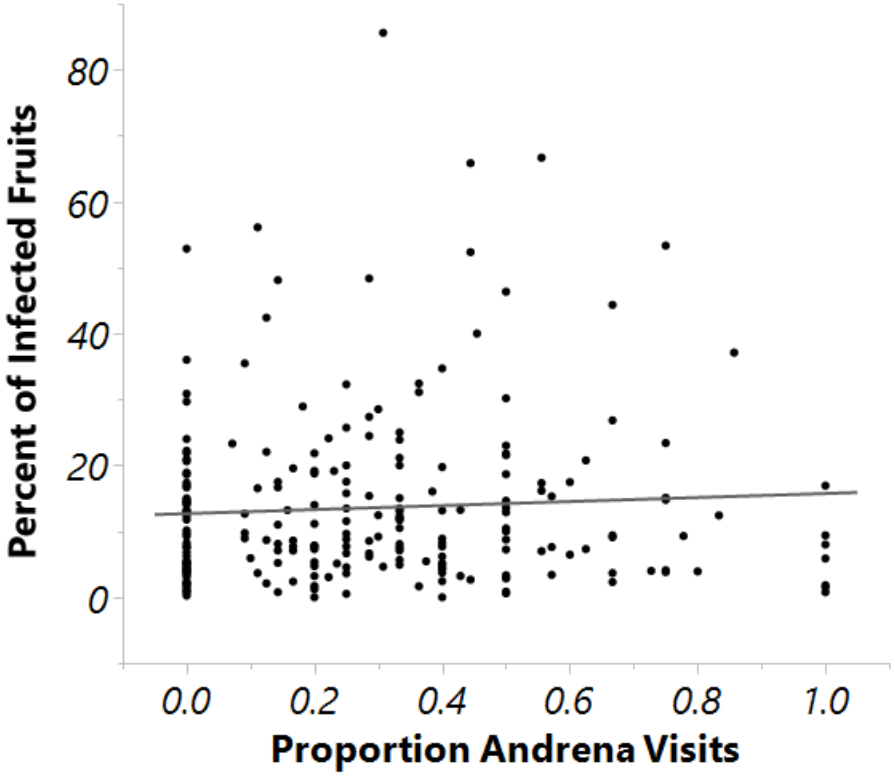
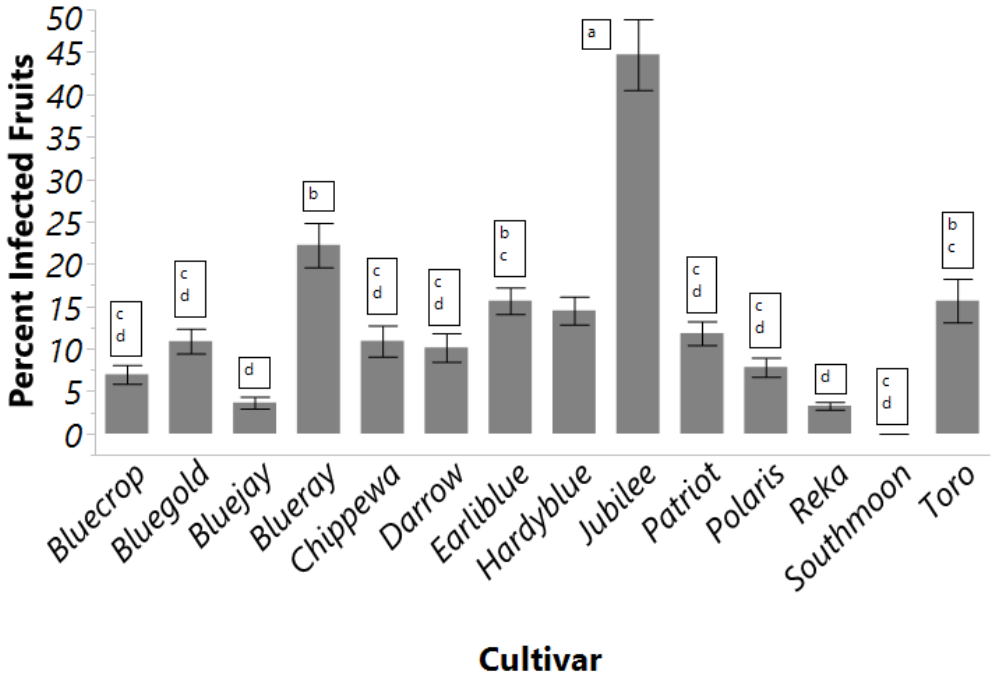


Figure 1-8. Percent of infected fruits varied by cultivar. $F_{13, 233} = 28.1975$, $p < 0.0001$. Cultivars not sharing letters are significantly different as determined by Tukey's HSD comparison.



CHAPTER 2

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

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, mummy berry 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 mummy berry during a single visit to a blighted stem and then a flower. This research demonstrates the

variety of floral visitors that carry mummy berry and that two common commercial pollinator species have similar potential to vector mummy berry to blueberry flowers during a single visit.

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) since both wild and managed insect taxa often vary widely in their efficiency as pollinators (Willmer et al. 2017).

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. 2014b). 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 avoidance.

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). 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). *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). 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.

Understanding which wild and managed pollinators are involved in the spread of the pathogen may help to minimize the damaging effects of mummy berry in environments where fungicide application is not feasible. 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 behavioral 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 behavioral differences in contact and interaction with floral reproductive structures.

Behavior 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 behavior, 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. To address Goal 1, we collected blueberry-visiting insects from a no-spray orchard infected with mummy berry disease. 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. To address Goal 2, we performed a cage trial to assess comparative transfer efficiency of mummy berry conidia by *Apis mellifera* and *Bombus impatiens*.

Methods

Study system

Monilinia vaccinii-corymbosi (MVC hereafter), or mummy berry disease, 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 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).

Insect field collection

During peak bloom from May 5 to June 4, 2014, insect visitors of highbush blueberry flowers were collected at Quonquont Orchard in Whately, Massachusetts, USA (42.444° N -72.639° W). Collection took place 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.

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 (Table 2-1; 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 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:

AAGGGCAGAACCTCTCCACCCTTTGTGTATTATT**ACTTTGTTGCTTTGGCGG**
GCCGCCTCCGGGCCTCGCGTGCCCGCCAATGACCTAACCCT

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

AAGGGCAGAACCTCTCCACCCTTTGTGTATTATTA**ATCGATCGATCGATCGA**
TCGATCGATCGGCCTCGCGTGCCCGCCAATGACCTAACCCT

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 17 through June 2, 2017, cage trials were conducted 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 (42.136, -72.434) 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 (42.444, -72.639) 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 did not differ 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.

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 4ºC 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.

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 analyzed two components of the potential to transmit MVC. First, we analyzed 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 compared 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. To compare pathogen load between species or functional groups as fixed effects, number of copies of MVC (both raw counts and values adjusted by insect bodyweight (ITS count/fresh body weight in g)) were compared using generalized linear models with negative binomial distributions (to adjust for overdispersion) and log link functions. Species with fewer than five samples (Table 2-3) were dropped from this analysis. To compare means of ITS sequence counts, a Tukey's post hoc comparison in the MultComp package for R was used (R Development Core Team 2018).

For the cage trials, foraging time on blights and flowers was analyzed 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 analyzed 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 (Table 2- 2). Of the

232 specimens 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 (Table 2-3). 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. 2-1). 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 (Figure 2-2) 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, $P = 0.9063$; Figure 2-3). *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 2001b), and an assortment of bee species have been observed visiting *V. corymbosum* flowers (MacKenzie & Eickwort 1996; Tuell et al. 2009). For example, one study investigating *V. corymbosum* bee communities in Oregon recorded 30 bee species spanning 5 families (Rao et al. 2009). Generally, bees are considered to be the primary pollinators (West & McCutcheon 2009; Isaacs & Kirk 2010); thus, little attention has been paid to non-bee visitors. We found 19 hymenopteran species visiting *V. corymbosum* spanning 5 families including Apidae, Andrenidae, and Halictidae, but also found Ichneumonids and seven species of Vespids visiting flowers, including common and aerial yellowjackets and Bald-faced Hornets (Table 2-2). However, the contribution of these species to blueberry pollination is uncertain as these taxa may be nectar robbers, thieves, or searching for prey species. We also identified 13 species of flies (Table 2-2), and 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 sampled in our study are considered key pollinators, the

broad community of insect visitors found may indicate that *V. corymbosum* crops will be resilient to decline of particular bee species.

We found that 25 of the 46 floral visitor species tested positive for MVC (Table 2-2), and of those species with six or more representatives, 9 out of 10 species tested positive (Table 2-3). 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). Of bees tested in our study, *A. mellifera* tested positive most often, with over 76% of samples returning positive results. *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.

We found a great disparity in the presence of MVC between fly species (Table 2-3). We identified ten specimens of *Chrysops carbonarius* (deer fly), with none of the samples testing positive for MVC. However, in *Mallota posticata*, a bee mimicking fly, all 6 samples tested positive for MVC. Behavioral differences are unlikely to explain this disparity since *M. posticata* and *C. carbonarius* are both floral visitors (Maier & Waldbauer 1979; Karolyi et al. 2014), but morphological differences may. 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. Recent camera trap work has shown that flies are more likely to visit blighted blueberry tissues than bees, although bees are more likely to carry MVC than flies (McArt et al. 2016). This suggests that both blight-visiting behavior and morphological features may work in tandem to maximize

vectoring potential. Further, flies are less commonly observed on blueberry flowers than bees (McArt et al. 2016), suggesting that although they may carry MVC, their role in transmission may be limited.

In our comparison of raw counts of MVC ITS regions, *D. maculata*, or the Bald-faced Hornet, carried the highest average MVC load of all insects sampled, when considering only insects that tested positive for the presence of MVC (Figure 2-1, Table 2-2). Prior work using PCR to determine presence/absence of MVC 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). This suggests that while incidence of Vespids carrying MVC may be relatively low on a presence-absence basis, 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 that recorded visiting taxa indicate 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 our comparison of ITS regions adjusted for insect body size, we found that *A. vicina* had the highest average MVC ITS count. *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.

Solitary bees carried more MVC than social bees, and social bees carried more than flies when comparing raw ITS counts (Figure 2-2A). 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. 2-2B). Congruent with prior work (McArt et al. 2016), we found that flies are less likely to carry any MVC than bees, and when they do they carry MVC, they carry less. The wide variety of behaviors exhibited by Dipteran taxa found in our study may help to explain the lower quantity of MVC found in flies. For example, taxa such as those in the Sarcophagidae 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 different intervals and with different behaviors than other Dipteran taxa. Given the vast diversity of Dipteran species, more study relating fly behavior to MVC transmission is needed.

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). We found no significant difference in raw or adjusted ITS counts (Figure 2-1), nor in number of conidia deposited by *A. mellifera* and *B. impatiens* (Figure 2-3). Our findings suggest that growers using honeybees

compared to bumblebees for supplemental pollination services, or environments with more of one species and less of the other, are unlikely to see a difference in pathogen transmission. *Bombus impatiens* are more effective extracting blueberry pollen due to their sonication behavior, 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 behavior (Delaplane & Mayer 2000). Despite the lack of sonication behavior, honeybees may incidentally release small amounts of pollen while retrieving nectar from blueberry flowers (Javorek et al. 2002). Although the amount of incidental pollen released by nectar harvesting is small in comparison 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 arguments may be made for the effectiveness of each species as a blueberry pollinator, our findings suggest that they may be equal vectors of mummy berry spores on a per-visit basis.

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 species behavior. Bees of either species may visit multiple blights before any given flower, or *vice versa*. Conidia deposition may rely on a cumulative

effect of multiple visits, in which case single visits may not be enough to determine differences between species. Field observations combined with fruit infection assessments have indicated that higher proportions of *A. mellifera* visitation are associated with greater incidence of fruit infection (Boyer, unpublished data). Thus, while our single-visit experiment demonstrated that conidial deposition per visit does not differ between these species, more comprehensive behavioral observations of visits to blights and flowers in the field may be necessary to understand transmission dynamics. *Bombus impatiens* may transfer fewer conidia in field conditions due to the overall aversion we observed for bumblebees landing on blights. *B. impatiens* had to be chilled to a state of immobility and allowed to revive on blighted tissues, while *A. mellifera* often preferred pseudoflowers to flowers, and needed no coaxing to visit blighted tissues. However, our presence/absence data indicate that *B. impatiens* do carry MVC (Table 2-3), but differences in floral manipulation may cause conidia to be present on bee body parts that do not often contact floral stigmas. Either way, these behavioral differences would be expected to impact pathogen transfer under natural conditions.

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 identifications of floral visitor community beyond bees 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. We found that there was 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.

Acknowledgements

We thank Quonquont orchard for the use of their blueberry crop for insect, flower, and blight collection. Kurt Lamour of Floodlight Genomics for DNA extraction, quantification, and processing. H. Danielle Capps and the Scherm Lab at the University of Georgia for providing extracted *Monilinia vaccinii-corymbosi* DNA samples, and BioBest Sustainable Crop Management for the donation of two bumblebee colonies. We also thank J. Lehman for his guidance working with this system and in fluorescence microscopy protocols. We thank G. Snyder for his assistance with field insect collection. Thanks to Elizabeth Connor and Katherine Dorfman for the use of their fluorescence microscopy equipment. This work was made possible by funding from the United States Department of Agriculture National Institute of Food and Agriculture award 2016-67011-24722, the Lotta M. Crabtree Fellowship, and the Organismic and Evolutionary Graduate Program at the University of Massachusetts Amherst.

Table 2-1. 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)

Table 2-2. 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 2-3. 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 melifera	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 2-1. Species comparison of MVC ITS regions for (A) raw counts and (B) counts 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. Error bars are +/- one standard error of the mean.

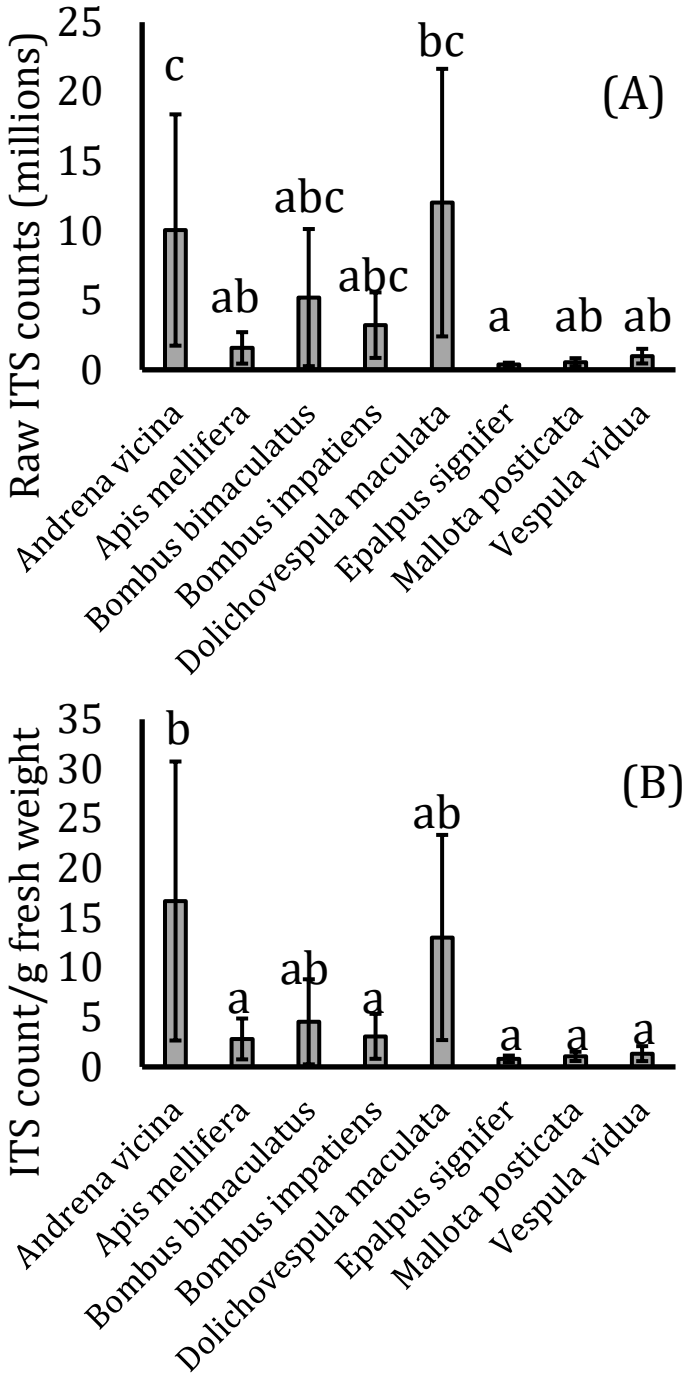


Figure 2-2. Functional group comparison of MVC ITS regions for (A) raw counts and (B) counts adjusted by body size (g fresh weight); 'social' and 'solitary' refer to bee species. Groups with different letters are statistically different as determined by Tukey's post hoc comparisons. Error bars are +/-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

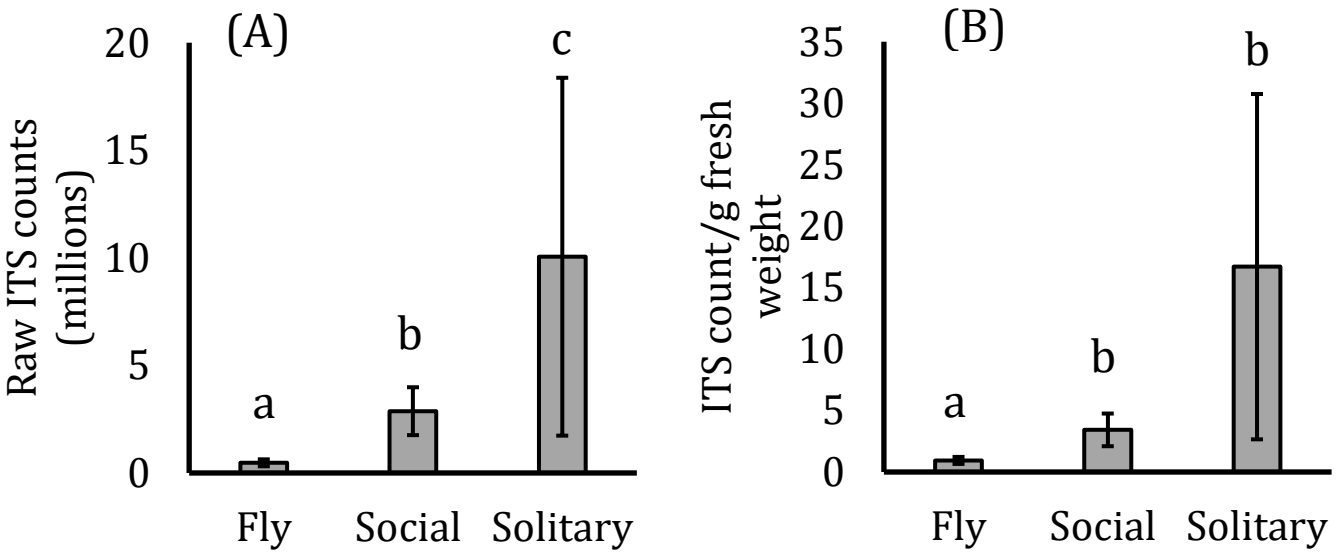
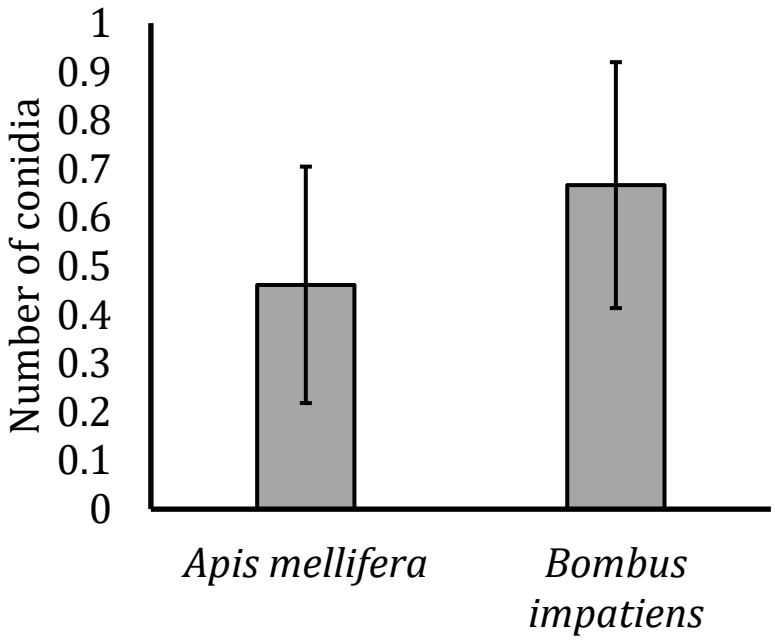


Figure 2-3. Mean conidia on stigmas visited by *A. mellifera* and *B. impatiens* in field cage trials. Bee species do not significantly differ in conidia deposited per single visit (see Results). Error bars are +/-1 standard error of the mean.



CHAPTER 3

NO TRADEOFF BETWEEN POLLEN GERMINATION AND RESISTANCE TO A FLORAL PATHOGEN (*Monilinia vacinii-corymbosi*) IN *Vaccinium corymbosum*

Abstract

Many plants must balance the need for pollination services with mediating the risk of pollinator-vectored pathogens. Highbush blueberry *(Vaccinium corymbosum)* plants require outcrossed pollen to maximize yield. *Monilinia vaccinii-corymbosi*, the cause of mummy berry disease, is the most damaging fungal pathogen affecting blueberry crops and is principally vectored by pollinators. Therefore, yield of blueberry crops relies on a balance between adequate pollination service and disease avoidance. Agricultural cultivars have varying levels of resistance to the pathogen, but the mechanisms of resistance are unclear. We examined whether resistance is related to inhibition of fungal spore germination at the floral stigma, and whether fungal spore germination is correlated with germination of pollen grains, creating a potential tradeoff between pollination and disease resistance. Flowers from 25 cultivars were hand inoculated with pollen and conidia that were allowed to germinate and then stained for fluorescence microscopy to determine germination success. Germinated pollen and conidia were counted and compared with published cultivar resistance rankings, and cultivars were evaluated for relationships between pollen and conidia germination. We did not find a tradeoff between pollen and fungal spore germination, suggesting that disease resistance traits mediated by stigma traits may not come at a cost of reduced pollination. We also did not find a relationship between spore germination and published

disease resistance. However, published disease resistance rankings are based on infection in the field, which would be a result of pollinator visitation as well as stigmatic interactions with conidia. With mounting concerns over the use and cost of fungicides, including negative effects on non-target organisms, adding to our understanding of disease resistance in economically important crops such as blueberry can help to inform breeders and growers to maximize yield while combating the negative effects of this pathogen.

Introduction

Trade-offs between reproduction and avoiding antagonists are ubiquitous in both animals and plants. Mobile animals must reconcile the challenges of attracting mates while avoiding predation (Andersson 1994). Similarly, sessile plants often must balance attracting pollinators with avoiding antagonists that use flowers as cues to find hosts, such as florivores, nectar robbers, and seed predators (Strauss and Whittall 2006, Adler 2007). For example, high concentrations of a volatile organic compound emitted by *Polemonium viscosum* flowers can deter nectar robbers but also reduce visitation by pollinators (Galen et al. 2011). Similarly, high concentrations of nectar alkaloids can deter nectar robbers, but also reduce pollination services (Adler and Irwin 2005). These examples highlight conflicting selection on floral traits driven by multispecies interactions, shaping selection in directions not expected in a simple pairwise interaction (Strauss and Irwin 2004).

For several plant species, reproduction can also carry the cost of disease transmission vectored by pollinators (McArt et al. 2014). Flowers may provide both signals and rewards to attract pollinators, but high visitation combined with a

microclimate conducive to infection can make flowers a point of entry for pathogens. In several cases, specific floral traits have been identified that mediate successful infection. For example, anther smut fungi (*Microbotryum violaceum*) infect floral tissues of *Silene spp.* (Alexander and Antonovics 1988), and floral traits such as bloom duration and morphology can influence the outcome of pathogen infection (Kaltz and Shykoff 2001). Also, when male *Silene latifolia* flowers senesced quickly following inoculation, these flowers were less likely to become infected (Kaltz and Shykoff 2001). Nectar provides a climate rich in resources that can promote microbial growth, but may also defend against pathogens via the nectar redox cycle (Carter and Thornburg 2004) Additionally, secondary metabolites in nectar may alter pathogen interactions (reviewed in McArt et al. 2014). For example, *Cucurbita* sp. nectar can inhibit the growth of the pathogen *Erwinia tracheiphilia* (Sasu et al. 2010), although the compounds mediating this inhibition are unknown. Infection may also be inhibited by volatile compounds exuded by floral stigmatic surfaces. Volatile compounds commonly produced by floral tissues, such as (E)-B-caryophyllene, can increase resistance to *Pseudomonas syringae* in *Arabadopsis thaliana* (Huang et al. 2012). Thus, a wide range of floral traits that can influence pollinator attraction may also mediate pathogen transmission through flowers.

Disease transmission vectored by pollinators can play a significant role in agricultural as well as wild systems. For example, both wild and cultivated highbush blueberry (*Vaccinium corymbosum*; Ericaceae) are frequently infected by *Monilinia vaccinii-corymbosi* (MVC hereafter), the cause of mummy berry disease (Batra 1983). A highly damaging pathogen affecting highbush blueberry crops, MVC causes major economic losses with infections that can reduce yield up to 80% (Stretch et al. 2001).

Floral visitors vector asexual spores, or conidia, to blueberry flowers (Batra 1983). Like pollen grains, spores germinate on stigmas and hyphae grow down the stylar canal to infect the ovary, resulting in infected and inedible fruits (Batra and Batra 1985). Thus, there is the potential for variation in stigmatic properties that could affect spore germination and growth as well as pollination.

Vaccinium corymbosum is susceptible to both sexually produced ascospores (primary spores), which are wind-disbursed and infect new shoots and stems, a process referred to as 'primary infection,' and by asexual conidia (secondary spores), wherein conidia are vectored from blighted vegetative tissues to flowers, which are subjected to 'secondary infection.' While many studies have demonstrated cultivar variation in resistance to both primary and secondary infection, little is known about the mechanisms of resistance (Stretch et al. 2001, Ehlenfeldt et al. 2010). One study found that hyphal growth rate in five cultivars roughly correlated with established resistance rankings of secondary infection (Lehman et al. 2007). Since subsequent hyphal growth requires germination success, traits influencing conidial germination may comprise a component of resistance. While this provides an important starting point to suggest possible mechanisms of resistance, over 150 cultivars have been evaluated for disease resistance, providing the opportunity to expand prior work to a greater number of cultivars. Additionally, no study to our knowledge has assessed trade-offs between conidial germination and pollen germination. If inhibitory stigmatic chemicals reduce conidia germination but also inhibit pollen germination, this may lead to a trade-off between reproduction and resistance to infection.

We hypothesized that published resistance to infection in cultivars (Ehlenfeldt 2010) will correlate with conidia germination, and that mechanisms inhibiting conidia germination may also reduce pollen germination. We used laboratory pollination and inoculation techniques and fluorescence microscopy to answer the following questions: 1) Do cultivars differ in conidia and pollen germination? 2) Does conidia germination correspond with published resistance rankings for cultivars, suggesting a mechanism of resistance? 3) Is there a trade-off such that cultivars with lower conidia germination also have lower pollen germination?

Methods

Study system

Monilinia vaccinii-corymbosi is an ascomycete fungal pathogen infecting *Vaccinium corymbosum*, highbush blueberry, across North America (Batra 1983). It employs a two-stage infection process, starting with infection through windborne ascospores disbursed from ascocarps emerging in the spring. This primary infection affects new shoot and stem tissues and creates blighted tissues known as 'pseudoflowers.' These co-opted tissues induce production of large amounts of asexual spores (conidia) and attract insect visitors by reflecting UV light (Batra and Batra 1985) and exuding volatile organic compounds that mimic floral scent (McArt et al. 2016). Although wind and rain can spread conidia, flower-visiting insects are the primary vectors (Ngugi et al. 2002), transferring conidia from pseudoflowers to floral tissues, resulting in secondary infection. Conidia mimic pollen grains in both their mode of delivery and germination on stigmatic surfaces (Ngugi and Scherm 2004). Hyphal growth, analogous to pollen tubes, extend down the stylar canal to the ovary and result in secondary infection (Ngugi and

Scherm 2004). Infection results in fungus-filled locules, with berries becoming grey and hard upon ripening. These inedible berries then drop and overwinter, emerging with apothecia in the spring and releasing spores that begin a new cycle of infection (Batra 1983, Batra and Batra 1985).

Highbush blueberry cultivars have varying levels of resistance to infection (Ngugi et al. 2002, Ehlenfeldt et al. 2010). Some cultivars may be highly resistant to primary infection while being susceptible to secondary infection, or *vice versa* (Lehman et al. 2007, Ehlenfeldt et al. 2010). While levels or resistance have been assessed in the field for more than 150 cultivars in multiple years (Ehlenfeldt et al. 2010), mechanisms underlying this resistance are largely unknown.

Cultivar selection and collection

On April 24th 2014, bud-bearing clippings were collected from 29 cultivars at the Rutgers University Phillip M. Marucci Center for Blueberry and Cranberry Research. Cultivars were selected to represent the available range of resistance to secondary infection, from highly resistant to highly susceptible, as ranked by Ehlenfeldt (2010). Between five and ten clippings, each bearing 10-25 bud clusters, were taken from each cultivar. When possible, clippings were taken from separate plants. For cultivars represented by few or a single plant, clippings were taken from different branches of the same plant. Cuttings were placed in water with Floralife (Smithers-Oasis Co., Kent, Ohio) plant cutting fertilizer powder mixed with tap water (10g /L) and driven to the University of Massachusetts at Amherst. On April 25, cuttings were stored in darkness to retard flowering. On May 1, cuttings were moved to cold storage at 11°C without light to further delay bloom. Cuttings were moved from cold storage to standard lab benches on

May 13 and exposed to ambient light at 24°C for inoculation. All cuttings were routinely re-wounded to aid in water uptake. Other research evaluating fungal species associated with blueberry stem canker has also used cuttings rather than live plants for logistical reasons, as we did here (Elfar et al. 2013).

To collect conidia for treatments, blighted tissues were collected haphazardly from the range of cultivars at the Marucci center (Chatsworth, New Jersey, USA) on May 9 and received at UMass Amherst on May 13. Blighted tissues were kept refrigerated until May 20. A sample of conidia was plated onto half-strength potato dextrose agar (PDA) every two days to ensure they were viable and capable of germination.

Inoculation and pollination

To determine whether trade-offs or differential germination occur on floral stigmas, flowers of all cultivars were inoculated with equal amounts of conidia and pollen grains. For pollen collection, one donor flower was chosen haphazardly from one plant per cultivar on each day and pollen was extracted by vibrating the flower with a spindle obtained by removing the brush components from an Oral-B battery-powered toothbrush (Procter & Gamble, Cincinnati, OH) onto a glass slide. Conidia were extracted in similar fashion from sporulating tissue received from the Marucci Center on May 13, to a separate glass slide on each day of inoculation. A subsample of conidia from each extraction was plated onto ½ strength PDA and allowed to germinate to ensure viability. Under a dissecting microscope, approximately 10 - 20 pollen grains and 10 - 20 conidia were collected with a dissecting needle by passing it through pollen and conidia on the glass slides, and this mixture was applied to the stigmas of newly opened flowers. Needles were visually spot-checked to ensure a relatively equal ratio of pollen and

conidia for each inoculation. Each cultivar was only treated with pollen from the same cultivar. Highbush blueberry cultivars are generally self-compatible, although fruit set is improved by outcrossing (Elagamy et al. 1981; Ehlenfeldt 2001a). Flowers were individually labeled to indicate date of inoculation, and after four days, stigmas were collected with forceps and immediately submerged in EtOH as a preservative and fixative. Due to the range of flowering phenology between cultivars, inoculations continued until May 19 as flowers opened. In total, 337 floral stigma samples were collected from 25 cultivars.

Fluorescence microscopy

Beginning on August 10, 2015, stigmas were processed for fluorescence microscopy following the protocol in Lehman (2007). Stigmas were removed from the 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 then 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 05101) excitation filter (300 – 400 nm, barrier filter 400 nm, emitter filter 410- 500 nm). Pollen tubes and fungal hyphae were counted from germinating grains and spores, respectively.

Statistical analysis

To determine if pollen or conidia germination varied by cultivar, we compared generalized linear models with and without cultivar as an explanatory fixed factor with

plant, branch and date sampled (stigma harvested) as random effects, with branch nested within plant. Separate analyses were conducted for pollen and conidia germination. ANOVA comparisons between models with and without cultivar were made using the ANOVA function and χ^2 test statistic in the lme4 package for R (R Core Team 2011, Version 2.13.1, The R Foundation for Statistical Computing, Vienna, Austria) with a Poisson distribution. Upon finding a significant cultivar effect, a Tukey's HSD post hoc test was run using the multcomp package to determine which cultivars differed from one another.

To assess trade-offs between pollen and conidia germination, Pearson product moment correlations were run within each cultivar and across cultivars using samples as replicates (JMP Pro 13.2.1, SAS Institute Inc., Cary, NC, 1989-2007). Cultivars with samples sizes of fewer than 5 were not included in correlation analysis.

To determine the relationship between published cultivar resistance ranking for secondary infection and observed conidia and pollen germination, a general linear model was used (JMP Pro 13.2.1, SAS Institute, Inc.) with relative resistance rankings as predictors and cultivar average values of germinated pollen and conidia as responses. Cultivars were assigned a resistance rank relative to the other cultivars used in this study (Table 3-1), adapting ranks first published in Ehlenfeldt (2010).

Results

Comparison between models with and without cultivar as a fixed factor revealed that conidia germination varied by cultivar ($\chi^2 = 36.103$, df = 20, $p = 0.015$), but pollen germination did not ($\chi^2 = 31.66$, df = 24, $p = 0.136$). In *post hoc* comparisons, Patriot and

Jersey cultivars had notably high numbers of conidia germinated, while Darrow had the fewest (Figure 3-1).

Correlation analysis revealed no relationship between published resistance rank and either pollen ($r^2 = 0.0038$, df = 21, p=0.261) or conidial germination ($r^2 = 0.0017$, df = 21, p=0.453; Figure 2). There was also no significant correlation between pollen and conidial germination across cultivars (Pollen: $r^2 = 0.0038$, df = 21, p=0.261, Conidia: $r^2 = 0.0017$, df = 21, p=0.453), indicating no support for the hypothesis that cultivars would experience tradeoffs. In within-cultivar analyses, Bounty and Weymouth were the only cultivars that had a significant negative relationship between pollen and conidia germination, indicating that individuals with high pollen germination also had low conidial germination, while Early Blue had a significantly positive relationship (Table 3-2), indicating that individuals of that cultivar with high pollen germination also had high conidial germination, showing a tradeoff.

Discussion

In highbush blueberry, although plants are self-compatible, fruit set is maximized by outcrossing (Isaacs et al. 2016), and therefore traits affecting pollen germination could have important impacts on agricultural production. Since pollinators vector fungal conidia in addition to pollen, plants must balance increasing pollen deposition against infection risk. Ideally, plants would benefit from maximizing outcrossed pollen germination while inhibiting the germination of fungal spores. If unrelated traits promote pollen versus conidia germination, no trade-off would be present. However, trade-offs may be expected if some of the same plant traits control pollen and conidial germination. In this study, we found little evidence of such a tradeoff, with most cultivars exhibiting

no relationship between pollen and conidia germination. This lack of trade-offs between conidia germination and pollen germination in most cultivars has agricultural implications, suggesting that breeding for traits that increase resistance by reducing conidial germination should not negatively impact pollination success, at least in terms of pollen germination.

The lack of trade-offs suggests that there are separate mechanisms mediating pollen and conidial germination. For example, pollen grains are generally dehydrated before leaving the anther, aiding in survival across environmental stressors, and proper stigmatic moisture level is essential for pollen grain rehydration and germination (Kerhoas et al. 1987). Further, proper balance of ions such as calcium, hydrogen, potassium, and chlorine are needed for pollen germination and navigation down the stylar canal (Song et al. 2009), and disruption of ion balances may affect germination. In lowbush blueberry (*Vaccinium augustifolium*), boron and calcium play an integral role in pollen germination success, and trials augmenting B and Ca levels through foliar spray increased pollen germination over untreated plants (Brewbaker and Kwack 1963, Chen et al. 1998). Some of these cues on stigmatic surfaces could also stimulate or inhibit germination of fungal spores. For example, calcium ions have long been studied as promoters of spore germination and appressorial formation in ascomycete plant pathogens (Griffin 1966, Warwar and Dickman 1996). However, because we found little relationship between pollen and conidial germination within or between cultivars, this suggests that separate mechanisms, or different synergistic combinations of mechanisms, cue germination of pollen and conidia in our system.

Aside from promoting pollen germination (Ylstra et al. 1992), many secondary metabolites inhibit conidial germination and fungal growth, and could explain differences in conidial germination between cultivars. For example, alkaloids such as beta-carbolines have an inhibitory effect on fungi such as *Botrytis cinerea* and *Penicillium digitatum,* and are ubiquitously found in nature (Olmedo et al. 2017). Similarly, some compounds inhibit germination of both plant pollen and fungal spores. For example, volatile hexenal applications inhibit germination of both apple pollen (Hamiltonkemp et al. 1991) and fungal plant pathogens such as *Colletotrichum coccodes* (Black Dot) and *Helminthosporium solani* (Silver Scurf) (Wood et al. 2012). A recent phytochemical analysis of blueberry flowers yielded 21 phenolic compounds (Wan et al. 2012), and phenolics are widely reported to inhibit growth and germination of fungal plant pathogens (del Río et al. 2004; Leontopoulos et al. 2015; Pizzolitto et al. 2015). Further, blueberry cultivars can vary widely in levels of secondary metabolites, including phenolics, in floral and leaf tissue (Egan et al. in review). Thus, variation of phenolic concentrations between cultivars is a possible mechanism that could explain differences in conidial germination.

While cultivars varied in conidial germination, our conidia germination rankings did not correspond with published resistance levels for secondary infection in Ehlenfeldt (2010; Table 1). This disparity is not necessarily surprising due to different methodology between the studies. First, our study was conducted in the lab under controlled conditions, whereas Ehlenfeldt assessed resistance to mummy berry using naturally pollinated plants in unmanipulated orchard over multiple years. Our study used hand inoculations of known fungal and pollen sources, while field-based plants were subject to

interactions with local pollinator communities that may vary widely in their capacity to carry and deposit pollen and conidia. Further, pollinator preferences may differ between cultivars, resulting in some cultivars receiving more conidial deposition than others. In addition, varying abiotic environmental conditions, such as temperature, relative humidity and moisture, would affect pathogen success. For example, May temperatures were the only significant environmental predictor of *Colletotrichum acutatum* (Anthracnose fruit rot), another ascomycete fungal pathogen, incidents from year to year (Polashock et al. 2005). Although our results indicate that conidial germination is unlikely to be a predictor of overall resistance to secondary infection, it may be an important component along with other factors. For example, in cultivars visited more often by pathogen vectors, conidial inhibition on the stigma may be important for reducing infection. Whatever trait, or combination of traits, is responsible, our result that cultivars vary in germination suggests an underlying genetic variation that could be selected for by breeders to aid in disease resistance.

In contrast with conidial germination, pollen germination did not vary across cultivars. This suggests that there is little genetic variation in the traits that control pollen germination, consistent with general evolutionary theory that traits most closely linked to fitness are under strong selection that reduces or eliminates genetic variation (Futuyma 2005). Similar artificial selection by breeders to maximize yield may have resulted in uniformly high pollen germination across cultivars. The lack of genetic variation in pollen germination suggests that breeders can continue to breed disease resistant cultivars with little concern for reducing pollen germination, which could be an obstacle to maximum yield in pollinator-limited environments.

Only one cultivar showed an individual-level tradeoff in our correlation analysis (Table 3-2), with two others exhibiting a negative relationship between conidia and pollen germination. Bounty and Weymouth had significant negative correlations between number of germinated pollen and conidia, indicating stigmatic conditions conducive to either pollen or conidia germination. Early Blue was the only cultivar with a significantly positive correlation (Table 3-2). These within-cultivar variations should indicate non-genetic variation in stigmas that have the potential to affect germination of pollen and conidia. Although the amount of time flowers were open was controlled to within one day, these cultivars may vary in how quickly stigmatic surfaces dry out or respond to stresses depending on the hour in which particular flowers open, perhaps comprising another component of resistance.

Our study was largely congruent with prior work measuring hyphal growth and infection of blueberry floral locules using fluorescence microscopy to measure the lengths of hyphae extending from germinated conidia down the stylar canal in five highbush cultivars (Lehman et al. 2007). Lehman's study concluded that differences in stylar growth are a likely component of resistance to infection, and it is possible that it is more indicative of resistance than conidial germination alone. We used three cultivars in common with that study: Weymouth, Coville, and Jersey. While using different metrics, in both studies Jersey had low conidial germination or growth and Weymouth had higher. Although Coville had intermediate hyphal growth in Lehman (2007), it displayed relatively low conidial germination in our study, suggesting fairly high resistance. Several factors may contribute to the disparity. First, Lehman measured both length of hyphae and locules infected, while we based our study on conidia germination alone. We

used clippings taken from mature and established plants, while Lehman et al. used young, potted specimens. Therefore, our results may be more relevant for established orchards and less so for young plants. Further, cut branches may not respond as living ones do, and suspension in floralife may alter the chemical responses produced by clippings.

As with many crop plants, highbush blueberry yield is improved with outcrossed pollination (Ehlenfeldt 2001a), but the key pollinators in this system can be carriers of fungal pathogens. While fungicides can be used to manage mummy berry, in situations where fungicide use is not ideal, such as 'no spray' or 'pick-your-own' farms, it is important to understand how the interactions of pollen and disease spores affect disease resistance across cultivars. We found little evidence suggesting tradeoffs between pollen and conidia germination. Although conidia germination varied by cultivar, germination did not correspond to published resistance rankings for secondary infection. Further studies seeking to understand the mechanisms behind resistance to secondary infection may benefit from examining interactions in the flower that occur post-inoculation, such as success rate of hyphal penetration of the locule and subsequent fruit infection. Considering the economic importance of this crop and growing concerns pertaining to the use of fungicides (Wightwick et al. 2010), adding to our understanding of resistance mechanisms can provide valuable information for breeders to combat the damaging effects of this pathogen.

Acknowledgements

We thank C. Rodriguez-Saona, R. Martin, and all the staff at the Rutgers New Jersey Agricultural Experiment Station for providing the clippings used in this study. We

also thank J. Lehman for his guidance working with this system and in fluorescence microscopy protocols. We thank G. Snyder and C. Howard for their assistance in the lab during inoculations. This work was made possible by funding from USDA NIFA award 2016-67011-24722, the Lotta M. Crabtree Fellowship, and the Organismic and Evolutionary Graduate Program at the University of Massachusetts Amherst.

Table 3-1. Cultivars are listed in order of resistance to secondary infection ranked from most to least resistant. Published rank values are from the Ehlenfledt (2010) list of 117 cultivars and the relative values are for cultivars compared in this study.

Cultivar	Published rank	Relative ranking	Avg Germinated Conidia	Resistance Rank (this study)	Plants sampled	Clippings per plant
Patriot	14	1	15.67	24	2	2
Weymouth	18	2	3.67	9	4	4
June	20	3	2.6	8	1	1
Harrison	30	4	4.42	13	2	4
Coville	43	5	2.5	6	1	1
Meader	46	6	0	1	1	1
Wareham	47	7	4	12	2	2
Duke	50	8	4	11	1	1
Collins	53	9	0	1	1	1
Bounty	56	10	8.03	20	2	3
Hanna's Choice	63	11	8.33	21	2	3
Pemberton	67	12	4.71	15	2	3
Darrow	74	13	1.37	3	5	6
Nelson	81	14	4.7	14	1	3
Jersey	83	15	13.22	23	2	2
Ivanhoe	86	16	2.58	7	3	3
Pender	88	17	1.83	4	1	1
Elizabeth	92	18	11.33	22	2	2
Earliblue	95	19	3.7	10	3	3
Bluechip	96	20	5.3	16	2	3
Stanley	100	21	7.71	19	3	3
Blueray	101	22	1	2	2	2
Elliot	107	23	7	18	1	2
Herbert	113	24	6.85	17	4	5
Atlantic	117	25	1.91	5	2	3

Table 3-2. Pearson's Correlation tests of mean pollen and conidia counts within each cultivar. Bold indicates significant value.

Cultivar	Mean Pollen (SD)	Mean Conidia (SD)	DF	r^2	*P*
Atlantic	8.364 (1.502)	1.909 (4.011)	10	0.312	0.074
Bluechip	10.20 (3.393)	5.3 (4.832)	9	0.003	0.873
Blueray	6 (1.690)	1 (2.450)	7	0.171	0.308
Bounty	5.8 (2.058)	8.033 (6.430)	29	0.157	**0.03**
Darrow	8.556 (3.755)	1.370 (2.133)	26	0.006	0.694
Early Blue	6 (4.0)	3.7 (4.596)	9	0.562	**0.013**
Elliot	8.933 (4.399)	7.0 (4.884)	14	0.016	0.654
Hanna's Choice	6.25 (8.51)	8.333 (6.429)	11	0.137	0.236
Herbert	6.923 (5.003)	6.846 (5.746)	25	0.008	0.674
Harrison	8.833 (3.460)	4.417 (6.789)	11	0.032	0.578
Ivanhoe	13.167 (8.387)	2.583 (3.825)	11	0.002	0.888
Jersey	7.189 (3.178)	13.216 (10.188)	36	0.07	0.115
June	6 (5.228)	2.6 (4.742)	9	0	1
Nelson	4.870 (3.935)	4.696 (5.397)	22	0.008	0.684
Pembroke	9.708 (5.353)	4.708 (6.182)	23	>0.001	0.995
Pender	6.333 (1.506)	1.833 (1.602)	5	0.049	0.674
Stanley	6.333 (3.136)	7.714 (7.484)	20	0.131	0.107
Wareham	5 (.633)	4 (3.286)	5	0.148	0.451
Weymouth	6.143 (4.234)	3.667 (5.257)	20	0.272	**0.015**

Figure 3-1. Columns indicate mean number of germinated conidia per cultivar, and error bars are +/- 1 standard error of the mean. Cultivars that do not share the same letter above the bar are significantly different as determined by Tukey's HSD test.

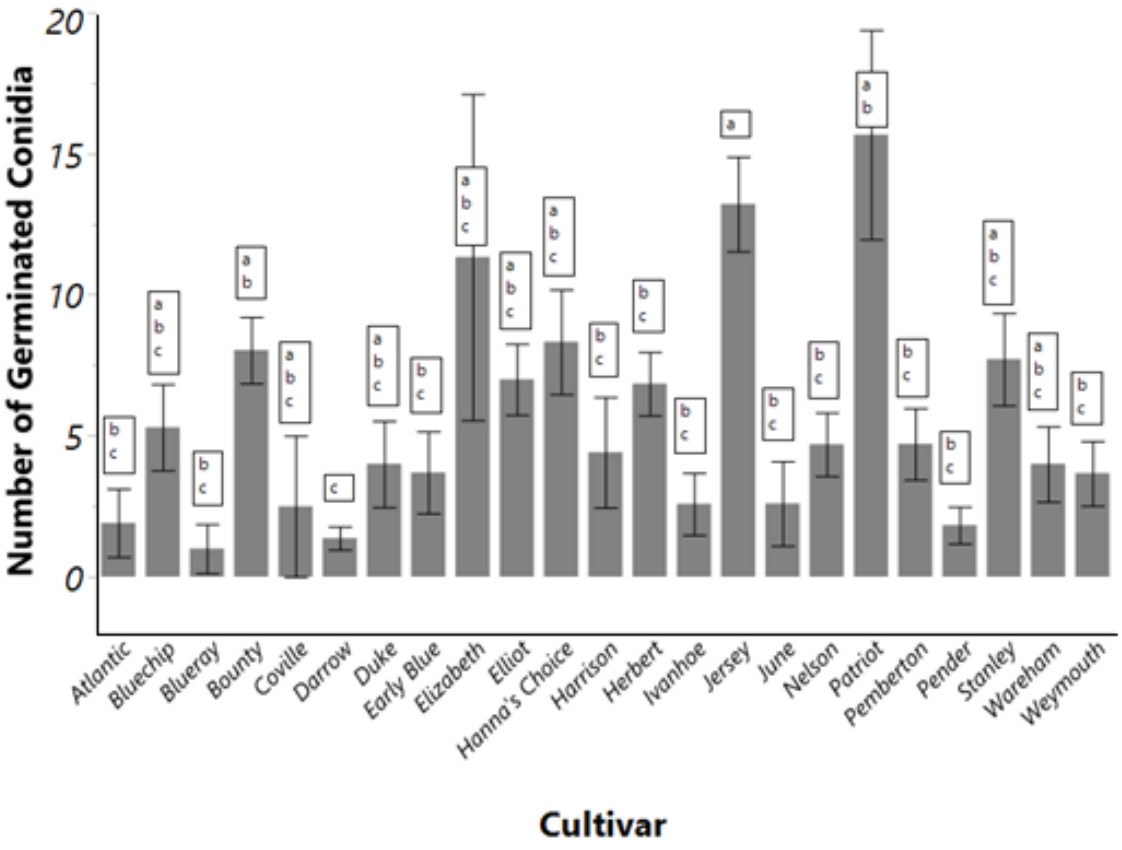
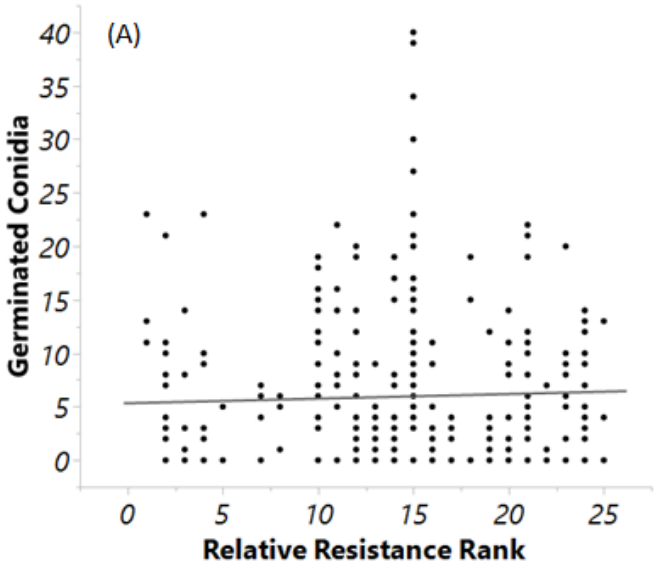
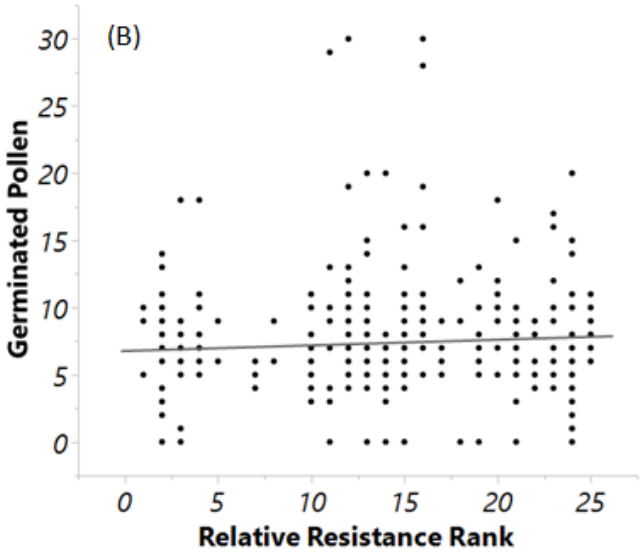


Figure 3-2. Number of (A) germinated conidia and (B) pollen grains plotted against the relative resistance ranking from Ehlenfeldt (2010). First rank is the most resistant and 25th is the least.





BIBLIOGRAPHY

Adler LS, Irwin RE (2006) Comparison of pollen transfer dynamics by multiple floral visitors: Experiments with pollen and fluorescent dye. Annals of Botany 97:141–150. [online] URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2000767/

Arrington M, Wasko DeVetter L (2018) Floral morphology differs among new northern highbush blueberry cultivars. Journal of Horticulture 5:1–4. [online] URL: https://www.omicsonline.org/open-access/floral-morphology-differs-among-new-northern-highbush-blueberry-cultivars-2376-0354-1000223-98651.html?aid=98651

Batra LR (1983) *Monilinia vaccinii-corymbosi* (Sclerotiniaceae) - Its biology on blueberry and comparison with related species. Mycologia 75:131–152.

Batra LR, Batra SWT (1985) Floral mimicry induced by mummy-berry fungus exploits host's pollinators as vectors. Science 228:1011–1013.

Bell G (1985) On the function of flowers. Proceedings of the Royal Society of London. Series B. Biological Sciences 224:223 LP-265. [online] URL: http://rspb.royalsocietypublishing.org/content/224/1235/223.abstract

Brosi BJ, Briggs HM (2013) Single pollinator species losses reduce floral fidelity and plant reproductive function. Proceedings of the National Academy of Sciences 110:13044 LP-13048. [online] URL: http://www.pnas.org/content/110/32/13044.abstract

Cameron SA, Lozier JD, Strange JP, Koch JB, Cordes N, Solter LF, Griswold TL (2011) Patterns of widespread decline in North American bumble bees. Proceedings of the National Academy of Sciences 108:662 LP-667. [online] URL: http://www.pnas.org/content/108/2/662.abstract

Cane J, Payne JA (1993) Regional, annual, and aeasonal variation in pollinator guilds: Intrinsic traits of bees (Hymenoptera: Apoidea) underlie their patterns of abundance at *Vaccinium ashei* (Ericaceae).

Chambers VH (1946) An examination of the pollen loads of Andrena: The species that visit fruit trees. Journal of Animal Ecology 15:9–21. [online] URL: http://www.jstor.org/stable/1621

Courcelles MDM, Button L, Elle E (2013) Bee visit rates vary with floral morphology among highbush blueberry cultivars (*Vaccinium corymbosum* L.). Journal of Applied Entomology 137:693–701. [online] URL: https://doi.org/10.1111/jen.12059

Dedej S, Delaphane KS, Scherm H (2004) Effectiveness of honey bees in delivering the biocontrol agent *Bacillus subtilis* to blueberry flowers to suppress mummy berry disease. Biological Control 31:422–427.

Delaplane KS, Mayer DF (2000) Crop pollination by bees. CABI Publishing. [online] URL: www.cabi-publishing.org

Dijksterhuis J, van Doorn T, Samson R, Postma J (2011) Effects of seven fungicides on non-target aquatic fungi. Water, Air, & Soil Pollution 222:421–425. [online] URL: https://doi.org/10.1007/s11270-011-0836-3

Dobson A, Crawley W (1994) Pathogens and the structure of plant-communities. Trends in Ecology & Evolution 9:393–398.

Egan PA, Adler LS, Irwin RE, Farrell IW, Palmer-Young EC, Stevenson PC Crop domestication alters floral reward chemistry with potential consequences for pollinator health. Frontiers in Plant Science

Ehlenfeldt MK (2001a) Self- and cross-fertility in recently released highbush blueberry

cultivars. Hortscience 36:133–135.

Ehlenfeldt MK (2001b) Self- and cross-fertility in recently released highbush blueberry cultivars. Hortscience 36:133–135.

Ehlenfeldt MK, Polashock JJ, Stretch AW, Kramer M (2010) Ranking cultivated blueberry for mummy berry blight and fruit infection incidence using resampling and principal components analysis. Hortscience 45:1205–1210.

Elagamy S, Sherman W, Lyrene P (1981) Fruit-set and seed number from self-pollinated and cross-pollinated highbush (4x) and rabbiteye (6x) blueberries. Journal of the American Society for Horticultural Science 106:443–445.

Elbrecht V, Leese F (2017) Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. Frontiers in Environmental Science 5:11. [online] URL: https://www.frontiersin.org/article/10.3389/fenvs.2017.00011

Elfar K, Torres R, Diaz G, Latorre B (2013) Characterization of *Diaporthe australafricana* and *Diaporthe spp*. associated with stem canker of blueberry in chile. Plant DIsease 97:1042–1050.

Fishbein M, Venable DL (1996) Diversity and temporal change in the effective pollinators of *Asclepias tuberosa*. Ecology 77:1061–1073. [online] URL: http://www.jstor.org/stable/2265576

Forup ML, Memmott J (2005) The relationship between the abundances of bumblebees and honeybees in a native habitat. Ecological Entomology 30:47–57. [online] URL: https://doi.org/10.1111/j.0307-6946.2005.00660.x

Futuyma DJ (2005) Evolution. Sinauer Associates, Inc.

Garibaldi LA, Steffan-Dewenter I, Winfree R, Aizen MA, Bommarco R, Cunningham

SA, Kremen C, Carvalheiro LG, Harder LD, Afik O, Bartomeus I, Benjamin F, Boreux V, Cariveau D, Chacoff NP, Dudenhöffer JH, Freitas BM, Ghazoul J, Greenleaf S, Hipólito J, Holzschuh A, Howlett B, Isaacs R, Javorek SK, Kennedy CM, Krewenka KM, Krishnan S, Mandelik Y, Mayfield MM, Motzke I, Munyuli T, Nault BA, Otieno M, Petersen J, Pisanty G, Potts SG, Rader R, Ricketts TH, Rundlöf M, Seymour CL, Schüepp C, Szentgyörgyi H, Taki H, Tscharntke T, Vergara CH, Viana BF, Wanger TC, Westphal C, Williams N, Klein AM (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. Science 339:1608 LP-1611. [online] URL: http://science.sciencemag.org/content/339/6127/1608.abstract

Gibbs J, Elle E, Bobiwash K, Haapalainen T, Isaacs R (2016) Contrasting pollinators and pollination in native and non-native regions of highbush blueberry production. PLOS ONE 11:e0158937. [online] URL: https://doi.org/10.1371/journal.pone.0158937

Hamiltonkemp T, Loughrin J, Archbold D, Andersen R, Hildebrand D (1991) Inhibition of pollen germination by volatile compounds including 2-hexenal and 3-hexenal. Journal of Agricultural and Food Chemistry 39:952–956.

Herrera CM (1987) Components of pollinator quality - Comparative analysis of a diverse insect assemblage. Oikos 50:79–90.

Isaacs R, Gibbs J, May E, Hanson E, Hancock J (2016) Invest in pollination for success with highbush blueberries. Michigan State University Extension [online] URL: http://msue.anr.msu.edu/news/invest_in_pollination_for_success_with_highbush_blueberries (accessed 1 September 2018).

Isaacs R, Kirk A (2010) Pollination services provided to small and large highbush blueberry fields by wild and managed bees. Journal of Applied Ecology 47:841–849.

Jacobs S (2015) Baldfaced Hornet Fact Sheet. PennState College of Agriculture Extension [online] URL: https://ento.psu.edu/extension/factsheets/baldfaced-hornet

Javorek SK, Mackenzie KE, Kloet SP Vander (2002) Comparative pollination effectiveness among bees (Hymenoptera: Apoidea) on lowbush blueberry (Ericaceae: *Vaccinium angustifolium*). Annals of the Entomological Society of America 95:345–351. [online] URL: http://dx.doi.org/10.1603/0013-8746(2002)095[0345:CPEABH]2.0.CO

Jennersten O (1988) Insect dispersal of fungal disease: Effects of *Ustilago infection* on pollinator attraction in *Viscaria vulgaris*. Oikos 51:163–170. [online] URL: http://www.jstor.org/stable/3565638

Karolyi F, Colville JF, Handschuh S, Metscher BD, Krenn HW (2014) One proboscis, two tasks: Adaptations to blood-feeding and nectar-extracting in long-proboscid horse flies (Tabanidae, Philoliche). Arthropod Structure & Development 43:403–413. [online] URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4175409/

Kerhoas C, Gay G, Dumas C (1987) A multidisciplinary approach to the study of the plasma membrane of Zea mays pollen during controlled dehydration. Planta 171:1–10. [online] URL: https://doi.org/10.1007/BF00395062

Klein AM, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T (2007) Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B-Biological Sciences 274:303–313.

Ladurner E, Bosch J, Kemp WP, Maini S (2005) Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* Say and *Apis mellifera*. Apidologie 36:449–460. [online] URL: https://doi.org/10.1051/apido:2005032

Larson BMH, Kevan PG, Inouye DW (2001) Flies and flowers: taxonomic diversity of anthophiles and pollinators. The Canadian Entomologist 133:439–465. [online] URL: https://www.cambridge.org/core/article/flies-and-flowers-taxonomic-diversity-of-anthophiles-and-pollinators/1F6DE0F9A4E5CEDBA5CBC74C7B085984

Legard DE, MacKenzie SJ, Mertely JC, Chandler CK, Peres NA (2005) Development of a reduced use fungicide program for control of botrytis fruit rot on annual winter strawberry. Plant Disease 89:1353–1358. [online] URL: https://doi.org/10.1094/PD-89-1353

Lehman JS, Igarashi S, Oudemans P V (2007) Host resistance to *Monilinia vaccinii-corymbosi* in flowers and fruits of highbush blueberry. Plant Disease 91:852–856.

Leonard AS, Dornhaus A, Papaj DR (2011) Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. The Journal of Experimental Biology 214:113 LP-121. [online] URL: http://jeb.biologists.org/content/214/1/113.abstract

Leontopoulos S V, Giavasis I, Petrotos K, Kokkora M, Makridis C (2015) Effect of different formulations of polyphenolic compounds obtained from OMWW on the growth of several fungal plant and food borne pathogens. Studies *in vitro* and *in vivo*. Agriculture and Agricultural Science Procedia 4:327–337. [online] URL: http://www.sciencedirect.com/science/article/pii/S221078431500100X

MacKenzie KE, Eickwort GC (1996) Diversity and abundance of bees (Hymenoptera: Apoidea) foraging on highbush blueberry (*Vaccinium corymbosum* L.) in central New York. Journal of the Kansas Entomological Society 69:185–194. [online] URL: http://www.jstor.org/stable/25085716

Maier CT, Waldbauer GP (1979) Diurnal activity patterns of flower flies (Diptera: Syrphidae) in an Illinois sand area.

McArt SH, Koch H, Irwin RE, Adler LS (2014a) Arranging the bouquet of disease: floral traits and the transmission of plant and animal pathogens. Ecology Letters:n/a-n/a. [online] URL: http://dx.doi.org/10.1111/ele.12257

McArt SH, Koch H, Irwin RE, Adler LS (2014b) Arranging the boquet of disease: floral traits and the transmission of plant and animal pathogens. Ecology Letters

McArt SH, Miles TD, Rodriguez-Saona C, Schilder A, Adler LS, Grieshop MJ (2016) Floral scent mimicry and vector-pathogen associations in a pseudoflower-inducing plant pathogen system. Plos One 11

Moisan-Deserres J, Girard M, Chagnon M, Fournier V (2014) Pollen loads and specificity of native pollinators of lowbush blueberry. Journal of Economic Entomology 107:1156–1162. [online] URL: http://dx.doi.org/10.1603/EC13229

Ngugi HK, Scherm H (2004) Pollen mimicry during infection of blueberry flowers by conidia of *Monilinia vaccinii-corymbosi*. Physiological and Molecular Plant Pathology 64:113–123.

Ngugi HK, Scherm H, Lehman JS (2002) Relationships between blueberry flower age, pollination, and conidial infection by *Monilinia vaccinii-corymbosi*. Phytopathology 92:1104–1109.

Nguyen-Dumont T, Pope BJ, Hammet F, Southey MC, Park DJ (2013) A high-plex PCR approach for massively parallel sequencing. BioTechniques 55:69–74.

Norelli JL, Jones AL, Aldwinckle HS (2003) Fire blight management in the twenty-first century: Using new technologies that enhance host resistance in apple. Plant Disease 87:756–765. [online] URL: https://doi.org/10.1094/PDIS.2003.87.7.756

Olsen KM (1997) Pollination effectiveness and pollinator importance in a population of *Heterotheca subaxillaris* (Asteraceae). Oecologia 109:114–121.

Orke EC, Dehne HW, Schonbeck F, Weber A (1994) Crop production and crop protection: Estimated losses in major food and cash crops. Amsterdam: Elsevier.

Pearson BL, Simon JM, McCoy ES, Salazar G, Fragola G, Zylka MJ (2016) Identification of chemicals that mimic transcriptional changes associated with autism, brain aging and neurodegeneration. Nature Communications 7:11173. [online] URL: http://dx.doi.org/10.1038/ncomms11173

Pinstrup-Andersen P (2000) The future world food situation and the role of plant diseases. Canadian Journal of Plant Pathology 22:321–331. [online] URL: https://doi.org/10.1080/07060660009500451

Pizzolitto RP, Barberis CL, Dambolena JS, Herrera JM, Zunino MP, Magnoli CE, Rubinstein HR, Zygadlo JA, Dalcero AM (2015) Inhibitory effect of natural phenolic compounds on *Aspergillus parasiticus* growth. Journal of Chemistry 2015:7.

Polashock JJ, Ehlenfeldt MK, Stretch AW, Kramer M (2005) Anthracnose fruit rot resistance in blueberry cultivars. Plant DIsease 89:33–38.

Primack RB, Silander JA (1975) Measuring relative importance of different pollinators to

plants. Nature 255:143–144.

R Development Core Team (2018) R: A language and environment for statistical computing.

Rao S, Stephen WP, White L (2009) Native bee pollinator diversity in Oregon blueberries. In: Acta Horticulturae. International Society for Horticultural Science (ISHS), Leuven, Belgium, pp 539–548. [online] URL: https://doi.org/10.17660/ActaHortic.2009.810.71

Rathman ES, Lanza J, Wilson J (1990) Feeding preferences of flesh flies (*Sarcophaga bullata*) for sugar-only vs. sugar-amino acid nectars. The American Midland Naturalist 124:379–389. [online] URL: http://www.jstor.org/stable/2426188

Rick CM, Holle M, Thorp RW (1978) Rates of cross-pollination in *Lycopersicon pimpinellifolium*: Impact of genetic variation in floral characters. Plant Systematics and Evolution 129:31–44. [online] URL: https://doi.org/10.1007/BF00988982

del Río JA, Gómez P, Báidez A, Fuster MD, Ortuño A, Frías V (2004) Phenolic Compounds have a role in the defence mechanism protecting grapevine against the fungi involved in petri disease.

Rodriguez-Saona CR, Rodriguez-Saona LE, Frost CJ (2009) Herbivore-induced volatiles in the perennial shrub, *Vaccinium corymbosum*, and their role in inter-branch signaling. Journal of Chemical Ecology 35:163–175.

Rogers SR, Tarpy DR, Burrack HJ (2013) Multiple criteria for evaluating pollinator performance in highbush blueberry (Ericales: Ericaceae) agroecosystems. Environmental Entomology 42:1201–1209. [online] URL: http://dx.doi.org/10.1603/EN12303

Rogers SR, Tarpy DR, Burrack HJ (2014) Bee species diversity enhances productivity and stability in a perennial crop. Blenau W (ed). PLoS ONE 9:e97307. [online] URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4016306/

Ross J, Foster D, Hillard P, Pendarvis S, Ray T (2017) Agricultural Statistics 2017. [online] URL: https://www.nass.usda.gov/Publications/Ag_Statistics/2017/Complete Ag Stats 2017.pdf

Roy BA (1994) The use and abuse of pollinators by fungi. Trends in Ecology & Evolution 9:335–339. [online] URL: http://dx.doi.org/10.1016/0169-5347(94)90154-6

Scott Z, Ginsberg H, Alm SR (2016) Native bee diversity and pollen foraging specificity in cultivated highbush blueberry (Ericaceae: *Vaccinium corymbosum*) in Rhode Island. Environmental Entomology 45:1432–1438. [online] URL: http://pubs.er.usgs.gov/publication/70179455

Shykoff JA, Bucheli E (1995) Pollinator visitation patterns, floral rewards and the probability of transmission of *Microbotryum violaceum*, a veneral disease of plants. Journal of Ecology 83:189–198. [online] URL: http://www.jstor.org/stable/2261557

Smith KM, Loh EH, Rostal MK, Zambrana-Torrelio CM, Mendiola L, Daszak P (2013) Pathogens, pests, and economics: Drivers of honey bee colony declines and losses. EcoHealth 10:434–445. [online] URL: https://doi.org/10.1007/s10393-013-0870-2

Song L-F, Zou J-J, Zhang W-Z, Wu W-H, Wang Y (2009) Ion transporters involved in pollen germination and pollen tube tip-growth. Plant Signaling & Behavior 4:1193–1195. [online] URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2819455/

Ssymank A, Kearns CA, Pape T, Thompson FC (2008) Pollinating flies (Diptera): A

major contribution to plant diversity and agricultural production. Biodiversity 9:86–89. [online] URL: https://doi.org/10.1080/14888386.2008.9712892

Stretch AW, Ehlenfeldt MK, Brewster V, Vorsa N, Polashock J (2001) Resistance of diploid *Vaccinium spp*. to the fruit rot stage of mummy berry disease. Plant Disease 85:27–30.

Suso MJ, Nadal S, Roman B, Gilsanz S (2007) *Vicia faba* germplasm multiplication – floral traits associated with pollen-mediated gene flow under diverse between-plot isolation strategies. Annals of Applied Biology 152:201–208. [online] URL: https://doi.org/10.1111/j.1744-7348.2007.00205.x

Tuell JK, Ascher JS, Isaacs R (2009) Wild bees (Hymenoptera: Apoidea: Anthophila) of the Michigan highbush blueberry agroecosystem. Annals of the Entomological Society of America 102:275–287. [online] URL: http://dx.doi.org/10.1603/008.102.0209

Vicens N (2009) Pollinating efficacy of *Osmia cornuta* and *Apis mellifera* (Hymenoptera: Megachilidae, Apidae) on "Red Delicious" Apple.

Wan C, Yuan T, Cirello AL, Seeram NP (2012) Antioxidant and α-glucosidase inhibitory phenolics isolated from highbush blueberry flowers. Food Chemistry 135:1929–1937. [online] URL: http://www.sciencedirect.com/science/article/pii/S0308814612010291

Watts S, Ovalle DH, Herrera MM, Ollerton J (2011) Pollinator effectiveness of native and non-native flower visitors to an apparently generalist Andean shrub, *Duranta mandonii* (Verbenaceae). Plant Species Biology 27:147–158. [online] URL: https://doi.org/10.1111/j.1442-1984.2011.00337.x

West TP, McCutcheon TW (2009) Evaluating *Osmia cornifrons* as pollinators of highbush blueberry. International Journal of Fruit Science 9:115–125. [online] URL: https://doi.org/10.1080/15538360902991303

Wightwick A, Walters R, Allinson G, Reichman S, Menzies N (2010) Fungicides: Environmental risks of fungicides used in horticultural production systems. Carisse O (ed). In-Tech.

Willmer PG, Cunnold H, Ballantyne G (2017) Insights from measuring pollen deposition: quantifying the pre-eminence of bees as flower visitors and effective pollinators. Arthropod-Plant Interactions 11:411–425. [online] URL: https://doi.org/10.1007/s11829-017-9528-2

Wilson P, Castellanos MC, Hogue JN, Thomson JD, Armbruster WS (2004) A multivariate search for pollination syndromes among penstemons. Oikos 104:345–361. [online] URL: https://doi.org/10.1111/j.0030-1299.2004.12819.x

Winfree R, Williams NM, Dushoff J, Kremen C (2007) Native bees provide insurance against ongoing honey bee losses. Ecology letters 10:1105–1113.

Wood E, Miles TD, Wharton P (2012) The use of natural plant volatile compounds for the control of the potato postharvest diseases, black dot, silver scurf and soft rot. Biological Control 64:152–159.

Ylstra B, Touraev A, Moreno RMB, Stöger E, van Tunen AJ, Vicente O, Mol JNM, Heberle-Bors E (1992) Flavonols stimulate development, germination, and tube growth of tobacco pollen. Plant Physiology 100:902 LP-907. [online] URL: http://www.plantphysiol.org/content/100/2/902.abstract