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Rna Virus Ecology In Bumble Bees (bombus Spp.) And Evidence For Disease Spillover

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RNA VIRUS ECOLOGY IN BUMBLE BEES (*BOMBUS* SPP.) AND EVIDENCE FOR DISEASE SPILLOVER

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ABSTRACT

The inadvertent spread of exotic pests and pathogens has resulted in devastating losses for bees. The vast majority of bee disease research has focused on a single species of managed bee, the European honey bee (*Apis mellifera*). More recently, pathogen spillover from managed bees is implicated in the decline of several bumble bee species (*Bombus* spp.) demonstrating a need to better understand the mechanisms driving disease prevalence in bees, transmission routes, and spillover events.

RNA viruses, once considered specific to honey bees, are suspected of spilling over from managed honey bees into wild bumble bee populations. To test this, I collected bees and flowers in the field from areas with and without honey bee apiaries nearby. Prevalence of deformed wing virus (DWV) and black queen cell virus (BQCV) as well as replicating DWV infections in *Bombus vagans* and *B. bimaculatus* were highest in bumble bees collected near honey bee apiaries ($\chi_1^2 < 6.531$, $P < 0.05$). My results suggest that honey bees are significant contributors of viruses to bumble bees. Flowers have been suspected as bridges in virus transmission among bees. I detected bee viruses on 18% of the flowers collected within honey bee apiaries and detected no virus on flowers in areas without apiaries, thus providing evidence that viruses are transmitted at flowers from infected honey bees. In controlled experiments using captive colonies in flight cages, I found that honey bees leave viruses on flowers but not equally across plant species. My results suggest that there are differences in virus ecology mediated by floral morphology and/or pollinator behavior. No bumble bees became infected in controlled experiments, indicating that virus transmission through plants is a rare event that is likely to require repeated exposure.

The few studies examining viruses in bumble bees are generally limited to virus detection, resulting in little understanding of the conditions affecting virus titers. In honeybees, infections may remain latent, capable of replicating under certain conditions, such as immunosuppression induced by pesticide exposure. I tested whether exposure to imidacloprid, a neonicotinoid pesticide, affects virus titers in bumble bees. In previous honey bee studies, imidacloprid exposure increased virus titers. In contrast, I found that bumble bee exposure to imidacloprid decreased BQCV and DWV titers ($\chi_4^2 < 20.873$, $p < 0.02$). My findings suggest that virus-pesticide interactions are species-specific and results from honey bee studies should not be generalized across other bee species.

Having found that honey bees are significant contributors of viruses to wild bees and flowers, I investigated how honey bee management practices affect disease spread and developed recommendations and tools to lesson the risk of spillover events. Honey bee disease may be exacerbated by migratory beekeeping which increases stress and opportunities for disease transmission. I experimentally tested whether migratory conditions contribute to disease spread in honey bees and found negative yet varying effects on bees suggesting that the effects of migratory practices may be ameliorated with rest time between pollination events. State apiary inspection programs are critical to controlling disease spread and reducing the risk of spillover. However, these programs are often resource constrained. I developed and deployed a toolkit that enables state programs to prioritize inspections and provide a platform for beekeeper education. Using novel data collected in Vermont, I discovered several promising avenues for future research and provided realistic recommendations to improve bee health.

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

Bees naturally host a broad range of parasites, parasitoids, and pathogens including bacteria, fungi, and viruses (Gillespie, 2010; Evans & Schwarz, 2011; Kissinger et al., 2011). Over the past five decades, the inadvertent spread of exotic pests and pathogens through long-distance travel of honey bees has resulted in devastating losses for beekeepers, particularly in the US and Europe (Neumann & Carreck, 2010; Evans & Schwarz, 2011). Although there are over 20,000 species of bees in the world, the vast majority of bee disease research has focused on a single species of managed bee, the European honey bee (*Apis mellifera*). More recently, disease has been implicated in the decline of several bumble bee species (*Bombus* spp.), (Potts et al., 2010; Cameron et al., 2011; Koch, 2011; Meeus et al., 2011; Colla et al., 2012) and broadened the focus of bee disease to include wild bees (albeit predominantly two model organisms, *B. terrestris* and *B. impatiens*). Despite this knowledge growth, the concurrent expansion of global trade has led to the emergence and host switching of bee pathogens, leaving in its wake, many unanswered questions and unexplored avenues of research.

Pathogen spillover can occur when heavily infected domesticated hosts interact with closely related novel populations. The spillover of pathogens has emerged as a major threat to both managed and wild bee species (Tentcheva et al., 2004; Klee et al., 2007; Rosenkranz, Aumeier & Ziegelmann, 2010; Meeus et al., 2011; Graystock et al., 2013a; Fürst et al., 2014; Graystock, Goulson & Hughes, 2014; Schmid-Hempel et al., 2014). Perhaps the best-known example in the honey bee literature is the *Varroa* mite (*Varroa destructor*), an ectoparasite that vectors numerous viruses, suppresses honey bee

immune systems, and causes colony losses. *Varroa* began its circumglobal invasion in the 1960s when it host jumped from the Asian honey bee (*Apis cerana*) to *Apis mellifera* in Africa and has since spread to Europe, the Americas, and New Zealand (Rosenkranz, Aumeier & Ziegelmann, 2010; Nazzi et al., 2012). In another similar example, *Nosema ceranae*, a microspordian implicated in high honeybee colony mortality in Spain (Higes et al., 2008), also reached high frequencies since its introduction from Asia to the Americas and Europe (Klee et al., 2007; Chen et al., 2008). The global trade of commercially available bumble bee colonies purchased primarily for the pollination of green house crops is also contributing to the spread of disease to wild bumble bee populations (Colla et al., 2006; Otterstatter & Thomson, 2008; Brown, 2017). Commercial colonies are commonly infected with *Nosema bombi*, *Crithidia*, and RNA viruses, likely, in part, a result of laboratory rearing conditions (Graystock et al., 2013b; Sachman-Ruiz, Narváez-Padilla & Reynaud, 2015). Honey bee-collected pollen is used as a food source and is implicated as a potential source of viruses to commercially reared bumblebee colonies (Singh et al., 2010). In light of these findings and global reports of wild bumble bee species declines (Goulson, Lye & Darvill, 2008; Grixti et al., 2009; Cameron et al., 2011; Colla et al., 2012; Goulson et al., 2015), there is a critical need to examine virus spillover from managed honey bees to *wild* bumble bee species.

Once considered to be honey bee specific, RNA viruses have been detected in numerous arthropod species including bumble bees (Levitt et al., 2013). With short generation times, RNA viruses are able to quickly mutate and are likely to switch hosts (Domingo & Perales, 2012), however host range and prevalence in wild bumble bee species are largely unknown (reviewed in Manley, Boots, & Wilfert, 2015). Only two

studies to date have conducted comprehensive surveys of virus prevalence in bumble bees, both of which focused on Old World species of the UK (Fürst et al., 2014; McMahon et al., 2015). No previous study has examined virus prevalence among New World species. In both UK studies, virus prevalence in bumble bees was linked to virus prevalence in honey bees, providing evidence of disease spillover, however directionality could not be elucidated (Fürst et al., 2014). To further investigate the evidence for virus spillover from honey bees, additional studies are needed to examine virus prevalence in bumble bees captured from sites both with and without neighboring honey bees. Conducting this work in New World bumble bee species would greatly add to our knowledge of virus prevalence in understudied organisms.

Although RNA viruses have been detected in non-*Apis* bee species, interspecies transmission routes are virtually unknown. It is hypothesized that viruses are spread to new hosts at flowers while pollinators forage and comingle (McArt et al., 2014). Although this hypothesis is largely accepted, no study to date has directly tested the role of flowers in virus transmission. Israeli acute paralysis virus (IAPV) was found in bumble bees that foraged and comingled alongside experimentally infected honey bee colonies (Singh et al., 2010). However, the role of direct contact verses indirect contact via floral resources in this study remains unclear. Flowers are dispersal platforms for the interspecies transmission of other bee pathogens including *N. ceranae*, *N. bombi,* and *Crithidia* (Durrer & Schmid-Hempel, 1994; Graystock, Goulson & Hughes, 2015). Although it is likely that flowers play a role in the spread of bee viruses, studies that directly test the transmission route are needed.

The few studies examining RNA viruses in bumble bees are generally limited to virus detection with only a handful testing for virus replication. Viruses have been detected in 11 bumble bees species, yet only three studies have confirmed the replication of viruses in seven species, three of which were New World species (Li et al., 2011; Levitt et al., 2013; Fürst et al., 2014). Furthermore, the effects and conditions leading to higher virus titers in bumble bees are virtually unknown. In honeybees, viruses may remain as latent infections capable of replicating under certain conditions, such as immunosuppression induced by *Varroa* mites and pesticide exposure (Yang & Cox-Foster, 2005; Di Prisco et al., 2013). Since bees naturally face a multitude of threats, it is critical to understand the effect of multiple interacting stressors. In particular, there is a paucity of studies examining pesticide-pathogen interactions, particularly for non-*Apis* bee species (Collison et al., 2016). To my knowledge, there are no studies examining pesticide-virus interactions in bumble bee species.

History has taught us that long-distance travel is a major contributor of disease spread (reviewed in Fèvre et al., 2006; Tatem, Rogers & Hay, 2006). For decades, we have known that travel will exacerbate bacterial and viral infections for vertebrate livestock (Yates, 1982), yet its effect on invertebrate livestock (honey bees) is understudied (Goulson et al., 2015). Over 1.3 million honey bee colonies, representing half of the US's commercial honey bee population, undergo long distance travel each year for large crop pollination events (USDA National Agricultural Statistics Service, 2017a). Known as migratory beekeeping, colonies are transported by truck to a series of monoculture crops. Conditions are stressful and opportunities for disease transmission are abundant as millions of colonies originating from across the county converge on a single

crop for a month at a time (Simone-Finstrom et al., 2016; Glenny et al., 2017). Globally, we have witnessed disease spread by the movement of managed honey bees (Goulson et al., 2015; Wilfert et al., 2016). However, the role of migratory travel in disease spread across the US is practically unknown. Previous studies, surveys typically focused on the collection of baseline data, have found high disease prevalence in migratory colonies (Welch et al., 2009), including four novel virus strains (Runckel et al., 2011). Through preliminary research I conducted as part of the National Honey Bee Survey, I found higher virus and *Varroa* loads in Vermont's migratory colonies as compared to stationary colonies (Vermont's Pollinator Protection Committee, 2017). While this work has pointed to migratory colonies as contributors of disease spread, previous studies have lacked a proper stationary control group and results have been confounded by sampling time and other beekeeping practices. To examine the role of migratory conditions in disease spread, experimental approaches that control for these confounding factors are needed. Understanding how beekeeping practices, such as migratory stressors affect disease is necessary to inform practical recommendations to reduce disease spread in both managed and wild pollinators.

Proper surveillance systems and beekeeper education may reduce disease incidence in honey bees and lower the risk of disease spillover to wild pollinators. In the US, state apiary inspection programs are often at the forefront of these campaigns, combating bee disease through colony inspections and providing education to beekeepers (Ellis, 2016). However, for many states, the programs are often understaffed and underfunded, leaving gaps in our defense against bee disease and our ability to understand the risk factors associated with colony loss at the local scale. To improve bee

health and lessen the risk of disease spread, a 'grassroots' approach should be taken whereby we improve the ability of our state apiary inspection programs to perform inspections, educate beekeepers and gather data to drive future research efforts.

Dissertation Overview

Question 1) Are RNA viruses spilling over from managed honey bees (*Apis mellifera)* to wild bumble bees (*Bombus* spp.) (Chapter 2)? I conducted a comprehensive field survey to examine the prevalence of RNA viruses in two understudied wild bumble bee species, *B. vagans* and *B. bimaculatus*. To test whether viruses are spilling over from managed honey bees into wild bee populations, I surveyed bumble bees from sites with and without a nearby honey bee apiary. To examine how honey bee virus loads impact virus prevalence in bumble bees, I also collected and tested honey bees from sites when present. At each site, I also conducted bee abundance surveys to examine how the relative abundance of bumble bees and honey bees influence patterns in bumble bee virus prevalence. Using real time reverse quantitative polymerase chain reaction (RT-qPCR) I tested all bees for three RNA viruses: deformed wing virus (DWV), black queen cell virus (BQCV) and Israeli acute paralysis virus (IAPV) and calculated virus loads for all bee species. I detected BQCV and DWV in both bumble bee species and species-specific differences with *B. bimaculatus* having significantly higher BQCV load and prevalence as compared to *B. vagans*. For both viruses, prevalence was significantly higher in bumble bees collected near managed honey bee apiaries and bumble bees were more likely to be infected with DWV when neighboring honey bees had high infection levels. Most notably, in sites completely absent of honey

bees, no DWV was detected in bumble bees. These results indicate that honey bees are significant contributors to viruses detected in bumble bees.

Next, to test whether the bumble bees hosted replicating viral infections, I amplified the negative RNA virus strand in all virus-positive bumble bees. I discovered virus replication for both DWV and BQCV in both bumble bee species with *B. bimaculatus* having significantly higher rates of replication as compared to *B. vagans*. Active replicating infections were more prevalent in bumble bees collected near honey bee apiaries for DWV but not BQCV.

Collectively, these results contribute to our understanding of virus ecology in bumble bees and provide strong evidence for RNA virus spillover from managed honey bees into wild bumble bees.

Question 2) What is the role of shared floral resources in bee-virus transmission (Chapter 2 and Chapter 3)? Having established that bumble bees are more likely to host RNA viruses near honey bee apiaries, I then hypothesized that flowers could serve as platforms in virus transmission between bee species. I predicted that if viruses were spilling over from managed honey bees, then flowers collected near honey bee apiaries would be more likely to harbor viruses. Additionally, due to differences in floral morphology and bee behavior while foraging for nectar and pollen, I hypothesized that plant species would differ in their propensity to harbor viruses. To test whether flowers host bee viruses, I collected flowering plant samples during my comprehensive field survey (Chapter 2) from sites both with and without honey bee apiaries. I discovered that a high proportion of flowers hosted bee viruses (18%), and the only positive-virus samples were collected in sites near honey bee apiaries. These findings indicate that

honey bees are significant contributors to RNA viruses on the landscape, and flowers may serve as bridges in bee-virus transmission. To further test the floral transmission route hypothesis, and examine whether plant species differ in their propensity to harbor viruses, I conducted a controlled experiment using captive bee colonies (Chapter 3). From seed, I grew three plant species (red clover, white clover, and birdsfoot trefoil) in the greenhouse and upon bloom, allowed infected honey bees to forage on arrays of inflorescences within tent enclosures. Next, in separate enclosures, I allowed uninfected bumble bees to forage on either the honey bee flowers or 'clean' flowers as a control group. After the foraging trials, all bumble bees and flowers were collected and tested for DWV and BQCV. Similar to the field collected plants, I detected DWV and BQCV on 25% and 21.8% of plant samples. There was a significant interaction effect of plant species and virus type such that DWV and BQCV were not equally distributed across plant species. These results suggest differences in viral ecology and/or differences in pollinator contact with flowers. No bumble bees became infected in this experiment, suggesting that virus transmission through flowering plants is a rare occurrence, with experimental detection contingent on many factors. Collectively, this work demonstrated, for the first time, that honey bees leave behind viruses on flowers while they forage. In addition, it provided several avenues for future work such as the dynamics governing virus deposition on flowers and whether transmission to bumble bees can occur under experimental conditions.

Question 3) How does pesticide exposure affect RNA virus titers, sucrose intake, and survivorship in bumble bees (*B. impatiens*) (Chapter 4)? Having found that RNA viruses are prevalent in wild bumble bee species, I hypothesized that virus

titers in bumble bee hosts will be affected by additional stressors (Chapter 4). Bees encounter multiple interacting stressors, yet few studies have examined these interactions (Collison et al., 2016). For example, in isolation, both pesticide exposure (Baron et al., 2017; Woodcock et al., 2017) and viruses (Genersch et al., 2006; Gauthier et al., 2011; Fürst et al., 2014; Piot et al., 2015) negatively impact bees. However, few studies have examined pesticide-virus interactions (Boncristiani et al., 2012; Di Prisco et al., 2013; Locke, Forsgren & De Miranda, 2014; Doublet et al., 2015) and none have focused on the question in bumble bee species. In honey bees, exposure of a common class of pesticides, neonicotinoids, affects immune related genes and causes increased virus titers (Di Prisco et al., 2013), however its effects on bumble bee virus titers are completely unknown. Using captive bumble bee colonies (*B. impatiens*) that arrived to our lab already infected with DWV and BQCV, I examined how chronic oral exposure to imidacloprid, a commonly used pesticide in the neonicotinoid family, affects virus load, food intake, and survivorship. Contrary to previous results derived from the few honey bee studies, I found that imidacloprid exposure reduced BQCV and DWV titers. In addition, at high doses, imidacloprid caused a reduction in food intake, yet survivorship was not affected. These food intake results corroborate previous work showing the negative effects of pesticide exposure. However, the reduction in virus titers was unexpected and indicates that pesticide-virus interactions are highly variable among bee species and underscores the danger of relying on honey bee studies to generalize results across the multitude of non-*Apis* species.

Question 4) Does migratory beekeeping contribute to disease load and spread in honey bees (Chapter 5)? In my previous chapters, I established that honey bees are

important contributors of RNA viruses and bumble bees are more likely to host viruses when neighboring honey bees have high virus loads. These results indicated that honey bees, as managed pollinators, could possibly be managed in such a way as to reduce disease loads and reduce the risk of pathogen spread. Although untested, previous work hypothesized that migratory beekeeping practices could play a role in disease spread (Glenny et al., 2017; Welch et al., 2009). To begin to establish the basis for practical management recommendations that would reduce the risk of spillover, I conducted a field experiment to examine the role of migratory beekeeping practices in disease spread among honey bees (www.experiment.com\beekeeping). I conducted an experiment in which I transported honey bee colonies from North Carolina to pollinate almonds in California and back to North Carolina. Before and after the pollination event, I compared the parasite and pathogen loads as well as population size of the migratory group to a stationary group of colonies in North Carolina. Upon the return of the migratory colonies, I measured subsequent disease spread to a separate group of stationary colonies. Migratory colonies returned from California with fewer bees and higher BQCV loads as compared to stationary colonies. However, one month later, BQCV loads of the two groups were similar. Colonies exposed to migratory bees experienced a greater increase of deformed wing virus prevalence and load compared to the isolated stationary group. The three groups had similar infestations of *Varroa* mites upon return of the migratory colonies. However, one month later, mite loads in migratory colonies were significantly lower compared to the other groups, possibly because of lower number of host bees. These results demonstrate that migratory practices have varying effects on honey bee

health and disease that may be ameliorated if colonies are allowed ample rest time between large pollination events.

Question 5) How can we overcome limitations of State Apiary Inspection Programs to ameliorate pollinator health (Chapter 6)? State apiary inspection programs play a critical role in the maintenance of healthy bee populations through colony inspections and beekeeper education. However, the programs often face a number of limitations and challenges. Working with state apiary inspection programs, I first identified common challenges and limitations they face. Next, I developed a toolkit designed to help overcome some of the common limitations, consisting of data collection tools as well as two online applications. *BeekApp* (https://apiarydata.shinyapps.io/BeekApp/) allows users to view and interact with apiary and beekeeper data specific to their state. *InspectApp* enables technology to apiary inspectors to prioritize inspections and aid in performing routine tasks. Using Vermont as a case study, I deployed the toolkit and in doing so, derived the first data set Vermont Apiary Inspection program has collected on colony losses and beekeeping practices. As a result, I developed informed recommendations to improve bee health in Vermont and identified several promising avenues for future research. Based on the success in Vermont, I believe the toolkit can be used as a template for other states with resource-constrained apiary inspection programs.

Conclusions

Despite nearly two decades of active research examining risk factors of bee population declines, many knowledge gaps remain. Although pests and pathogens are cited among the top threats to bees, studies that examine pathogens in wild bees remain

rare. Diseases are likely to spillover from managed bees to wild bee populations, and data are needed to inform recommendations that will lessen spillover risk. The results of my dissertation provide evidence that honey bees are significant contributors of viruses to wild bumble bees, and to floral resources that could potentially serve as bridges in virus transmission. In addition, my work demonstrates that multi-stressor interactions are host specific; indicating that results derived from honey bee research cannot be generalized across other pollinator species such as bumble bees. I also found that honey bee management regimes, such as migratory beekeeping, can have negative impacts on honey bee disease and population size. Lastly, with the aim to improve pollinator health, my work identified common limitations of state apiary inspection programs and provides a framework for improving the programs' ability to conduct inspections and provide beekeeper education.

Although I experimentally demonstrated that viruses are left behind on flowers by foraging honey bees, the next step is to further investigate whether bumble bees can become infected after visiting inoculated flowers (currently being examined by S. A. Alger and P. A. Burnham). To reduce the risk of disease spillover to wild bees, I am working to collaborate with other apiary inspection programs to improve their ability to combat disease spread in honey bees.

In all, my dissertation research has provided new insight into the spillover of bee pathogens among managed and wild bees and the role of flowers in bee virus transmission, as well as pathogen-pesticide interactions in a wild bumble bee species. In addition to these important contributions to virus ecology and science, my work also aids in applied efforts to improve bee health by developing technology for apiary inspection

programs and beekeeper education and providing informed recommendations for management practices.

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CHAPTER 2: RNA VIRUS SPILLOVER FROM MANAGED HONEY BEES TO WILD BUMBLE BEES

Abstract

The decline of many bumble bee species (*Bombus* spp.) has been linked to an increased prevalence of pathogens likely caused by spillover from managed bees (Colla et al., 2006; Otterstatter & Thomson, 2008). Although poorly understood, RNA viruses are suspected of moving from managed honey bees (*Apis mellifera*) into wild bumble bees through shared floral resources (Singh et al., 2010; Fürst et al., 2014). We examined evidence for RNA virus spillover from managed honey bees, the extent to which viruses are replicating within bumble bee hosts, and the role of flowers in transmission. We surveyed bees and flowers from sites either with or without managed honey bee apiaries and found that viruses detected in bumble bees and on flowering plants were strongly correlated with the presence of neighboring honey bees. Prevalence and replicating infections of deformed wing virus (DWV) as well as prevalence of black queen cell virus (BQCV) were higher in bumble bees collected near apiaries. Additionally, bumble bees were more likely to be infected with DWV when neighboring honey bees had high infection levels and no DWV was detected in bumble bees where honey bees were absent. Furthermore, we detected viruses on a high proportion of flower samples (18%), all of which were collected within apiaries. Our results show that honey bees are significant contributors of viruses to wild bumble bees and flowering plants. Collectively, our results support the hypothesis that viruses are spilling over from managed honey bees to wild bumble bees and that flowers may be an important route for transmission.

Significance Statement

Many species of bumble bees are declining worldwide. Disease spillover from managed bees is among the primary listed threats to these important pollinators. Although widely hypothesized, it has not been demonstrated that RNA viruses are spilling over into wild bumble bee populations through shared floral resources. By screening bumble bees, honey bees, and flowers from sites both with and without managed honey bee apiaries, we provide evidence that honey bees are significant contributors of viruses to wild bumble bees via flowering plants. Moreover, we demonstrate viral replication in two New World bumble bee species. Our study highlights the need to improve disease-monitoring and reduction efforts for managed bees to reduce spillover events.

Introduction

Many diseases are caused by generalist pathogens that infect multiple host species (Fenton & Pedersen, 2005). For pathogens capable of infecting multiple hosts, spillover occurs when the pathogen is introduced and transmitted from a reservoir population into a naive host population. Pathogen spillover between managed and wild animals causes species declines, threatens global biodiversity, and alters ecosystem function and services (Daszak, Cunningham & Hyatt, 2000; Power & Mitchell, 2004). Due to the complexity of multi-host systems, the principal directionality of spillover events is oftentimes difficult to determine.

Given recent declines in managed honey bees (*Apis mellifera*), the importance of native pollinators and their ability to provide effective pollination services has risen to global attention (Klein et al., 2007a; Winfree et al., 2007). Many of the threats to managed honey bees are also affecting native bees (Naug, 2009; Potts et al., 2010; González-Varo et al., 2013;

Hopwood et al., 2013), most notably the increased prevalence of pathogens caused by spillover events from managed bees. Disease spillover from managed honey bees to wild bees has been examined in several bee pathogens including the microsporidian parasites *Nosema ceranae* and *N. bombi,* a trypanosome *Crithidia bombi,* and a parasitic protozoan *Apicytis bombi* (Colla et al., 2006; Otterstatter & Thomson, 2008; Williams & Osborne, 2009; Graystock et al., 2013a; Graystock, Goulson & Hughes, 2014; McMahon et al., 2015).

Although poorly understood, RNA viruses are also suspected of moving from managed honey bees into other insect species including wild bees (Singh et al., 2010; Fürst et al., 2014). Once considered to be specific to European honey bees, RNA viruses have now been detected in a wide range of insects including bumble bees, solitary bees, hoverflies, wasps and ants (Singh et al., 2010; Li et al., 2011; Peng et al., 2011; Evison et al., 2012; Levitt et al., 2013; Fürst et al., 2014; Ravoet et al., 2014; McMahon et al., 2015; McMenamin & Genersch, 2015). Due to their high mutation rates and short generation time, RNA viruses are likely to cross species barriers and adapt rapidly to new environments (Singh et al., 2010; Li, Cornman & Evans, 2014). Both relatedness and shared foraging habits have been proposed to increase the risk of disease transfer among managed bees and native bumble bees (Goulson, 2003; Li et al., 2011). In the United Kingdom (UK), sympatric bumble bees and honey bees are infected by the same deformed wing virus (DWV) strains (Fürst et al., 2014) and virus prevalence in honey bees is a significant predictor of virus prevalence in bumble bees (McMahon et al., 2015). A phylogeographic analysis of DWV attributes its global distribution to the European honey bee and the spread of the *Varroa* mite which vectors the virus (Wilfert et al., 2016); however, other bee species are not hosts for the *Varroa* mite. Although there is some evidence of virus spillover from managed honey bees into wild bees, more work is needed to elucidate transmission routes,

the principal directionality of virus transmission, and whether, once contacted, viruses replicate in bumble bee hosts (Tehel, Brown & Paxton, 2016).

Horizontal transmission routes for viruses among bee species are currently suspected but largely unconfirmed. One potential route of transmission is through the use of shared floral resources (Singh et al., 2010; Zhang et al., 2012; Fürst et al., 2014; McMahon et al., 2015). Viruses have been detected in the feces and glandular secretions of worker honey bees as well as in the pollen loads they carry (Chen et al., 2006; Yue et al., 2007; Singh et al., 2010). Thus, viruses may be directly transmitted through salivary secretions or feces while bees are mingling on flowers or indirectly through infected nectar and/or pollen. To our knowledge, only one study examined bee viruses in pollen directly collected from flowers in a single apiary. Pollen was collected from both unvisited (netted) flowers and flowers that had been visited by foraging bees. Viruses were detected on pollen from visited flowers only, however pollinator visitations were not measured (Mazzei et al., 2014). Overall, the degree to which viruses can be horizontally transmitted with flowers acting as a bridge is poorly understood (McMahon et al., 2015).

The purpose of this study was to assess if there is evidence for the spillover of RNA viruses from managed honey bees into wild bumble bees, and if so, whether transmission may be mediated by the shared use of floral resources. First, we examined the prevalence of RNA viruses in two bumble bee species, and the extent to which bumble bees had active replicating infections. We then examined if virus prevalence, load, and virus infection in bumble bees is related to the presence of neighboring managed honey bee colonies and their virus loads. We also investigated horizontal

transmission through shared floral resources by examining how bee abundance, honey bee colony presence, and landscape level floral density influences patterns of virus prevalence and by testing flowers collected from our field sites for RNA viruses. Our results provide evidence that honey bees are significant contributors of RNA viruses to both wild bumble bee species and flowers.

Results

To determine the prevalence of RNA viruses in bumble bees and to assess if there is evidence for virus spillover from managed honey bees, we surveyed bumble bees, honey bees, and flowering plants across Vermont from 19 sites either with (7) or without (12) a commercial honey bee apiary (Table 1). We detected BQCV in 75% and DWV in 8% of bumble bees tested. We did not detect Israeli acute paralysis in any of the bees. Bumble bees collected within 1 km of a commercial honey bee apiary had significantly higher prevalence of both viruses compared to bumble bees collected from sites without a commercial apiary nearby (BQCV: $\chi_1^2 = 3.959$, $P = 0.047$; DWV: $\chi_1^2 = 6.531$, $P < 0.012$) (Fig. 1). In sites both without a commercial apiary and completely absent of honey bees (no honey bee foragers were observed during visitation surveys), all bumble bees were negative for DWV (Fig. 2). Virus load for both viruses in bumble bees was not significantly affected by apiary presence. By amplifying the negative strand of RNA viruses, we detected actively replicating virus for BQCV and DWV in both bumble bee species. In bumble bees with viruses detected, we found BQCV replication in 20% and DWV replication in 16% of bumble bees. Replicating DWV was more prevalent in bumble bees collected near honey bee apiaries ($\chi_1^2 = 4.013$, $P = 0.045$). However, this was not the case for BQCV infections ($\chi_1^2 = 0.968$, $P = 0.325$) (Table 2).

Prevalence of BQCV was significantly higher in *B. bimaculatus* (85%) compared to *B. vagans* (65%) (χ_1^2= 15.671, P < 0.001) but there was no difference in prevalence of DWV between species (Table 2). Virus loads in bumble bees ranged from 10^3 to 10^6 for DWV and 10^4 to 10^8 for BQCV. *Bombus bimaculatus* had significantly higher virus loads than *B. vagans* for BQCV (χ_1^2= 18.662, P < 0.001) but not DWV. Actively replicating BQCV infections were higher in *B. bimaculatus* (28%) compared to *B. vagans* (11%) (χ_1^2 = 19.828, P < 0.001). DWV was actively replicating in 23% of *B. bimaculatus* and 12% of *B. vagans* (χ_1^2 = 0.027, P = 0.87).

Honey bee virus loads (measured in average virus genome copies per bee) ranged from 10^3 to 10^9 for DWV and 10^6 to 10^9 for BQCV. No Israeli acute paralysis virus was detected in the honey bees. Honey bee DWV loads followed a bimodal distribution (Fig. S1) with clear separation between two groups which we designated as either having "low" (< 10^7 genome copies) or "high" (> 10^7 genome copies) virus loads. The prevalence of DWV in bumble bees was significantly higher in sites with high honey bee DWV loads compared to bumble bees collected from sites where DWV load in honey bees was low (χ_1^2 = 8.068, P = 0.018; full model fit: χ_4^2 = 17.375, P = 0.002) (Fig. 2).

We found no evidence for spatial autocorrelation for DWV prevalence (Moran's I: 0.018, p = 0.29) or BQCV load (Moran's I: -0.045, p = 0.88). However, there was significant clustering for DWV virus load (Moran's I: 0.083, p = 0.01) and weak clustering for BQCV prevalence (Moran's I: 0.120, p = 0.03).

Overall, we detected viruses on 18% (n=6) of the flower samples. Virus loads on flowers ranged from 10^4-10^5 genome copies per gram of flower material. All positive samples came from flowers collected from sites with honey bee apiaries (Fig. 3) and

included the following plant species: (*Asclepias syriaca,* milkweed; n=2), (*Monarda spp*., bee balm, n=2), (*Trifolium pratense*, red clover; n=1), (*Melilotus albus,* white-sweet clover; n=1). Of the samples collected in apiaries, 30% (n=4) were positive for DWV, 23% (n=3) were positive for BQCV; one of these were positive for both viruses. Because bumble bees and honey bees may be contributors to viruses on plants, we examined whether the abundance of each species were significant predictors of virus detection of flowers. Based on the bee abundance surveys, honey bee abundance but not bumble bee abundance was greater in sites where we detected viruses on plants ($\chi_1^2 = 7.567$, P = 0.006; full model fit: $\chi_2^2 = 14.729$, P = 0.006) (Table 3).

Site-level floral density was significantly positively correlated with DWV prevalence in bumble bees ($\chi_1^2 = 6.025$, P = 0.014). However, floral density was not correlated with BQCV prevalence, BQCV load, or DWV load (Table 2).

Discussion

By examining viruses in both bumble bees and on flowers from sites with and without honey bees, we show that managed honey bees are significant contributors of RNA viruses to both wild bumble bees and floral resources. We also show that the occurrence of replicating DWV infections was highest in bumble bees collected near apiaries. Together, our results support the hypothesis that RNA viruses are spilling over from managed honey bees into wild bumble bee populations through the use of shared floral resources.

In the bees we sampled, BQCV prevalence and replication was higher for *B. bimaculatus* than *B. vagans*. Although both species are medium sized long-tonged bees belonging to the *Pyrobombus* subgenus, *B. bimaculatus* queens emerge earlier and

establish colonies before *B. vagans*. By emerging earlier, *B. bimaculatus* may have an increased opportunity of foraging overlap with honey bees and contacting virus particles on flowers. In spring, honey bees must intensify their pollen foraging activities to sustain their colony's dramatic increase in brood rearing. If viruses are transmitted among bees through pollen, early-emerging bumble bees could be at a higher risk for contacting contaminated pollen grains left behind by honey bees. Understanding the temporal variation of virus prevalence among bumble bee species and flowers would help to understand the ecological factors driving virus transmission and infectivity.

We detected bee viruses on flowers of four different plant species and only found viruses on flowers we collected within honey bee apiaries. These results support the hypothesis that viruses are likely left behind by foraging honey bees and provide evidence that sites near honey bee apiaries could be hotspots for disease transmission between honey bees and wild bees through the use of shared floral resources.

If transmission of bee viruses occurs through the shared use of flowers, we predicted virus prevalence patterns to be shaped by landscape level floral composition. The prevalence of DWV in bumble bees was lower in sites with high floral density. In areas or times with a high abundance of floral resources, foraging overlap among bees and competition for the available flowers may be reduced. Our results of DWV support a dilution phenomenon whereby the risk of infection was lessened for individual foragers in areas of high floral abundance. However, we did not find an effect of floral density on BQCV prevalence. Other factors besides transmission from honey bees at floral resources may be more important for the spread of BQCV in bumble bees. It is likely that BQCV is vertically transmitted, as with honey bees (Chen, Evans & Feldlaufer, 2006), or highly

transmissible among nest mates. In captive lab colonies that are positive for BQCV, prevalence within a colony is near 100% (Alger, unpub. data) indicating that rapid dissemination within a colony may occur. This may also explain our observations of high BQCV prevalence as compared to DWV as well as the occurrence of replicating BQCV infections, regardless of apiary presence.

Here, we homogenized and pooled flowers for virus assays. Separately testing petals, nectaries, pollen etc. could help understand where viruses are deposited on flowers and lead to experiments testing how different floral traits influence a plant species' propensity to harbor and transmit viruses. For example, if viruses are detected in nectaries, antiviral secondary metabolites expressed in the nectar of some plants could reduce virus survivability (Aurori et al., 2016). Further, flowers with deep nectaries could exclude some pollinators and reduce transmission between bee species. Floral morphology that influences bee-flower contact or forager handling time could also affect virus deposition (McArt et al., 2014). Future controlled experiments should elucidate how differences in floral traits influence the likelihood for virus deposition and transmission.

Several bumble bee species of Europe, North America, and Asia have suffered dramatic declines. Particularly in North America, pathogens appear to be a chief threat to this group (Williams & Osborne, 2009). Overall, we detected DWV in 8% of all bumble bees tested which falls between other estimates from Europe where reported prevalence ranged from 3% to 11% (Fürst et al., 2014; McMahon et al., 2015). However, BQCV prevalence (75%) in the bumble bees we tested was 12.5 times higher than UK reports (6%) (McMahon et al., 2015). Although it is often difficult to directly compare results among studies, we believe this substantial difference is notable given the similarities of

sample sizes and sampling efforts between the studies. These differences could be due to bumble bee species susceptibility and/or life history traits that affect exposure to the viruses.

By detection of the negative virus strand, our study confirms viral replication in two bumble bee species and adds to the growing list of bee species that may be affected by RNA viruses. Despite the burgeoning interest in viruses among wild bees and the confirmation of replicating viral infections, the effects of viruses on non-*Apis* species physiology and fitness are almost completely unknown (but see Genersch et al., 2006; Fürst et al., 2014; Meeus et al., 2014). If bumble bees are greatly affected, RNA viruses may be contributing to observed declines. Conversely, bumble bees may serve as a tolerant reservoir host, facilitating the maintenance of viral infections within the pollinator community at large. Improving knowledge of RNA virus effects is critical to protecting vulnerable species.

Compelling evidence for pathogen spillover from managed bees to wild bumble bees indicates a need for management guidelines that reduce the introduction and spread of bee pathogens. For example, developing robust apiary inspection programs is a priority. By monitoring bee disease and providing beekeeper education, these programs serve as a first line of defense against honey bee disease outbreaks. We recognize that virus detection often involves molecular techniques unavailable to most apiary inspection programs due to funding constraints. However, visual inspections can detect *Varroa* mites, which through proper monitoring and treatment, can reduce virus loads (Martin, Ball & Carreck, 2010) thus reducing the risk of virus spillover to wild populations. For example, in Vermont, where this study was conducted, only 36% of beekeepers reported

monitoring mite populations in their colonies and only 67% of beekeepers reported using mite treatments (Vermont Department of Agriculture Food and Markets & University of Vermont, 2018), indicating a specific opportunity for apiary inspection programs to improve beekeeper education regarding mite monitoring and treatments. In addition, since viruses can spread in honey bees, even at low virus titers (Francis, Nielsen & Kryger, 2013), state level management guidelines should limit apiary activity or increase disease monitoring in critical habitat of sensitive wild bee populations, such as the federally endangered *B. affinis* (Rusty patch bumble bee) (Fish and Wildlife Service, 2017). Although our study focused on two RNA viruses, the spillover of numerous other pests and pathogens from commercial bees is well documented (Colla et al., 2006; Otterstatter & Thomson, 2008; Graystock et al., 2013a; Manley, Boots & Wilfert, 2015; Sachman-Ruiz, Narváez-Padilla & Reynaud, 2015). With the increase in global transportation of commercial pollinators, introduced pests and pathogens will continue to pose problems for conservation efforts underlining the need to prevent the introduction of disease through robust monitoring and management practices. In all, the conditions under which transmission among bee species occurs need to be further explored to develop a predictive understanding and thus mitigation measures.

Materials and Methods

FIELD SAMPLING

All field surveys were conducted June 18th- August 26th 2015 across 19 field sites in Northern Vermont. We chose seven sites with commercial managed honey bee apiaries within 300 m. For these sites, the apiaries were managed by a commercial beekeeper and number of honey bee colonies ranged from 19-48 (mean=28.7+/- 9.6 colonies). We chose

twelve sites with no nearby commercial apiaries within 1 km. Sites were located within one of the following landcover types: urban, farmland (crops and wildflower meadows), forest, and wetlands. At each sampling location, we made collections of bumble bees by walking haphazard trajectories among flowering plants within at least a 15,000 m^2 area and catching all visible workers as they foraged on flowers with sweep nets. We identified each netted bee and identified and recorded the plant on which it was collected. At each site, we collected up to 15 bumble bees of each target species: *Bombus vagans* and *B. bimaculatus* (Table 1). To reduce the likelihood of collecting multiple samples from the same colony, collections were made throughout the entire sampling area. Honey bees were found in sites with and without apiaries nearby. In sites without a commercial apiary within 1 km, we collected up to 10 honey bee foragers from flowers. In sites with commercial apiaries, we sampled bees from eight randomly chosen colonies by netting forager honey bees directly from hive entrances. We combined honey bees into a single composite sample for that site. Honey bees were entirely absent in four sites (Table 1). We placed all bees on dry ice in the field to preserve RNA until lab storage at -80°C. Overall pollinator abundance could influence the likelihood of bee-to-bee contact. Therefore, we measured bee abundance, with a focus on bumble bees and honey bees. For each site, we walked a 100 m transect over a 10 minute period and recorded all bee individuals by morphotype within 5 m of either side of the transect.

Because shared flowers are suspected bridges for spillover of viruses from honey bees to wild bumble bees (Singh et al., 2010; McArt et al., 2014), we surveyed flowering plants at each field site. To test if flowers can harbor viruses, we collected 20-60 inflorescences from the most highly visited and locally common flower species at

each site. Samples were collected and stored on dry ice and stored in the lab at -80°C to later be tested for viruses. For each site, all flowering plants were identified and counted within a 1m x 1m quadrat that was placed every 10 m along the 100 m bee survey transect (Jha & Kremen, 2012). For each site, average flowering plant density was calculated as inflorescences/m^2.

Apiary Data

In 2015, Vermont began a mandatory apiary registration program whereby all beekeepers are required to report the location of each apiary. We obtained this apiary registration dataset from the Vermont Agency of Agriculture and used ArcGIS (v 10.3.1) to confirm our field observations of apiary and honey bee colony presence within a 1 km buffer zone from each bumble bee collection site.

Virus Detection and Quantification.

We extracted total RNA following Qiagen RNeasy mini kit protocols. After flash freezing individual bumble bees in liquid nitrogen, we homogenized each sample into 600 µl of GITC buffer in 1.5 ml vials using a pestle for 2 minutes. For honey bees, we pooled together samples of up to 10 bees from each site, flash froze the sample in liquid nitrogen and then homogenized it together in an extraction bag (Bioreba, Switzerland) with 2 mL of GITC buffer. For both bumble bees and honey bees, we centrifuged the resulting homogenate and mixed 100 µl of the lysate with RLT buffer (10% β-mercaptoethanol) and used Qiagen protocols thereafter. For plants, we transferred 1.5 g of flower material to an extraction bag and flash froze the sample in liquid nitrogen prior to grinding it to a powder using a ceramic pestle on the outside of the extraction bag for 30 seconds. After adding 3 mL of GITC buffer to the bag, we used

the pestle again on the outside of the bag to mix the homogenate into the buffer for 2 minutes. We centrifuged the resulting homogenate and used 200 µl tn RNA extractions following Qiagen RNeasy mini kit protocols. We assessed all RNA quantity and quality using a Spectrometer (Nanodrop, Thermo Scientific).

For bumble bees and honey bees, we diluted all RNA extractions to 20 ng/µl prior to virus assays. RNA recovered from plants was not diluted prior to further analyses. For reverse transcription of RNA and absolute quantification of each virus target in bees and plants, we performed duplicate reverse transcription quantitative polymerase chain reactions (RT-qPCR) for each sample using SYBR green one-step RT-qPCR kit in 10 ul reactions. We used the following thermal cycling program: 10 min at 50°C (RT) followed by 1 min at 95°C, and 40 amplification cycles of 95°C for 15 s, 60°C for 60s and derived melt-curves using the following program: 65-95°C (0.5°C increments, each 2s). We used primers specific to the following RNA virus targets: DWV, BQCV and IAPV, and a housekeeping gene (ACTIN) as a positive control of RNA extraction efficiency (Appendix A). To quantify virus load, we used triplicate standard curves of gBlocks Gene Fragments (Integrated DNA Technologies) (Appendix B). Efficiencies were 91 % (DWV), 95 % (BQCV), 90 % (IAPV), and 90 % (Actin), with correlation coefficients (R^2) ranging from 0.993-0.999. We tested a total of 15 composite honey bee samples and 342 bumble bee workers consisting of 180 *B. vagans* and 162 *B. bimaculatus*. We tested 33 flower samples of which 13 were collected from sites with apiaries and 20 were collected from sites without apiaries.

NEGATIVE STRAND DETECTION.

To test for actively replicating viruses in the bumble bees, we conducted strand specific RT-PCR (Boncristiani et al., 2009) on extracted RNA samples that tested positive for a virus. Each RNA sample was transcribed to cDNA using iScript cDNA Synthesis Kit (BioRad). To increase specificity, we used PAGE purified, biotinylated forward and reverse primers (Integrated DNA Technologies) during reverse transcription and purified the resulting cDNAs using magnetic beads coated with a monolayer of streptavidin following manufacturers protocols (New England BioLabs). We diluted each cDNA tenfold and then conducted PCRs with non-biotinylated primers in separate reactions for both for forward and reverse strands.

SEQUENCING

To confirm the identity of the viruses, we sequenced virus fragments from bumble bees and honey bees. qPCR product was cleaned (ExoSAP-IT PCR Product Cleanup) and sequencing was performed using the 3130xl Genetic Analyzer in the University of Vermont Cancer Center Advanced Genome Technologies Core. Sequence data were viewed for quality assessment (FinchTV 1.4) and aligned by eye to genome references using Geneious v 6.0.6 (BQCV: GenBank: KY243932.1; DWV: GenBank: KJ437447.1).

DATA REPORTING

We use "prevalence" to refer to the percentage of bumble bees positive for a virus. Virus load results in bees are presented in average virus genome copies/bee. Virus load results for flowers are presented as virus genome copies/gram of flower material. To measure honey bee and bumble bee abundance at each site, we calculated the number of bees observed per m^2 for each site. We calculated floral density as the number of

flowering inflorescences per m^2. We binned sites as either 'high' ($>10^7$) or 'low' ($<10^7$) honey bee virus loads based on the clear bimodal distribution of the logarithmic value of the virus genome copies/bee at for each site (Fig. S1).

Data processing and Analysis

We analyzed data from the qPCR runs using Thermo Fisher Cloud Software, v 1.0 (Life Technologies Corporation), and R v 0.99.903 (R Core Team 2016). We selected six ten-fold dilutions for each target (DWV, BQCV, IAPV, and Actin) and used a regression analysis to derive a standard curve for quantification. We quantified virus loads by using the slope and intercept to estimate genome copies from known Ct values. We converted RNA concentration to copies using the equation [copies=(cXN)/M] where c = concentration in g, N = avogrado's contant and M = molecular mass of the amplicon in Daltons. The baseline for qPCR runs was automatically set and thresholds were manually set for each virus and used for all runs (BQCV: 0.149, DWV: 0.137, IAPV: 0.25, ACTIN: 0.267). Samples with incorrect melting curve profiles or with Ct values outside our limit of detection were given a value of zero.

Statistics

We performed all analyses in R v 0.99.903 (R Core Team 2016). To test if virus prevalence or load was spatially clustered, we computed the spatial autocorrelation coefficient Moran's I with an inverse spatial distance matrix (R library ape, v 4.1, function Moran.I). To analyze virus load data, we first log transformed all virus loads to improve normality. To investigate whether honey bee apiary presence, floral density, or bumble bee species affected the prevalence or load of RNA viruses in bumble bees (DWV and BQCV were tested in separate models), we used separate general linear mixed

models (GLMMs) (R library lme4, v 1.1.13, functions lmer and glme) with virus load and virus prevalence as our response variables. Virus load was analyzed using a Gaussian distribution and the presence of virus as a binomial distribution. In each model we used the fixed effects apiary absent/present, site level floral density, and bumble bee species with site, latitude and longitude as random effects.

Site average honey bee virus loads were bimodally distributed (Fig. S1) and, therefore, we used a separate GLMM with binomial distribution to test if DWV virus prevalence in bumble bees is affected by the virus load in honey bees (high: $>10^7$ genome copies; low: $< 10^7$ genome copies) or honey bee abundance. We used honey bee viral load, honey bee abundance, and floral density as fixed effects and site as a random effect.

To investigate whether honey bee or bumble bee abundance affects virus deposition on plants, we used a GLMM with binomial distribution with the fixed effects honey bee abundance, bumble bee abundance and virus (DWV, BQCV) and site as a random effect. To calculate the effect of the presence of apiaries and bumble bee species on the prevalence of replicating viruses, separate chi-square tests for independence were conducted for each virus. To calculate the significance of each fixed effect for all models, we created a reduced model by removing the effect, and compared each reduced model to our full model using a log likelihood ratio test.

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Figure and Table Captions

Table 1. **Collection site data.** Site IDs were assigned for each collection site. Location is provided with latitude and longitude. Sites either had a commercial apiary present (Y) or no apiary nearby (N). Total sampling sizes are given for each of two bumble bee species (*Bombus bimaculatus* and *B. vagans*) and honey bees (*Apis mellifera*).

Table 2. Results of the GLMMs showing each model and the fixed effects tested. Table shows chi squared value, degrees of freedom (Df) and p-value. Apiary presence refers to whether the site had a commercial apiary present or no apiary nearby. Floral density was calculated as the number of inflorescences per m^2. Bee species was either *Bombus bimaculatus* or *B. vagans.* Asterisks represent significance.

Figure 1. Percent prevalence of infected bumble bee individuals for black queen cell virus (BQCV) and deformed wing virus (DWV). Bumble bees were either caught in sites with honey bee apiaries present or no apiary nearby. BQCV and DWV were more prevalent in bumble bees caught in sites with a honey bee apiary present than in sites without an apiary nearby. Standard error bars are shown. Asterisks represent significance.

Figure 2. Honey bee DWV loads predict DWV prevalence in bumble bees. Percent prevalence for bumble bees infected with deformed wing virus (DWV) at sites where honey bees had high and low viral loads, and sites where no honey bees were present and therefore could not be collected. DWV was more prevalent in bumble bees caught at sites with honey bees with high average viral loads, than sites with honey bees with low average viral loads. Standard error bars are shown.

Figure 3. Percentage of plant samples with viruses detected. Percentage of virus positive flower samples collected either at sites with or without apiaries. For all sites with an apiary, flowers were collected within 300 meters of the honey bee colonies. All virus positive samples were collected at sites with apiaries. Standard error bars are shown.

Table 3. Results of the GLMM for virus prevalence on flowering plants showing fixed effects tested. Prevalence is reported as the percentage of flowering plants with viruses detected. Bee abundance was measured as the number of bees (either honey bees or bumble bees) observed per m^2. Virus species is either deformed wing virus (DWV) or black queen cell virus (BQCV). Floral density was calculated as the number of inflorescences per m^2. Table shows chi squared value and p-value. Asterisks represent significance.

Figure S1. Distribution of site average honey bee DWV load (log transformed). Distribution follows a bimodal distribution with sites either have high ($> 10^7$ genome copies) or low ($< 10^7$ genome copies) viral loads.

Table S2. Results of the GLMM for DWV prevalence in bumble bees as a function of virus loads in honey bees (high/low), honey bee abundance, and floral density. Prevalence in bumble bees is the percentage of bumble bees with DWV detected. Honey

bee virus loads were calculated as the number of virus genome copies per bee and log transformed. Virus loads in honey bees were considered "high" if above 15 ($>10^7$ genome copies) and "low" if below 15 ($<10^7$ genome copies). Bee abundance was measured as the number of honey bees observed per m^2. Floral density was calculated as the number of inflorescences per m^2. Table shows chi squared value, degrees of freedom (Df) and p-value. Asterisks represent significance.

Tables and Figures

Table 1. Collection site data.

site ID	latitude	longitude	Apiary present?	*Bombus bimaculatus*	*Bombus vagans*	*Apis mellifera*
JOSH	44.859642	-72.408081	Y	0	13	10
FERL	44.948248	-73.082924	Y	10	10	10
ROCK	44.849911	-72.942441	N	11	8	10
MART	44.736855	-73.086848	Y	10	13	10
ONE	44.336968	-73.150093	Y	10	8	10
BOST	44.369755	-73.242064	Y	11	10	10
PAT	44.158423	-73.339091	Y	10	0	10
SAND	44.654202	-73.16209	N	10	10	5
FLAN	44.237572	-73.231302	N	10	0	0
SWAN	44.931132	-73.091239	N	10	10	10
WHAL	44.326216	-73.278147	Y	0	11	10
COL	44.550141	-73.12475	N	10	11	10
CLERK	44.807917	-72.447151	N	11	10	0
MUGE	44.672081	-72.599161	N	10	8	10
CIND	44.50658	-72.626181	N	10	0	9
HOGB	44.682381	-72.773484	N	10	10	10
NEK	44.950872	-71.830196	N	0	13	0
TIRE	44.87368	-72.051344	N	8	12	0
RICE	44.925435	-72.969001	N	10	15	9

Table 2. Results of each model and the fixed effects tested

Model/Parameter	χ^2	Df	p
BQCV Prevalence	-	-	-
Apiary Presence	3.959	1	**0.047***
Floral Density	0.273	1	0.601
Bombus Species	15.67115	1	**<0.001***
DWV Prevalence	-	-	-
Apiary Presence	6.531	1	**0.012***
Floral Density	6.025	1	**0.014***
Bombus Species	0.263	1	0.608
BQCV Load	-	-	-
Apiary Presence	0.943	1	0.331
Floral Density	2.902	1	0.0884
Bombus Species	18.662	1	**<0.001***
DWV Load	-	-	-
Apiary Presence	1.064	1	0.302
Floral Density	0.263	1	0.608
Bombus Species	0.089	1	0.765
BQCV Negative Strand	-	-	-
Apiary Presence	0.968	1	0.325
Bombus Species	17.177	1	**<0.001***
DWV Negative Strand	-	-	-
Apiary Presence	4.013	1	**0.045***
Bombus Species	0.368	1	0.544

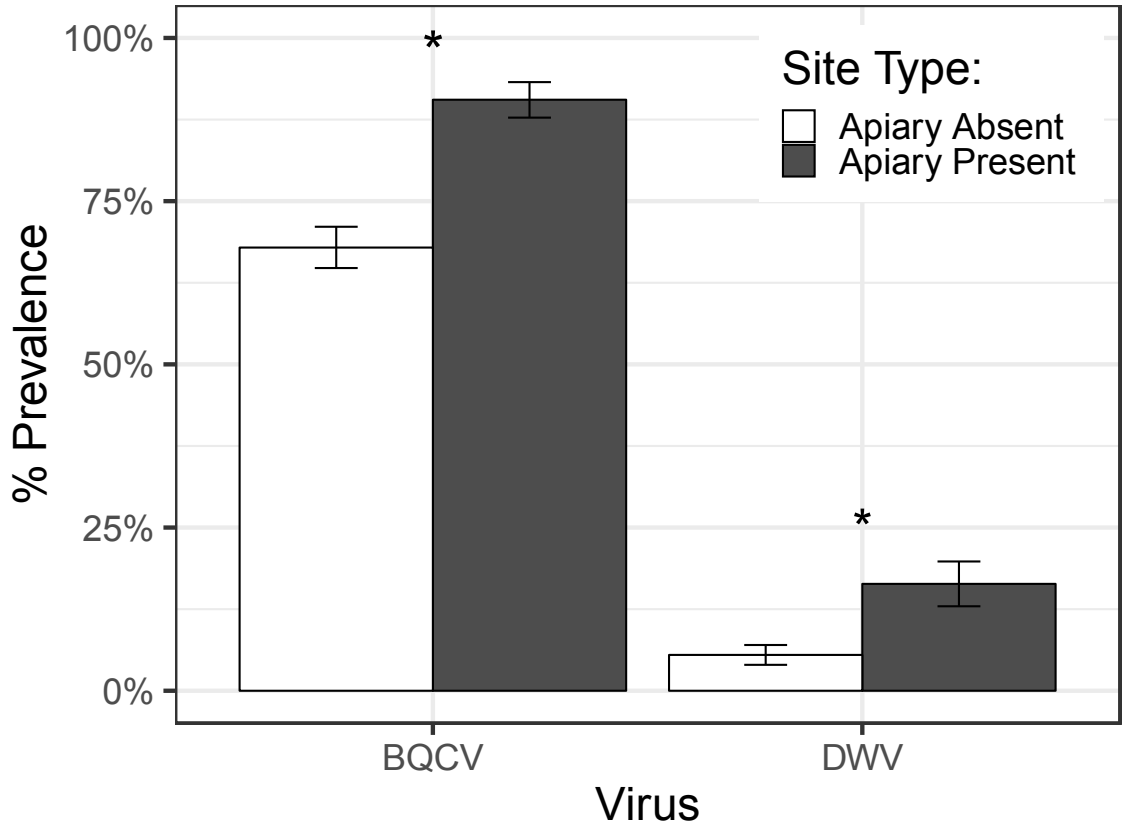

Figure 1. Percent prevalence of infected bumble bee individuals for black queen cell virus (BQCV) and deformed wing virus (DWV).

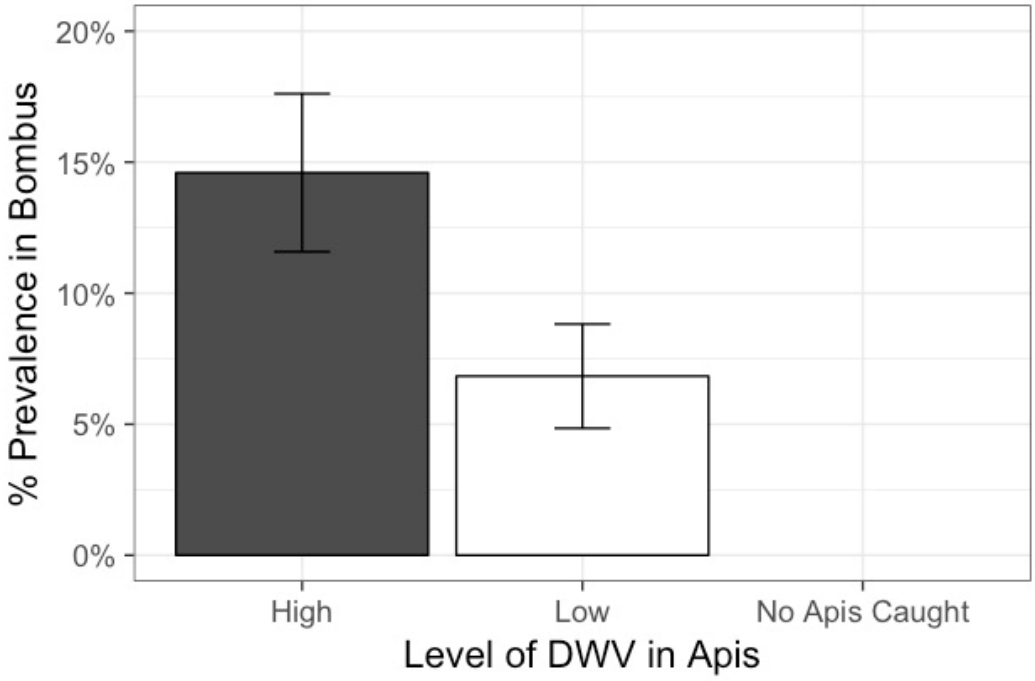

Figure 2. Honey bee DWV loads predict DWV prevalence in bumble bees.

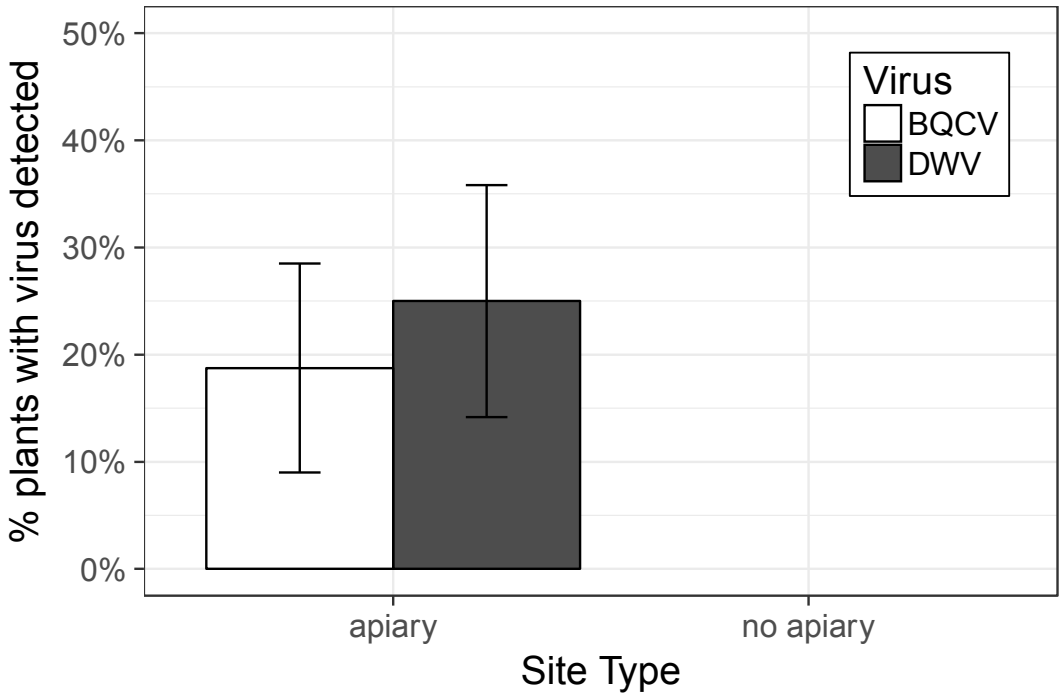

Figure 3. **Percentage of plant samples with viruses detected.**

Table 3. Results of the GLMM for virus prevalence on flowering plants showing fixed effects tested.

Model/Parameter	χ^2	p
Virus Prevalence on Flowers	-	-
Bombus Abundance	2.455	0.117
Apis Abundance	15.303	**<0. 001***
Virus Species	0.2801	0.596
Floral Density	3.315	0.069

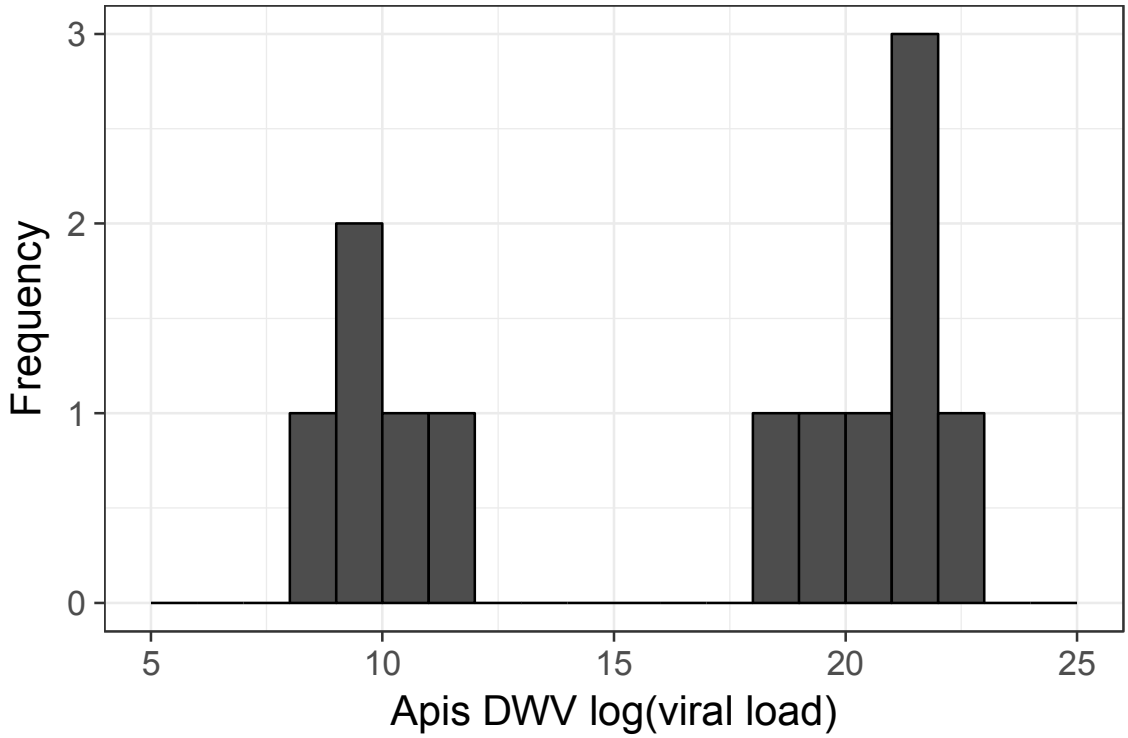

Figure S1. Distribution of site average honey bee DWV load (log transformed).

Table S2. Results of the GLMM for DWV prevalence in bumble bees as a function of virus loads in honey bees (high/low), honey bee abundance, and floral density.

Model/Parameter	χ^2	Df	p
DWV Prevalence by *Apis*	-	-	-
Apis Abundance	3.786	1	0.052
Apis DWV load	8.068	2	**0.018***
Floral Density	3.323	1	0.068

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CHAPTER 3: SHARED FLORAL RESOURCES AS HOT SPOTS FOR BEE VIRUS TRANSMISSION

Abstract

Managed bees pose a spillover risk to wild pollinator species. RNA viruses, once considered specific to honey bees, are suspected of spilling over from managed honey bees to wild pollinators; however, transmission routes are largely unknown. A widely accepted yet untested hypothesis posits that flowers serve as bridges in the transmission of viruses between bees. Here, using a series of controlled experiments with captive bee colonies, we examined the role of flowers in bee virus transmission. First, we examined if honey bees deposit viruses on flowers and whether bumble bees become infected after visiting infected flowers. Next, we examined whether plant species differ in their propensity to harbor viruses and if bee visitation rates increase the likelihood of virus deposition on flowers. Our experiment demonstrated, for the first time, that honey bees deposit viruses on flowers. However, the two viruses we examined, black queen cell virus (BQCV) and deformed wing virus (DWV), were not equally distributed across plant species, suggesting that differences in floral morphology, virus ecology and/or foraging behavior may mediate the likelihood of deposition. Bumble bees did not become infected after visiting flowers previously visited by honey bees suggesting that, if it occurs, transmission via flowers is contingent on numerous factors and may require multiple exposures. Our study is among the first to examine the role of flowers in bee disease transmission and uncovers promising avenues for future research.

Introduction

Pathogens are among the top threats to bees, causing colony losses, population declines, and a growing concern for food security and ecosystem function (Williams & Osborne, 2009; Potts et al., 2010; Evans & Schwarz, 2011; Goulson et al., 2015). Although the importance of pathogens to bees has garnered much attention over the past two decades, there are many unanswered questions regarding the dispersal mechanisms and transmission dynamics of bee pathogens (McArt et al., 2014). Numerous pathogens have been detected across broad host ranges including solitary bees, bumble bees, honey bees, ants, wasps, and beetles (Li et al., 2011; Levitt et al., 2013; Ravoet et al., 2014). Shared floral resources, which might act as dispersal platforms among comingling pollinator species, have been implicated as routes through which these pathogens may be acquired (Durrer & Schmid-Hempel, 1994; Singh et al., 2010; McArt et al., 2014; Graystock, Goulson & Hughes, 2015). Two studies have directly examined this route for parasites of bees. *Crithidia bombi*, a trypanosome parasite of bumble bees, was transmitted among bumble bees after visiting flowers that were inoculated by hand or previously visited by infected bumble bees (Durrer & Schmid-Hempel, 1994). More recently, the parasites *Apicysistis bombi, Nosema spp.*, and *Crithidia bombi* were vectored from host bees to flowers and between bee species through shared flowers (Graystock, Goulson & Hughes, 2015). Evidence suggests that flowers may also serve as dispersal platforms for RNA viruses (Singh et al., 2010). Positive sense single strand RNA viruses, once thought to be specific to honey bees, have been detected in a number of pollinating arthropod species including beetles, flies, solitary bees, and bumble bees (Levitt et al., 2013). Detected in the feces and glandular secretions of worker bees as well

as in pollen loads carried by bees, RNA viruses are likely left behind on flowers by foraging visitors (Chen, Higgins & Feldlaufer, 2005; Singh et al., 2010; Mazzei et al., 2014). Thus, flowers may serve as platforms for RNA virus spread to visiting arthropods. But, to our knowledge, only one previous study has tested the transmission of RNA viruses between bee species as a result of using the same flowers. In a controlled flight cage experiment, Israeli acute paralysis virus (IAPV) was transmitted between honey bee and bumble bee colonies that foraged alongside each other for several weeks. Although shared flowers may have provided the transmission route, bees could also have become infected by direct contact either by comingling or if bees entered each other's hives through robbing of resources (Singh et al., 2010). Although Singh et al. (2010) were instrumental in demonstrating transmission between bee species, the role of flowers in RNA virus transmission remains unclear.

The ability of flowers to serve as conduits for pathogens may be facilitated or constrained by plant species or floral morphology (McArt et al., 2014). In previous studies, parasites were unequally dispersed across plant morphotypes (Durrer & Schmid-Hempel, 1994) and plant species (Graystock, Goulson & Hughes, 2015), suggesting that floral architecture may influence dispersal and transmission rate. Alternatively, a plants' propensity to harbor pathogens could be a function of pollinator visitation rates, with highly attractive plants more likely to act as fomites. The role of flowers in RNA virus transmission have been widely proposed but largely untested. More research is needed to fill these knowledge gaps in virus transmission (McArt et al., 2014; Manley, Boots & Wilfert, 2015).

Here, we conducted a series of controlled flight cage experiments to test if flowers can act as bridges in virus transmission between bee species. Specifically, we examined if honey bees deposit viruses on flowers and whether bumble bees become infected after visiting infected flowers. To further examine the role of flowers in the transmission of RNA viruses, we examined whether plant species differ in their propensity to harbor viruses and if honey bee visitation rates increase the likelihood of virus deposition on flowers. Our results demonstrate that honey bees deposit viruses on flowers. However, the two viruses we examined, black queen cell virus (BQCV) and deformed wing virus (DWV), were not equally distributed across plant species, suggesting that both plant species and differences in virus ecology may mediate the likelihood of deposition. Bumble bees did not become infected after visiting flowers previously visited by honey bees. We discuss the conditions under which transmission and active replication may occur in the field.

Methods

Experiment Overview

To test for viral deposition on flowers by honey bees and transmission of viruses between bee species using shared floral resources, we conducted a series of experiments. First, we allowed infected honey bees to forage on arrays of flowering plants within a screened enclosure and later transferred these plants to enclosures where non-infected bumble bees were allowed to forage. We tested all bees and flowers after each experiment. We examined if virus deposition on flowers and/or virus transmission between bee species is influenced by plant species, plant diversity, and multiple exposure to infected plants. Lastly, by allowing honey bees and bumble bees to forage together in

the same enclosure, we tested if direct contact or co-mingling is necessary for viral transmission.

Setup and Pre-screening

We grew plants from seed and maintained them in a greenhouse until the start of the experiment. Beginning in mid-May, we broadcast seeds of *Trifolium pretense* (red clover), *Trifolium repens* (white clover), and *Lotus corniculatus* (birdsfoot trefoil) in 8 in. diameter, 6.5 in. deep plastic pots filled with Miracle Grow Potting Mix to achieve ca. 100 seeds per pot (Figure 1A-C). To encourage flowering, we trimmed the *T. repens* and *T. pratense* plants once and twice, respectively, and used grow lights to maintain 14 hours of sunlight. To verify that plants were virus-free at the start of the experiment, we haphazardly collected composite samples of each flowers species and tested them for DWV and BQCV using RT-qPCR protocols.

From each of two five-frame honey bee colonies, we tested composite sample of 50 bees for DWV and BQCV using RT-qPCR. Both viruses were detected in each colony. We received seven bumble bee colonies from a commercial supplier. To verify these bees were not infected with DWV, we pollen-starved 10 bees from each colony for 72 hours to rid the gut of any infected pollen and tested each bee using RT-qPCR. All bumble bee colonies tested negative for DWV and BQCV. From the seven colonies, we created microcolonies of 12 adult bees, provided them with 30% sucrose solution *ad libitum* and allowed them to acclimate for up to 5 days in a growth chamber maintained at 26 °C and 52-55% RH. We made new microcolonies every three days to ensure each microcolony used in the experiment was about the same age.

We carried out all experiments in three 3 x 3 x 3 m. screened tents with tarp bottoms (Figure 1D). We assigned each to one treatment: honey bee tent, bumble bee control tent, or bumble bee treatment tent. We used one additional tent as a plant holding area to keep unwanted insects from visiting the plants during the experiment. To restrict bumble bees to a smaller foraging area, we set up three hoop houses within each of the two bumble bee control and treatment tents. Hoop houses (1 x 1 x 0.7 m) were constructed of white fabric stretched and stapled over two pieces of arching PVC tubes that were screwed to a wooden frame (Figure 2).

Experimental Design

On each day of the experiment, we transported plants from the greenhouse to the plant holding tent and watered them. To ensure a standard abundance of flowers across replicates and treatments, we counted all inflorescences and assigned them accordingly. To acclimate the honey bees to their enclosure, the two colonies (consisting of 5 frames each) were placed in the honey bee tent 24 hours prior to the experiment. To infect the flowering plants, we placed plants within the screened enclosure with the two honey bee colonies and allowed bees to visit the flowers. After 9 hours, we transferred plants to a holding tent to allow for nectar to be replenished. After 15 hours, we transferred plants visited by honey bees to the treatment bumble bee tent and evenly distributed them among the three hoop houses. For the control bumble bee tent, we transferred clean flowering plants from the greenhouse directly into each of three hoop houses.

We allowed micro colonies of 12 bees each to forage on flowers that had or had not been exposed to honey bees. After six hours, we collected all inflorescences and bumble bees. We stored inflorescences at -80°C. We placed the bumble bee micro

colonies into new containers and fed 30% sucrose *ad libitum* for one week in a growth chamber. If bumble bees were exposed to infective virus during the experiment, the one week 'incubation' period allowed for the onset of viral infection. Previous work has shown that virus particles on pollen grains can remain infective for 6 months in ambient conditions (Singh et al., 2010). Thus, we did not feed bees pollen during this period to clear their guts of pollen that could have inactive virus particles, resulting in a false positive result during the viral assays. After one week, we collected all bees and stored them in -80°C until RNA extraction and virus assays.

To test if plant species influences the transmission of DWV between bee species, we conducted the above-described foraging trials three times for each plant species: *T. repens*, *T. pratense*, and *L. corniculatus* ("single plant" trials). We standardized the number of inflorescences used in each replicate: 15-20 *T. repens* inflorescences, 13-15 *T. pratense* inflorescences, and 31-40 *L. corniculatus* inflorescences. Because *L. corniculatus* inflorescences contain less than half the number of florets as *T. pratense* and *T. repens*, we used about twice as many inflorescences. If plant morphology affects virus transmission, we would expect results to be similar between *T. repens* and *T. pretense*, but different between the two clover species and *L. corniculatus*.

To test if plant diversity affects virus transmission, we allowed bees to forage on floral arrays containing all three plant species at once ("diversity" trials). Each diversity array consisted of 7-8 *T. repens* inflorescences, 6 *T. pratense* inflorescences, and 15-21 *L. corniculatus* inflorescences.

To test if multiple exposure to infected plants is necessary for virus transmission, we repeated the experiment using *T. repens* in "chronic exposure" trials. Six bumble bee

micro colonies were either assigned the treatment group or control group and allowed to forage on exposed or unexposed *T. repens* plants on three consecutive days. We allowed plants to replenish nectar between honey bee and bumble bee foraging bouts as in the other experiments. A new *T. repens* plant was used each day. After the three exposure events, we collected all bumble bees, transferred them to new containers, provided 30% sucrose ad libitum, and 'incubated' them for one week as in the previous experiments and then transferred them at -80°C. We also collected flowers each day of the multiple exposure experiment and stored them at -80°C.

To test if direct exposure, or co-mingling, on flowers is necessary for transmission of DWV between bee species, we used bumble bee colonies comprised of 75-100 workers and *T. repens* arrays consisting of 41-47 inflorescences ("comingling" trials). We placed two honey bee colonies, a single bumble bee colony, and pots of *T. repens* plants into a tent enclosure. For the control, we placed a single bumble bee colony with plants into a separate tent enclosure. We allowed all bees to forage on the plants for a total of 7 hours, during which we observed normal floral visitation by both bee species. After 7 hours, we returned all foraging bumble bees back to their colony box and transferred them back to the growth chamber. This was repeated three times over the course of three days using the same honey bee colonies but different bumble bee colonies. We fed the bumble bee colonies pollen and 30% sucrose *ad libitum* for three weeks in growth chambers to encourage the spread of DWV throughout the colony. After three weeks, we made pollen-starved micro colonies consisting of 12 bees. After a one-week pollen starvation period, we collected these bees and stored them at -80°C.

MEASURING VISITATION

We visually observed bumble bees to visit all flowering plant species. To examine how honey bee visitation may influence virus deposition on flowers, we filmed each trial for ~3 hours. We viewed the videos and recorded the number of honey bee visits to each plant species and computed the total foraging time on each plant species over the course of the three hours.

RNA EXTRACTION

We extracted total RNA following Qiagen RNeasy mini kit protocols. The abdomen of individual bumble bees were dissected and flash frozen on N_2 and homogenized into 600 ul of RLT buffer (10% β-mercaptoethanol) and Qiagen protocols were used thereafter. For honey bees, samples of 50 bees were pooled, flash frozen in N_2 and homogenized together in an extraction bag with 10 mL of GITC buffer. The resulting homogenate was centrifuged and 100 ul of the lysate was mixed with RLT buffer (10% β-mercaptoethanol) and Qiagen protocols were used thereafter. For plants, 1.5 g of flower material was transferred to an extraction bag (Bioreba, Switzerland) and flash frozen in N_2. Plant material was ground to a powder using a ceramic pestle on the outside of the extraction bag for 30 seconds. Three mL of GITC buffer was added to the bag and the pestle again was used on the outside of the bag to mix the homogenate into the buffer for 2 minutes. The resulting homogenate was centrifuged and 200 ul was used in RNA extractions following Qiagen RNeasy mini kit protocols. All RNA quantity and quality were assessed on a Spectrometer (Nanodrop, Thermo Scientific).

VIRUS DETECTION AND QUANTIFICATION

For bumble bees and honey bees, all RNA extractions were diluted to 20 ng/ul prior to virus assays. RNA recovered from plants was not diluted prior to further

analyses. For reverse transcription of RNA and absolute quantification, duplicate reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed for each sample with SYBR green one-step RT-qPCR kit in 10 ul reactions using the following thermal cycling program: 10 min at 50°C (RT) followed by 1 min at 95°C, and 40 amplification cycles of 95°C for 15 s, 60°C for 60s. Last, the melt-curve was obtained starting at 65-95°C (0.5°C increments, each 2 s). We used primers specific to the positive strand of the following RNA virus targets: DWV, BWCV and IAPV, and a housekeeping gene (ACTIN) as a positive control of RNA extraction efficiency (Appendix A). Quantification was calculated using duplicate standard curves of gBlocks Gene Fragments (Integrated DNA Technologies) that were developed using double-stranded, sequence verified genomic blocks consisting of the four targets of interest separated by ten random base pairs (Appendix B). Sequences of random base pairs consisting of at least 50% G and Cs were used at the beginning and terminal ends of the fragment. Efficiencies were 91.06% (DWV), 95.21% (BQCV), 90.27% (IAPV), and 90.12% (Actin), with correlation coefficients (R^2) ranging from 0.993-0.999. Virus loads on plants were calculated to virus genome copies/gram of flower material.

SEQUENCING

To confirm the identity of the viruses, we sequenced virus fragments from honey bees and flowers. qPCR product was cleaned (ExoSAP-IT PCR Product Cleanup) and sequencing was performed using the 3130xl Genetic Analyzer in the University of Vermont Cancer Center Advanced Genome Technologies Core. Sequence data were

viewed for quality assessment (FinchTV 1.4) and aligned by eye to genome references using Geneious v 6.0.6 (BQCV: GenBank: KY243932.1; DWV: GenBank: KJ437447.1).

Data Analysis

Analyses were performed in R v 0.99.903 (R Core Team 2016). To test for differences in visitation rate and foraging time across flower species, we used separate Kruskal-Wallis rank sum tests (function kruskal.test). We used a general linear model with a binomial distribution to examine the effect of flower species, virus type, and trial type on virus prevalence on flowers (glm, link = "logit"). We log transformed virus load data to improve normality and used a linear model to examine the effect of plant species and virus type on the virus loads detected on plants. Significance for all models was determined using Type II Wald Chi-Square tests (function anova, car package).

Results

At the onset of the experiment, all plant species were negative for DWV and BQCV. RNA virus loads (measured in average genome copies per bee) in the two honey bee colonies were 10^4 and 10^9 for DWV and 10^8 and 10^6 for BQCV. All bumble bees were negative for both viruses at the onset and conclusion of the experiment.

All flowers visited only by bumble bees were negative for both viruses. Of the flowers visited by both honey bees and bumble bees, we detected DWV and BQCV on 25% and 21.8%, respectively (Table 1, Figure 3). Virus loads on flowers ranged from 10^3-10^5 genome copies and there was a significant effect of plant species on virus load ($F= 10.517$, $df = 2$, $p = 0.003$, Table 1) with virus loads being significantly lower on *T. repens* as compared to *T. pratense* and *L. corniculatus* (Figure 4).

We found a significant effect of flower species ($\chi_2^2 = 9.759$, $p < 0.01$) and a significant interaction effect of plant species and virus type ($\chi_2^2 = 7.618$, $p = 0.022$); DWV and BQCV were not equally distributed across plant species (Table 2, Figure 5). We also found a significant interaction of trial type and plant species ($\chi_2^2 = 23.818$, $p < 0.001$, Table 2). When single species of plants were offered to bees, we detected viruses on all three species. However, in the diversity trials, where all three plant species were offered together, we only detected viruses on *T. pratense*. The number of visits ($\chi_2^2 = 5.693$, $p = 0.058$) and the sum foraging time ($\chi_2^2 = 4.2$, $p = 0.1225$) did not differ across the plant species.

Discussion

Although flowers have been implicated as bridges in the spread of bee diseases (McArt et al., 2014), controlled experiments are necessary to understand the role of flowers in the transmission of RNA viruses among pollinator species. Bee viruses have been detected on flowering plants in field (Mazzei *et al.* 2014), however the factors influencing virus deposition on flowers are virtually unknown. Using a series of foraging trials with captive honey bee colonies and arrays of flowering plant species, we experimentally demonstrated that honey bees deposit viruses on flowers. We also found evidence that flowering plant species and/or bee behavior may influence the likelihood of virus deposition. Our study is among the few to closely examine the role of flowers in pollinator disease transmission and is the first to demonstrate virus deposition on flowers by honey bees.

Deformed wing virus and BQCV were differentially deposited across the three plant species, indicating that modes of deposition may vary for virus species and that

deposition may be mediated by floral traits. In other bee-pathogen systems, plant traits such as floral morphology mediated deposition on flowers (Durrer & Schmid-Hempel, 1994; Graystock, Goulson & Hughes, 2015). To our knowledge, our results present the first experimental evidence of this phenomenon in the bee-virus system. Previous work suggests that plant species may differ in their likelihood to harbor viruses. For example, when comparing virus infection in honey bees and their corresponding pollen loads, viruses differ considerably (Singh et al., 2010); suggesting differences in viral ecology, and/or differences in pollinator contact with contaminated pollen. If a virus is deposited by feces, floral morphology that encourages 'hovering' behavior, may reduce the likelihood of viral deposition (Mcart *et al.*, 2014). In contrast, for viruses deposited through oral secretions, floral morphology that excludes bees from accessing floral nectaries may reduce the likelihood of viral deposition.

When bees foraged on single-species floral arrays, viruses were deposited on all three species. However, when bees were offered diverse arrays consisting of three plant species, we only detected viruses on *T. pratense* despite similar visitation rates and foraging times across plant species. These results could be explained if honey bee colonies hosted both infected and uninfected individuals that foraged differently. Foraging differs for parasite infected bees than for those that are uninfected, suggesting that bees seek benefits from the medicinal properties of secondary plant metabolites (Manson, Otterstatter & Thomson, 2010; Simone-Finstrom & Spivak, 2012; Richardson et al., 2015; Richardson, Bowers & Irwin, 2016; Annoscia et al., 2017). Compared to *T. repens, T. pratense* has substantially higher concentrations of isoflavonoids (Chang et al., 1969), a group of phenolic compounds that possess antiviral properties against a wide

range of viruses (Andres, Donovan & Kuhlenschmidt, 2009). However, we were unable to distinguish between infected and uninfected bees at the outset of the experiment.

Under our experimental conditions, bumble bees did not develop an infection after direct contact with honey bees through co-mingling or indirect contact through shared flowers. These results could indicate that transmission of viruses between bee species through flowers is a rare occurrence, with experimental detection contingent on numerous factors. For example, factors such as immunocompetence, virus virulence, virus load, and the probability a bumble bee will contact a virus particle on a flower may all contribute to detection. Thus, although we did not demonstrate virus transmission to bumble bees in our experiment, we remain cautious to exclude the possibility under different experimental conditions and with greater sample sizes.

Our findings present several promising avenues for future research. We were successful in demonstrating virus deposition to flowers. Thus, future experiments should focus on the second half of the transmission cycle and examine whether bumble bees can acquire virus particles or become infected after visiting inoculated flowers. Our results suggest that flowering plant species may differ in their propensity to harbor viruses. Future experimental studies should closely examine the mechanisms of virus deposition in conjunction with floral morphology. Lastly, additional behavior studies are needed to examine how foraging behavior may be affected by virus status.

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Figure and Table Captions

Figure 1. Flower species used in foraging trials. Three plant species were grown from seed in the greenhouse: *Trifolium pretense* (red clover) (A), *Trifolium repens* (white clover) (B), and *Lotus corniculatus* (birdsfoot trefoil) (C). Tent enclosures where bees were allowed to forage on plants (D).

Figure 2. Inside the flight enclosures where bees foraged on flowers. Honey bee tent where infected honey bees foraged on plants (A). One of two bumble bee tents where bumble bees were allowed to forage on plants either infected by honey bees or clean control plants (B).

Table 1. Summary table showing the detection rate of deformed wing virus (DWV) and black queen cell virus (BQCV) on three plant species across all foraging trials where both honey bees and bumble bees foraged. Plants foraged by bumble bees only were all negative for viruses and are therefore excluded from this table. In 'Single spp.' trials, bees foraged on arrays consisting of only one species at a time. In 'Diversity' trials, bees foraged on arrays consisting of all three plant species at once. In the 'Chronic' trials, only *Trifolium repens* was used. In the 'Comingle' trials, both honey bees and bumble bees were allowed to comingle and forage together on *Trifolium repens*. Virus detection on honey bee visited plant species across all trials. Proportions are presented as the percentage of flower samples with virus detected out of the total number (n) of flower samples tested for each trial.

Figure 3. Percentage of flower samples with virus detected across all trials. Bars color coded for virus type: Black queen cell virus (BQCV) and deformed wing virus (DWV). We detected viruses only on flowers foraged on by bumble bees and honey bees (HB + *Bombus*). All plant samples prior to the start of the experiment were negative for viruses (Pre Experiment). All plants foraged on by bumble bees only were also negative for viruses (*Bombus* Only).

Table 2. Summary statistics for models. Virus prev, virus prevalence. Plant species are *Lotus corniculatus* (Birdsfoot trefoil), *Trifolium pretense* (red clover), or *Trifolium repens* (white clover). Virus is the virus type, deformed wing virus (DWV) or black queen cell virus (BQCV). Trial is 'single spp.', 'diversity', 'chronic', or 'comingle'. Virus load is presented as virus genome copies/flower sample.

Figure 4. Virus load for virus positive flower samples by plant species. Box plots color coded by plant species. Deformed wing virus (DWV), black queen cell virus (BQCV). Plant species are *Lotus corniculatus* (Birdsfoot trefoil), *Trifolium pretense* (red clover), or *Trifolium repens* (white clover).

Figure 5. Percentage of flower samples with virus detected by plant species. Bars are color coded by plant species: *Lotus corniculatus* (birdsfoot trefoil), *Trifolium pretense*

*(*red clover)*, Trifolium repens* (white clover). Black queen cell virus (BQCV) and deformed wing virus (DWV) were not equally distributed across plant species

Tables and Figures

Figure 1. Flower species used in foraging trials and tent enclosures.

A

B

Figure 2. Inside the flight enclosures where bees foraged on flowers.

Table 1. Summary table showing the detection rate of deformed wing virus (DWV) and black queen cell virus (BQCV) on three plant species across all foraging trials where both honey bees and bumble bees foraged.

Virus	Plant species	Proportion with virus detected, n				
		Single spp.	Diversity	Chronic	Comingle	Total
DWV	*L. corniculatus*	0, 3	0, 3	-	-	0, 6
	T. pratense	33.3, 3	100, 3	-	-	66.6, 6
	T. repens	66.6, 3	0, 3	11.1, 9	20, 5	20, 2
BQCV	*L. corniculatus*	100, 3	0, 3	-	-	50, 6
	T. pratense	0, 3	100, 3	-	-	50, 6
	T. repens	0, 3	0, 3	11.1, 9	0, 5	5, 2

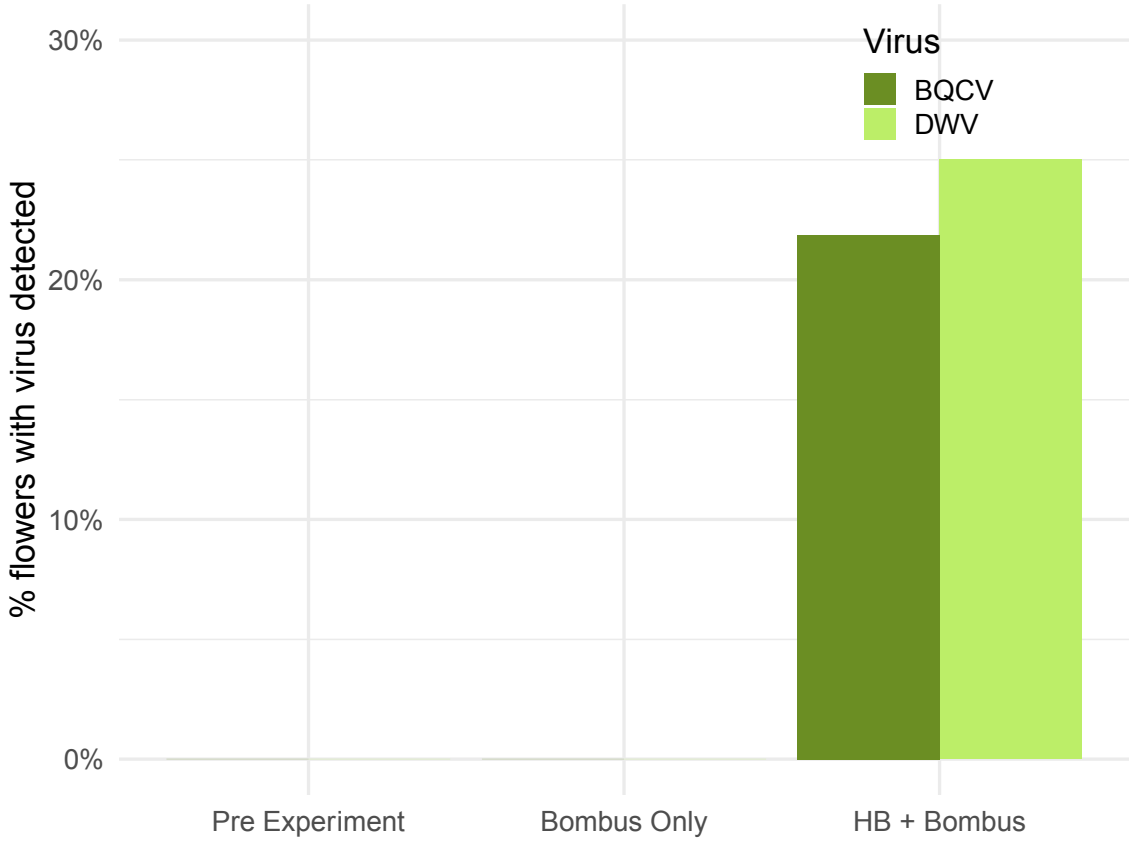

Figure 3. Percentage of flower samples with virus detected.

Table 2. Summary statistics for models.

Variable	Effect	Test statistic [a]	df	P [b]	Sig.[c]
Virus prev.	Flower spp.	9.759	2	0.008	**
	Virus	0.105	1	0.746	
	Trial	0.269	3	0.966	
	Plant spp: Virus	7.618	2	0.022	*
	Plant spp.: Trial	23.818	2	<0.001	***
Virus load	Flower spp.	10.517	2	0.003	**
	Virus	0.698	1	0.423	
	Flower spp.: Virus	0.021	1	0.887	

[a] Test statistics reported are χ^2 values for virus prevalence and F for virus load

[b] Significance was assessed using analysis of deviance for virus prevalence and ANOVA for virus load.

[c]Asterisks represent level of significance.

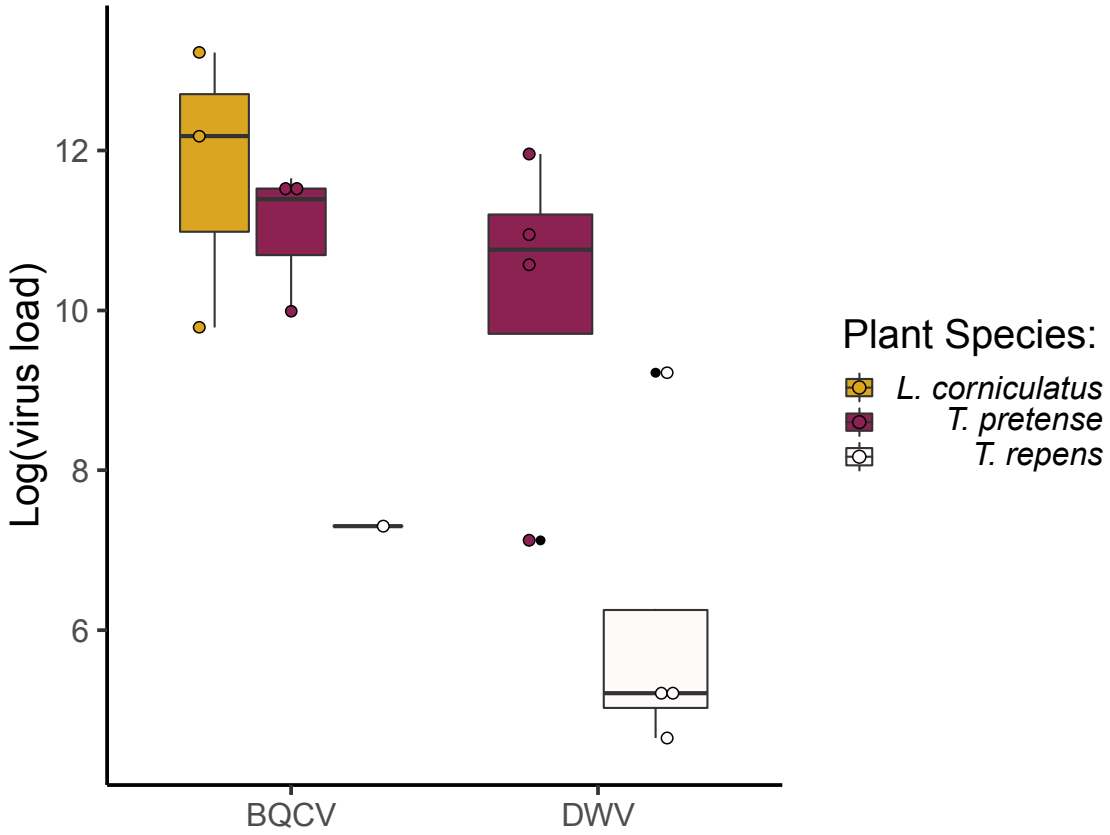

Figure 4. Virus load for virus positive flower samples by plant species.

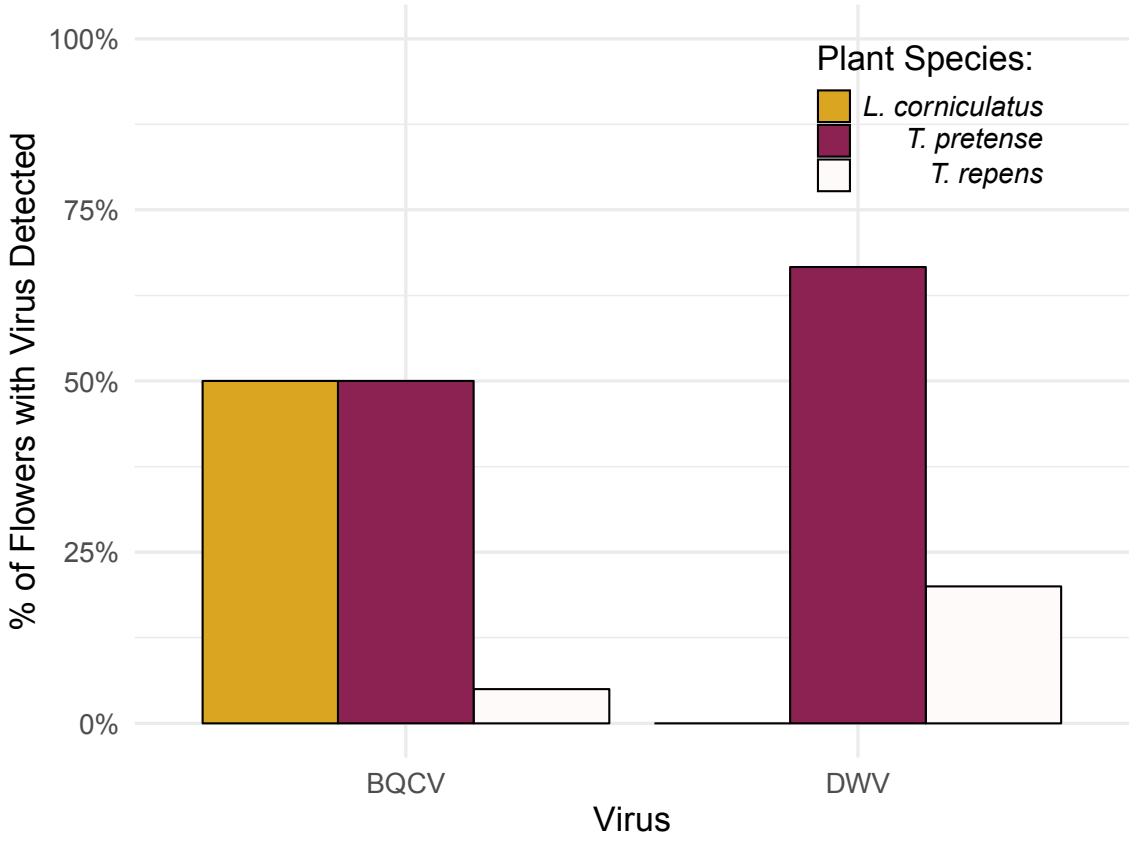

Figure 5. Percentage of flower samples with virus detected by plant species across all trials.

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CHAPTER 4: NEONICOTINOID IMIDACLOPRID REDUCES VIRUS TITERS AND SUCROSE CONSUMPTION IN BUMBLE BEES (*BOMBUS IMPATIENS*)

Abstract

1. Multiple interacting stressors including pesticide exposure, the spread of pests and pathogens, and the loss of floral resources, are among the putative causes of global pollinator declines. Although studies have examined the effects of these stressors in isolation, little is known regarding their combined impacts, particularly in wild bee species such as bumble bees.
2. We experimentally investigated how chronic oral exposure to different doses of the neonicotinoid pesticide imidacloprid affects the titers of two RNA viruses (deformed wing virus and black queen cell virus), sucrose intake, and survivorship in bumble bees (*Bombus impatiens*).
3. Imidacloprid significantly reduced both black queen cell virus titers and deformed wing virus titers. Bees exposed to high levels of imidacloprid consumed significantly less sucrose-water, yet survivorship did not differ among treatment groups.
4. *Synthesis and applications.* Our findings confirm that chronic oral exposure to imidacloprid impacts bumble bee foraging behavior. In studying how virus loads respond to imidacloprid exposure in bumble bees, our findings are contrary to results from previous experiments with honey bees that show increased viral titers in response to imidacloprid, presumably due to neonicotinoid-induced

immunosuppression. Our results suggest that the effects of pesticides on RNA viruses are host species specific. We speculate that neonicotinoid-induced apoptosis, rather than immunosuppression, may have a greater impact on virus replication in bumble bees. In light of these results, future pesticide risk assessments should investigate interactive effects for common pesticide-pathogen combinations and include non-*Apis* bee species. Lastly, to reduce the spread of pathogens, we suggest that all commercially-available pollen feed for honey bees and bumble bees undergo gamma irradiation treatment.

Keywords: neonicotinoid, pesticide, imidacloprid, RNA virus, pollinator, bumble bee, disease, pesticide

Introduction

There is widespread concern over the declines of insect pollinators, as their pollination services are fundamental for a third of agricultural crops and valued at about $200 billion worldwide (Gallai et al., 2009). For nearly a decade, beekeepers have reported losing a third (30%) of their honey bee (*Apis mellifera*) colonies each winter on average (Lee et al., 2015a). At the same time, wild bumble bee species (*Bombus* spp.) world-wide have experienced severe range contractions and, in some areas, extirpation (Goulson, Lye & Darvill, 2008; Grixti et al., 2009; Williams & Osborne, 2009; Cameron et al., 2011). The threats to both managed and wild bees include nutritional deficits as a result of decreased forage and land use change, climate change, pesticide application, and the spread of disease and parasites (Williams & Osborne, 2009; Potts et al., 2010; Singh et al., 2010; González-Varo et al., 2013; Goulson et al., 2015).

Bees host numerous diseases associated with a number of different viral, bacterial and fungal pathogens (Evans & Schwarz, 2011; Meeus et al., 2011; Evison et al., 2012). As pollinators of crops, bees may encounter a wide range of agricultural chemicals including fungicides, insecticides, herbicides, and inert surfactants used to enhance the spread and penetration of active ingredients (Mullin et al., 2010; Fine, Cox-Foster & Mullin, 2017). Because bees are likely to be exposed to a multitude of stressors, pollinator protection efforts necessitate an understanding of how stressors interact. However, the impact of different stressors in combination has only recently gained attention with the majority of studies focusing on managed honey bees (*Apis mellifera*) and a markedly few in wild bee species (reviewed in Collison, Hird, Cresswell, & Tyler, 2016). Studies that examine these stressors in isolation have documented negative health consequences for many pathogens and pesticides and it is proposed that in combination, pesticides may affect susceptibility and disease tolerance (Goulson et al., 2015; Sánchez-Bayo et al., 2016). Central to this postulation and a topic of debate is whether pesticide exposure influences pathogen load in bees. Pesticide exposure alters the expression of genes associated with immune response in bees (Boncristiani et al., 2012; Gregorc et al., 2012; Garrido et al., 2013). Therefore, it follows that pesticide exposure may enable conditions that promote the replication and resulting active infection of some pathogens.

The few studies examining pesticide-pathogen combinations have yielded variable results. For example, in honey bees, thiacloprid exposure resulted in higher *Nosema ceranae* spore loads while fipronil exposure lowered spore loads (Vidau et al., 2011). Also in honey bees, exposure to a pyrethroid acaricide treatment for *Varroa* infestations resulted in increased titers of RNA viruses including deformed wing virus

(DWV), black queen cell virus (BQCV) and sacbrood virus (SBV) (Locke et al., 2012). However, in a test of five different acaricide treatments, Boncristiani et al., (2012) found no effects on the titers of these same viruses. Exposure to neonicotinoids, a class of systemic pesticides identified as particularly harmful to bees and other pollinators (Desneux, Decourtye & Delpuech, 2007; van der Sluijs et al., 2013), increases both DWV and BQCV titers in honey bees (Di Prisco et al., 2013; Doublet et al., 2015), likely through the suppression of the innate immune response (Di Prisco et al., 2013).

Despite bumble bees hosting a wide range of pathogens, studies examining pesticide-pathogen combinations in bumble bees are few and have only focused on the trypanosome parasite, *Crythidia bombi.* These previous studies have found no significant impact of pyrethroids (Baron, Raine & Brown, 2014) or neonicotinoids on *C. bombi* loads (Fauser-Misslin et al., 2014). However, neonicotinoid exposure and *C. bombi* in combination reduced queen longevity (Fauser-Misslin et al., 2014) yet had no effect on queen hibernation mortality (Fauser et al., 2017). Since bees are susceptible to a wide range of pathogens and may be exposed to an ever-increasing number of pesticide chemicals, broad generalizations regarding pathogen-pesticide combinations may be difficult to conclude and underscore the need for more studies examining the multitude of combinations.

RNA viruses, once considered specific to honey bees, have been detected in bumble bees of several species (Fürst, McMahon, Osborne, Paxton, & Brown, 2014; Genersch, Yue, Fries, & De Miranda, 2006; Singh et al., 2010, Alger et al., unpublished). Although viral replication has also been demonstrated in seven bumble bee species, the effects of these viruses are understudied and the factors affecting virus titers within these

hosts is unknown (reviewed in Manley, Boots, & Wilfert, 2015). Stressors that adversely affect insect immunity such as neonicotinoid exposure may induce virus replication in honey bees (Di Prisco et al., 2013; Doublet et al., 2015) but the effect of neonicotinoid exposure on virus titers in bumble bees is completely unknown (reviewed in Collison et al., 2016). Thus, we conducted a controlled laboratory experiment to test whether chronic oral exposure to the neonicotinoid, imidacloprid, impacts the titers of two RNA viruses DWV and BQCV, in bumble bees (*Bombus impatiens)*. To further examine the impacts of imidacloprid on bee health and behavior, we tested whether imidacloprid exposure affects feeding behavior and bee survivorship.

Methods

We obtained three commercial bumble bee (*Bombus impatiens*) colonies and tested five individuals from each for RNA viruses BQCV and DWV and found all to be infected with both viruses upon arrival.

We tested the effect of chronic oral exposure to different concentrations of imidacloprid on two RNA virus titers (BQCV and DWV), bee feeding behavior measured as sucrose consumption, and mortality. We assigned twenty bees from colonies infected with DWV and BQCV to one of each of 5 treatments. We placed individual bumble bees in 18.5 mL snap cap containers (Fisherbrand) and housed them in a growth chamber maintained at 26°C and 48% relative humidity and allowed to acclimate for 24 hours prior to start of the experiment. We provided each bee with 30% sucrose-water solution *ad libitum* inoculated with different concentrations of imidacloprid: 0.1, 1, 10, and 20 parts per billion (ppb) for 8 days. Bees assigned to the control treatment received

30% sucrose only. We chose this range of imidacloprid concentrations to capture the range of field realistic doses found in a variety of crops and nectar (0.7-10 ppb) (Cresswell, 2011). The highest concentration (20 ppb) is above field realistic levels found in nectar, yet we included it as an extreme exposure level. We provided sucrose to bees via a 1.5 mL centrifuge vial equipped with a dental cotton wick and administered through each cage lid. On each day of the experiment, we transferred individual bees to new cages and provided a new sucrose feeder. We calculated sucrose consumption by weighing each centrifuge tube feeder daily and converting to mL consumed. We recorded mortality and sucrose consumption for 6 days, after which all surviving bees were transferred to -80°C and later tested for RNA viruses. Bees that died prior to the end of the experiment were excluded in virus assays due to RNA degradation after death.

VIRUS ASSAYS

We tested individual bees for RNA viruses using reverse transcription quantitative polymerase chain reactions (RT-qPCR). We flash froze each individual bee in liquid nitrogen, dissected the abdomen and homogenized it with a pestle in 500 uL of GITC buffer. For RNA extraction, we used 100 uL of the resulting homogenite and Qiagen RNeasy mini kit protocols were used thereafter. The quality and quantity of RNA was tested using a Spectrometer (Nanodrop, Thermo Scientific) and diluted to 20 ng/uL prior to PCRs.

We conducted duplicate RT-qPCRs for each individual bee using primers for viruses DWV and BQCV, and a housekeeping gene, Actin as a positive control of RNA extraction efficiency (Appendix A). For reverse transcription and amplification of

amplicons, we used SYBR green one-step RT-qPCR kit in 10 ul reactions. We used the following thermal cycling program: 10 min at 50°C (RT) followed by 1 min at 95°C, and 40 amplification cycles of 95°C for 15 s, 60°C for 60s and derived melt-curves using the following program: 65-95°C (0.5°C increments, each 2s). We quantified virus titers using triplicate standard curves of gBlocks Gene Fragments (Integrated DNA Technologies) to derive the total number of virus genome copies/bee (Appendix B). Efficiencies were 91% (DWV), 95% (BQCV), 90% (IAPV), and 90% (Actin), with correlation coefficients (R^2) ranging from 0.993-0.999. We confirmed the identity of the viruses by sequencing using the 3130xl Genetic Analyzer in the University of Vermont Cancer Center Advanced Genome Technologies Core.

STATISTICS

All statistical analyses were conducted in R v 0.99.903 (R Core Team 2016). To analyze virus titer data, we first log transformed all virus data to improve normality. To ensure the virus titers of the original colonies did not differ at the start of the experiment, we conducted an ANOVA (function aov). To examine whether imidacloprid exposure affects BQCV and DWV titers in bumble bees, we conducted separate linear mixed models (R library lme4, v 1.1.13, function lmer) with virus titer as the response variableS, treatment group (control, 0.1, 1, 10, 20 ppb) as a fixed effect and colony of origin as a random effect. We used a Gaussian distribution for virus titer models.

To examine whether imidacloprid exposure affects daily sucrose consumption, we conducted a repeated measures GLMM using the gamma distribution family with sucrose consumption as the response variable, treatment, time and treatment x time as fixed

effects, and colony of origin as a random effect. To investigate differences in total amounts of imidacloprid consumed by each treatment group, and to test whether groups received the treatment regardless of differences in daily sucrose consumption, we used a general linear model (function glm) with a gamma(link=log) distribution using imidacloprid consumption as a response variable and treatment group and colony as predictor variables. We examined pairwise comparisons using Tukey contrasts (R library multcomp, functions glht and mcp). To test for differences in survival among our treatment groups we visualized survivorship curves using Kaplan Meier plots and conducted a log-rank test to compare survivorship curves (R library survival, functions survfit and survdiff).

Results

Prior to the start of the experiment, all three original colonies arrived infected with BQCV and DWV at 100% prevalence for both viruses. DWV titers ranged from 10^4-10^6 and BQCV titers ranged from 10^6-10^7 and there were no differences in virus titers among colonies at the start of the experiment (DWV: $F_{2,12} = 3.073$, $p = 0.083$; BQCV: $F_{2,12} = 2.342$, $p = 0.138$). However, after five days of imidacloprid exposure, BQCV and DWV titers were significantly affected by imidacloprid exposure (BQCV: $\chi_4^2 = 20.873$, $p < 0.001$; DWV: $\chi_4^2 = 11.782$, $p = 0.019$). For BQCV, bees that received 1, 10, and 20 ppb of imidacloprid had significantly lower virus titers compared to the control group (1 ppb: $p < 0.001$; 10 ppb: $p = 0.002$; 20 ppb: $p = 0.003$; Fig. 3). For DWV, bees that received 10 ppb of imidacloprid had significantly lower virus titers compared to the control group (p

= 0.028; Fig. 3). Bees that received 20 ppb had marginally lower virus titers compared to the control group ($p = 0.05$; Fig. 3).

Sucrose consumption was significantly lower in bees treated with imidacloprid ($\chi_4^2 = 225.386$, $p < 0.001$). Bees in the 20 ppb group consumed significantly less sucrose compared to the control, 0.1, and 1 ppb groups ($p < 0.001$, Fig. 4). Bees in the 10 ppb group consumed significantly less sucrose than bees in the 0.1 ppb group ($p = 0.012$). Sucrose consumption changed over time ($\chi_4^2 = 42.324$ $p < 0.001$), but we found no time x treatment interaction effect on sucrose consumption. Despite differences in daily sucrose consumed, the mean total amount of imidacloprid consumed differed among groups ($\chi_3^2 = 1969.30$, $p < 0.001$) and incrementally increased according to treatment (Fig 4). We found significant differences among all pairwise comparisons ($p < 0.001$).

Survivorship was high (80-100%) across the 6 days and did not differ among groups ($\chi_4^2 = 4.3$, $p = 0.4$; Supplemental Fig. 1).

Discussion

To date, few studies have examined pesticide-pathogen combinations in bumble bee species, and none have focused on RNA viruses. Our study thus contributes to an understudied area of research and presents novel results demonstrating important interactions between imidacloprid exposure at field-realistic doses and RNA virus titers in bumble bees. Chronic exposure to imidacloprid significantly reduced both BQCV and DWV titers. Our study demonstrates that neonicotinoids can have a negative effect on virus levels. These findings are contrary to results from previous studies in honey bees where virus loads increased with exposure to neonicotinoids (Di Prisco et al., 2013;

Doublet et al., 2015), thus demonstrating that the impacts of pesticides on pathogens differ among host species.

Neonicotinoid exposure impacts the immune system in both honey bees (Boncristiani et al., 2012; Gregorc et al., 2012; Garrido et al., 2013) and in bumble bees (Czerwinski & Sadd, 2017) and has been suggested as a possible driver of increased virus titers in honey bees. However, the opposite trends we observed in bumble bees suggest other underlying mechanisms driving pathogen-pesticide interactions in bumble bees.

Apoptosis, or cell death, is a common symptom of neonicotinoid exposure in a broad range of organisms including mammals (Hsiao et al., 2016), birds (Tokumoto et al., 2013), and insects (Benzidane, Lapied & Thany, 2011). Neonicotinoid exposure induces apoptosis in the brains and midgut tissue of adult honey bees (Wu et al., 2015; Catae et al., 2018), as well as in the midguts, salivary glands, and ovaries of honey bee larvae (Gregorc & Ellis, 2011). As obligate intracellular pathogens, viruses cannot replicate without the organelles and metabolism of a host cell and thus, are often harmed by natural apoptosis elicited by a host. Many viruses encode proteins that inhibit apoptosis while, in other cases, viruses utilize apoptosis as part of their replication cycle to increase dissemination (reviewed in Clem, 2016; Hay & Kannourakis, 2002). Additional studies are needed to investigate the relationship of RNA virus replication and neonicotinoid-induced apoptosis in bees, and whether replication strategies employed by viruses differ among bee species.

Our results corroborate studies examining the impacts of plant secondary compounds on consumers and their pathogen loads. Despite the toxic effects of secondary metabolites, consumption may benefit herbivores and pollinators by reducing

parasitism (Singer, Mace & Bernays, 2009; Manson, Otterstatter & Thomson, 2010; Simone-Finstrom & Spivak, 2012). Neonicotinoids are chemically related to nicotine, a plant secondary metabolite that also acts on insect nicotinic acetylocholine receptors and reduces the survivorship of its consumers (Matsuda et al., 2001; Köhler, Pirk & Nicolson, 2012). In bumble bees, nicotine reduces parasitic infections of *Crithydia bombi* potentially through apoptosis or upregulation of the bee immune response (Richardson, Bowers & Irwin, 2016). Understanding the role of apoptosis and immune response in the mediation of RNA virus replication in honey bees and bumble bees is an important area for future research.

Here, daily sucrose consumption was reduced in bees that received the highest imidacloprid dose (20 ppb), indicating that high concentrations of imidacloprid may have negative effects on foraging behavior. Previous studies have also found reduced food consumption in bees exposed to neonicotinoids including lower doses of imidacloprid (Laycock et al., 2012; Kessler et al., 2015; Thompson et al., 2015). Although 20 ppb is above field realistic concentrations reported in nectar and pollen (Cresswell, 2011), bees can be exposed to much higher concentrations through leaf guttation, a natural phenomenon causing plants to excrete xylem fluid at leaf margins. Through guttation, plants grown from neonicotinoid-treated corn seeds will excrete droplets containing insecticides consistently higher than 10 mg/L (10,000 ppb) with maximum concentrations of imidacloprid reaching 200 mg/L (200,000 ppb) (Girolami et al., 2009). Therefore, the effects we observed may have important implications for wild bees foraging on treated crops.

We found no increased mortality for imidacloprid-fed bees. However, our experiment was conducted over the course of only five days over which we observed reduced sucrose consumption. Reduced foraging activity caused by pesticide exposure can affect worker size or other measures of colony success (Whitehorn O'Connor, S., Wackers, F. & Goulson, D., 2012; Gill & Raine, 2014; Arce et al., 2017). Other sublethal effects of neonicotinoid exposure include reduced brood production (Laycock et al., 2012), immune function (Czerwinski & Sadd, 2017), colony initiation by queens (Baron et al., 2017) and learning deficits (Phelps et al., 2018). Therefore, we suggest future studies examining the effects of pesticide-pathogen interactions over the lifetime of colonies. In addition, our bees were individually housed. Yet, for social insects, immunity is comprised of both the individual immune system as well as social immunity such as the removal of dead adult bees or diseased brood from the nest (Wilson-Rich et al., 2009). Future studies should investigate the effects of imidacloprid exposure on virus levels in full bumble bee colonies to examine the role of social immunity in this system.

Guidelines for assessing pesticide exposure risk to bees have greatly improved over the past decade and include a tiered structure for assessing both chronic and acute exposure in laboratory and field realistic settings (Environmental Protection Agency, 2016). However, in light of our findings and other recent research, we strongly suggest: 1) The inclusion of common pesticide-pathogen combinations in both lab and field realistic experiments, and 2) Mandatory pesticide risk assessment tests for non-*Apis* bees such as bumble bees and solitary bee species.

The bumble bee colonies we obtained for our experiment arrived infected with RNA viruses BQCV and DWV. Thus, we were unable to examine the effect of

imidacloprid without virus exposure. The virus status of these commercial bumble bee colonies is also alarming since they could be contributing to RNA virus spread. In captive rearing conditions, honey bee collected pollen is used as a food source for commercial bumble bee colonies and is implicated as a potential source of viruses (Singh et al., 2010). Fortunately, gamma irradiation inactivates RNA virus particles on pollen grains (Meeus et al., 2014). To reduce the risk of disease spread to both managed and wild bees, we suggest that all commercially available pollen feed for both honey bees and bumble bees undergo gamma irradiation treatment.

In conclusion, our results show that chronic oral exposure of imidacloprid reduces foraging behavior and reduces titers of two RNA viruses in bumble bees. We suggest future studies to examine the extent to which virus replication is mediated by insect immunity and/or apoptosis. Our results suggest that pesticide-virus interactions are highly variable among bee species and we caution against relying on honey bee studies to generalize results across the multitude of non-*Apis* species.

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Figure and Table Captions

Figure 1. Black queen cell virus (BQCV) titers in bumble bees in response to different concentrations of imidacloprid. Bees received chronic oral exposure to sucrose with varying concentrations of imidacloprid (0.1, 1, 10, and 20 parts per billion (ppb)). BQCV titers were significantly lower for bees in the 1, 10, and 20 ppb group as compared to bees in the control group that received sucrose only.

Figure 2. Deformed wing virus (DWV) titers in bumble bees in response to different concentrations of imidacloprid. Chronic oral exposure to imidacloprid at varying concentrations (0.1, 1, 10, and 20 parts per billion (ppb)) had a significant effect on DWV titers. Compared to the control group that received sucrose only, DWV titers were significantly lower for bees in the 10 ppb group ($p < 0.05$) and marginally lower for bees in the 20 ppb group ($p = 0.05$).

Figure 3. Sucrose consumption per bee per day for each treatment group. Sucrose consumption was measured daily over five days of chronic oral exposure to imidacloprid. Bees in treatment groups received sucrose that contained 0.1, 1, 10, or 20 ppb of imidacloprid. The control group received sucrose only. Bees that received the most imidacloprid (20 ppb group) consumed significantly less sucrose compared to the control. Bees in the 10 ppb group consumed significantly less sucrose compared to the bees in the 0.1 ppb group. All other pairwise comparisons were not significant.

Figure 4. Total imidacloprid consumed by bumble bees of each treatment group. Total amount of imidacloprid (ng) consumed by bees in each treatment group over the duration of the experiment. Bees in the 10, and 20 ppb groups consumed significantly more imidacloprid as compared to the 0.1 and 1 ppb group.

Supplemental Figure 1. Survivorship curves. Survivorship for each treatment group over the course of the five-day experiment. Survivorship did not differ among treatment groups.

Figures and Tables

Tables and Figures.

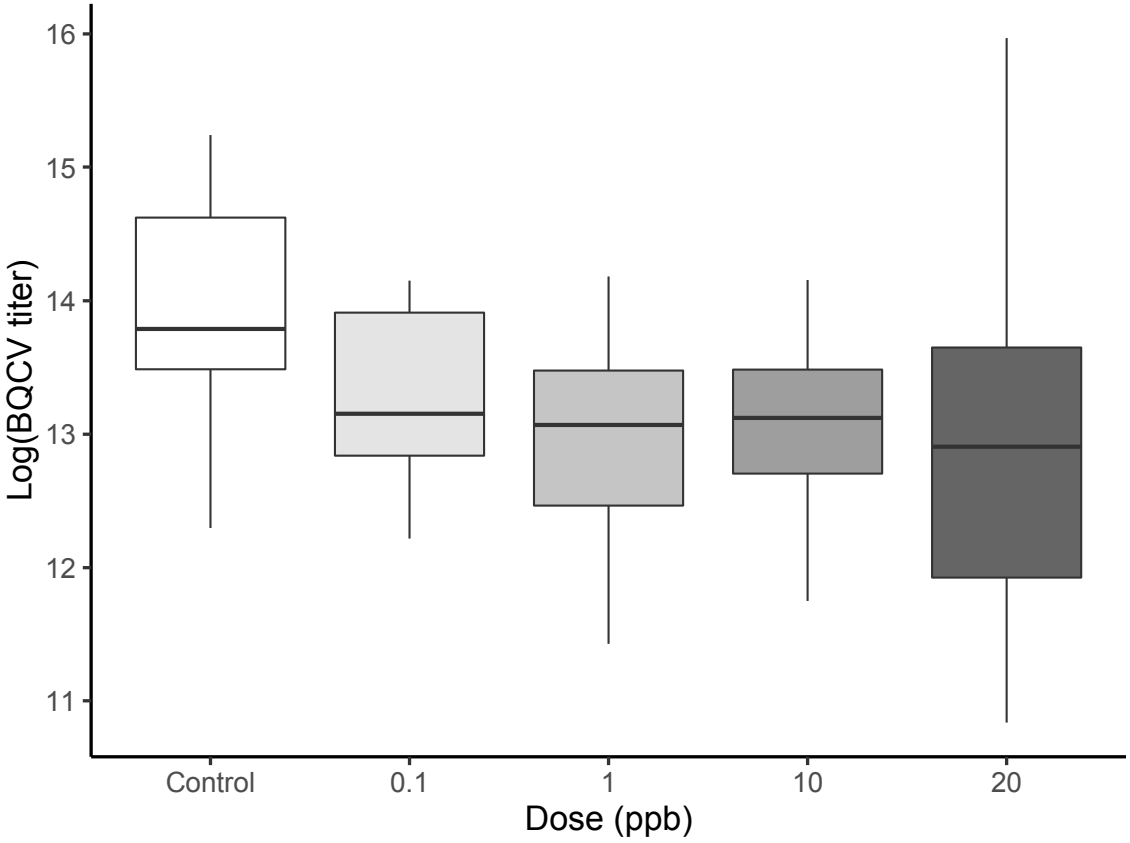


Figure 1. Black queen cell virus (BQCV) titers in bumble bees in response to different concentrations of imidacloprid

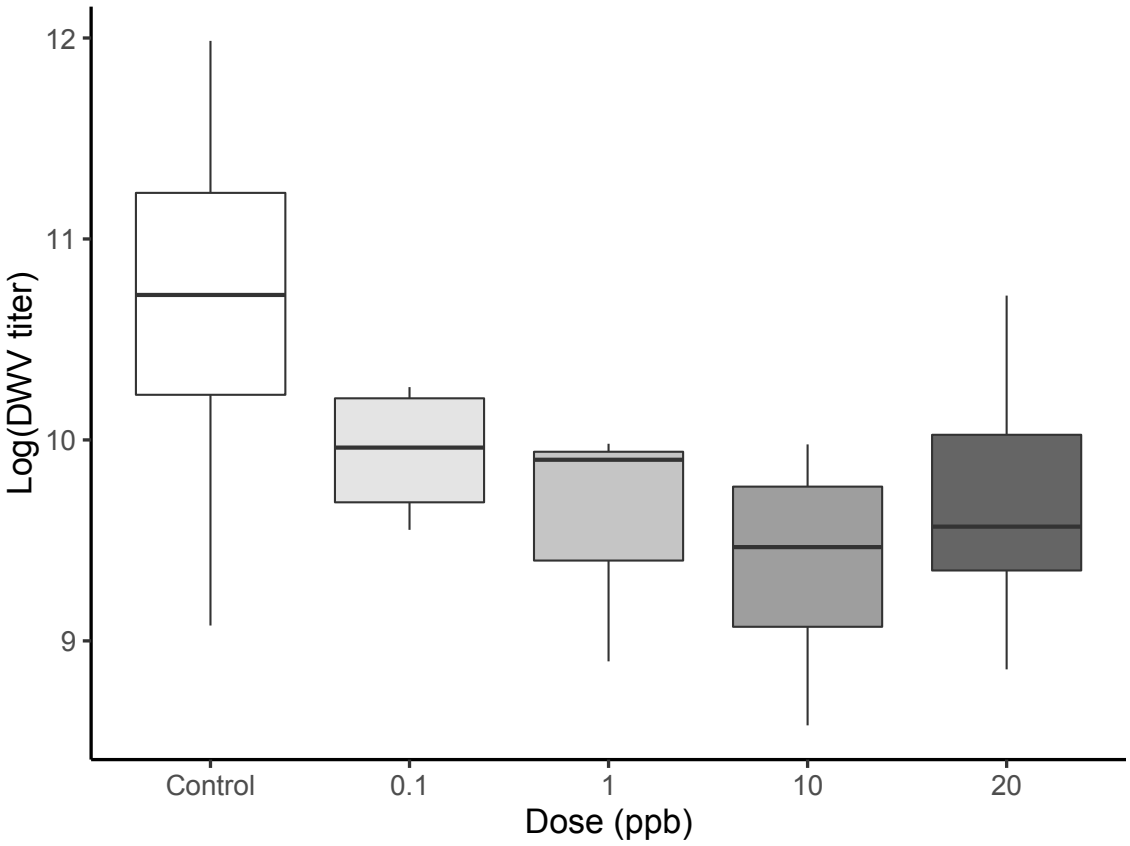


Figure 2. Deformed wing virus (DWV) titers in bumble bees in response to different concentrations of imidacloprid

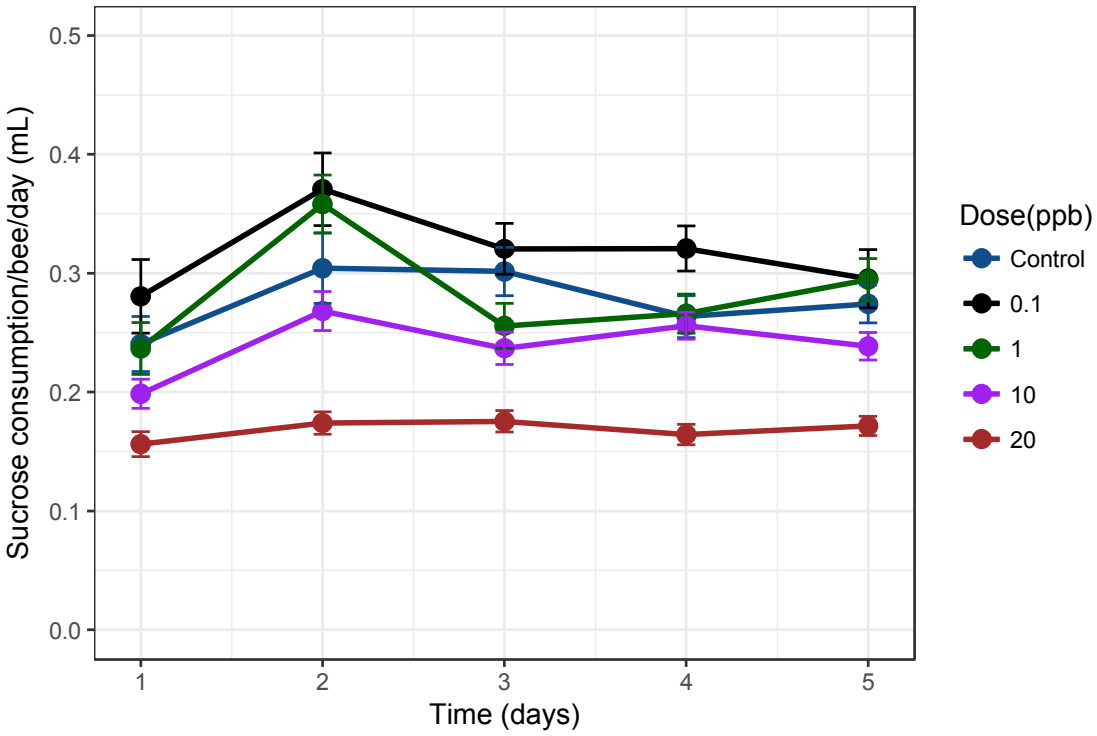


Figure 3. Sucrose consumption per bee per day for each treatment group.

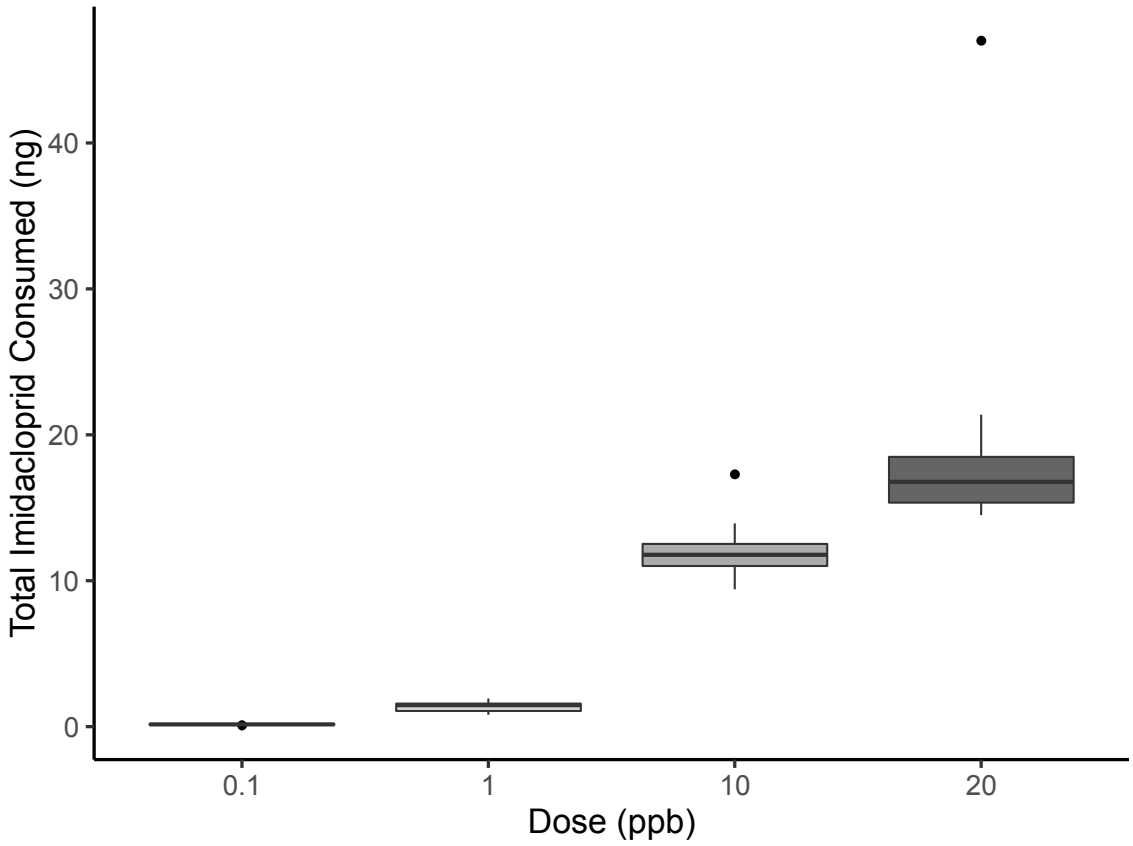
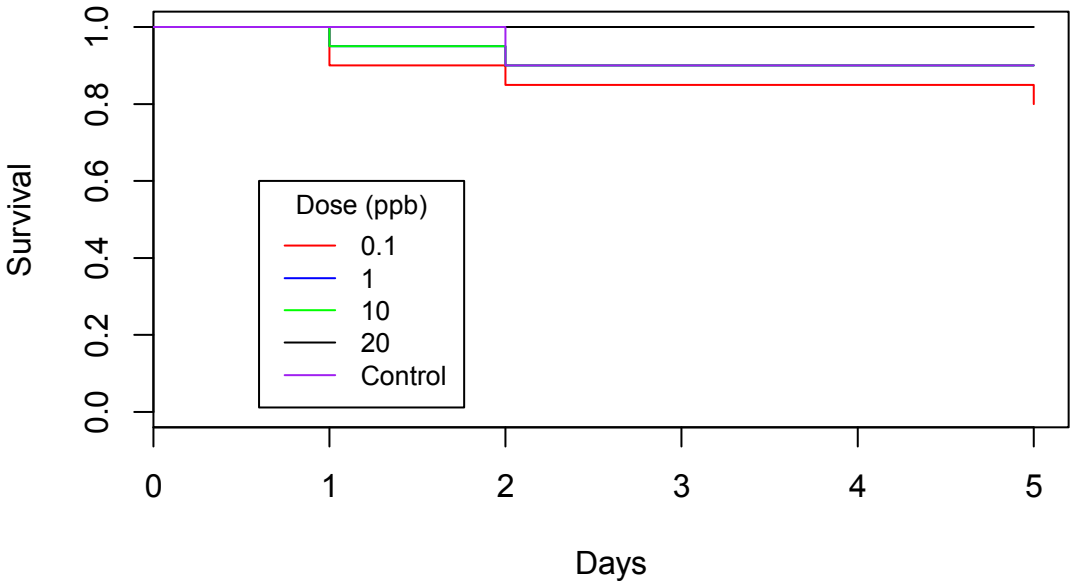


Figure 4. Total imidacloprid consumed by bumble bees of each treatment group.



Supplemental Figure 1. Survivorship curves.

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CHAPTER 5: HOMESICK: IMPACTS OF MIGRATORY BEEKEEPING ON HONEY BEE (*APIS MELLIFERA*) PESTS, PATHOGENS, AND COLONY SIZE

Abstract

Honey bees are important pollinators of agricultural crops and the dramatic losses of honey bee colonies have risen to a level of international concern. Potential contributors to such losses include pesticide exposure, lack of floral resources and parasites and pathogens. The damaging effects of all of these may be exacerbated by apicultural practices. To meet the pollination demand of US crops, bees are transported to areas of high pollination demand throughout the year. Compared to stationary colonies, risk of parasitism and infectious disease may be greater for migratory bees than those that remain in a single location, although this has not been experimentally established. Here, we conducted a manipulative experiment to test whether viral pathogen and parasite loads increase as a result of colonies being transported for pollination of a major US crop, California almonds. We also tested if they subsequently transmit those diseases to stationary colonies upon return to their home apiaries. Colonies started with equivalent numbers of bees, however migratory colonies returned with fewer bees compared to stationary colonies and this difference remained one month later. Migratory colonies returned with higher black queen cell virus loads than stationary colonies, but loads were similar between groups one month later. Colonies exposed to migratory bees experienced a greater increase of deformed wing virus prevalence and load compared to the isolated group. The three groups had similar infestations of *Varroa* mites upon return of the migratory colonies. However, one month later, mite loads in migratory colonies were

significantly lower compared to the other groups, possibly because of lower number of host bees. Our study demonstrates that migratory pollination practices has varying health effects for honey bee colonies. Further research is necessary to clarify how migratory pollination practices influence the disease dynamics of honey bee diseases we describe here.

Introduction

Animal-mediated pollination, provided primarily by bees, is required for the production of 75% of agricultural food crops (Klein et al., 2007b) and provides an estimated annual value of $200 billion worldwide (Gallai et al., 2009). Managed honey bees (*Apis mellifera*) are the most important commercially available pollinator and contribute approximately $17 billion in pollination services revenue annually to the United States (US) alone (Calderone, 2012). However, for over a decade, honey bees have experienced elevated colony losses (Neumann & Carreck, 2010; Potts et al., 2010; van der Zee et al., 2012, 2013; Kulhanek et al., 2017) attributed to multiple threats including pesticide exposure (Tsvetkov et al., 2017; Woodcock et al., 2017), forage availability (Decourtye, Mader & Desneux, 2010), and numerous pests and pathogens (vanEngelsdorp & Meixner, 2010). The numerous threats affecting honeybees may be exacerbated by practices inherent to the apicultural industry and required for large-scale crop pollination, specifically migratory beekeeping (Royce & Rossignol, 1990; Traynor et al., 2016a).

To meet the pollination demand of a variety of US agricultural crops, large numbers of bees are moved among crops at regional and national scales. Conditions for

migratory colonies vary greatly depending on the distance traveled and the crops visited. In the most extreme cases, colonies are transported by truck to a series of monoculture crops including blueberries, cranberries, almonds, and citrus (VanEngelsdorp et al., 2013) for months at a time. At each stop along the journey, millions of bees from different origins converge on a single crop for the duration of bloom, which typically lasts approximately one month and may offer little forage diversity (Decourtye, Mader & Desneux, 2010; Colwell et al., 2017). Nectar, comprised of sugars and amino acids, is required to fuel flight and feed the colony while pollen, high in protein and fats, provisions developing brood (Brodschneider & Crailsheim, 2010). To ensure survival en route or when crops are not in bloom, colonies may be supplemented with sucrose syrup and artificial pollen, temporary but poor substitutes for the diverse array of nectar and pollen types bees obtain in natural landscapes (Huang, 2012). Thus, compared to their stationary counterparts, migratory colonies experience greater stress (Simone-Finstrom et al., 2016), greater exposure to pesticides (Mullin et al., 2010; Traynor et al., 2016a), and lower quality forage, all of which may increase susceptibility to disease (Di Pasquale et al., 2013; Sánchez-Bayo et al., 2016). It is well known that stress from long distance travel results in heightened bacterial and viral infections in vertebrate livestock (Yates, 1982). However, despite the importance of large-scale pollination events for agriculture, few studies have examined how migratory conditions may contribute to disease incidence or spread in bees (Zhu, Zhou & Huang, 2014; Simone-Finstrom et al., 2016).

In the US, there are an estimated 2.62 million commercial honey bee colonies of which over half are contracted for crop pollination (USDA National Agricultural Statistics Service, 2017b). California almond pollination is the largest annual event for

the migratory beekeeping industry, requiring nearly 1.5 million honey bee colonies (USDA National Agricultural Statistics Service, 2017a). It is the largest convergence of honey bee colonies in the US, providing conditions in which pathogens are likely to be introduced, transmitted, and subsequently spread as colonies move along their human-imposed migration route (Bakonyi et al., 2002; Welch et al., 2009; Runckel et al., 2011; Goulson et al., 2015). Each acre of almonds requires an average of two honey bee colonies (Carman, 2011) and as bees will forage 3 km from their colonies (Visscher & Seeley, 1982; Beekman & Ratnieks, 2000; Couvillon et al., 2015), bees in large orchards could theoretically share flowers with bees from nearly 56,000 other colonies. While almond flowers may produce a large quantity of nectar and pollen, there is evidence that it is relatively low quality (and possibly toxic) forage for honey bees (London-Shafir, Shafir & Eisikowitch, 2003; Kevan & Ebert, 2005); moreover, the vast fields provide little forage diversity for bees and are heavily sprayed with pesticides (California Department of Pesticide Regulation, 2017), exposing bees to additional stress.

The spread of the most devastating honey bee parasites and pathogens has mainly occurred as a result of transporting honey bees long distances. For example, the *Varroa* mite (*Varroa destructor*), an ectoparasite and known vector of numerous RNA viruses, became a major contributor to colony losses in both North America and Europe after its introduction from Asia (Rosenkranz, Aumeier & Ziegelmann, 2010; Nazzi et al., 2012). *Nosema ceranae*, a microspordian implicated in high colony mortality in Spain (Higes et al., 2008), has also reached high frequencies since its introduction from Asia to the Americas and Europe (Klee et al., 2007; Chen et al., 2008). Despite the role of long-

distance travel in disease spread, there is a surprising lack of studies examining the role of migratory beekeeping in disease spread.

A limited number of observational surveys have compared disease loads of colonies belonging to migratory and stationary operations and found a higher prevalence of some pathogens in migratory colonies (Traynor et al., 2016b) including *Nosema ceranae* (Zhu, Zhou & Huang, 2014) and RNA viruses (Welch et al., 2009), some of which were not previously described in honey bees (Runckel et al., 2011). However, the focus of previous studies has been the collection of baseline disease data to characterize diseases in migratory colonies and, as such, rarely control for migratory conditions, management practices, and sampling times, all of which can significantly affect disease loads and colony health (Runckel et al., 2011; Glenny et al., 2017). Furthermore, studies examining the impact of migratory conditions on bees rarely include a control group of stationary colonies for comparison (but see Zhu, Zhou & Huang, 2014; Simone-Finstrom et al., 2016). Although migratory honey bee colonies are implicated as disease sources and could serve to introduce disease to local stationary honey bee colonies (Welch et al., 2009) we are unaware of previous studies that explicitly test the role of migratory colonies in the spread of diseases or parasites. Here, we conducted a two-pronged experiment in which we controlled for migratory conditions, sampling time, and beekeeper management practices. We first tested the effects of migration on honey bee colony population size, *Varroa* mite parasites, and pathogens including *Nosema* (a microsporidian) and three RNA viruses: black queen cell virus (BQCV), deformed wing virus (DWV), and Israeli acute paralysis virus (IAPV). We examined differences in the parasite and pathogen prevalence and load as well as colony size of migratory and

stationary colonies. Second, we examined if there is evidence for the transmission of diseases from migratory colonies to stationary colonies. If migration exposes bees to stressors that increase disease susceptibility, we predicted that migratory colonies would have greater pathogen prevalence and loads when compared to their stationary counterparts, and that pathogen loads in sympatric stationary colonies would increase after foraging alongside the migratory colonies for one month.

Materials and Methods

In February 2017, we selected 48 colonies from a North Carolina apiary that is used for the production of products (honey, colonies, etc.) rather than pollination services, and assigned each to one of the following groups: *migratory* (n=16), *isolated stationary* (*isolated*) (n=16), and *exposed stationary* (*exposed*) (n=16; Fig. 1). We transported colonies in the migratory group from Whiteville (Columbus County), North Carolina to Coalinga (Fresno County), California (36°21'N, 120°12'W) to pollinate almonds for the duration of the bloom (approximately one month). They were then transported back to North Carolina. As typical of migratory beekeeping practices, the migratory colonies were covered by netting during transport (to reduce escapees) and temporarily brought to a nearby holding yard in California before and after pollinating almond orchards. The isolated stationary group remained in North Carolina (34°22'N, 78°36'W) and outside the flight distance from returning migratory colonies for the entirety of the experiment. To maintain similar colony densities at the isolated stationary and migratory yards, there were an additional 15 stationary colonies in the isolated yard. These colonies originated from the same North Carolina apiary and were not tested as part of the experiment.

At the start of the experiment in February 2017, all colonies had 7-9 frames of bees, and 7-8 frames with brood. To measure bee population size, we counted frames of adult bees (FOB) by assessing the coverage of adult bees on each frame and summing the estimates for all frames in the brood chamber (the lower hive body containing the queen and brood) (DeGrandi-Hoffman et al., 2016). Frames with brood were assessed by counting the total number of frames containing 30% capped brood. Each colony was provided a new queen by replacement with open-mated Italian (*A. mellifera ligustica*)/ Carniolan (*A. mellifera carnica*) queens in summer 2016. Colonies were matched in triplicate by frames of bees and frames of brood and randomly assigned a treatment group (migratory, isolated stationary, or exposed stationary) to ensure equal distribution across groups. Prior to the start of the experiment, in October 2016, we treated all colonies for *Varroa* mites with fluvalinate, a synthetic pyrethroid commonly used as an acaricide in honey bee colonies. No other mite or pathogen treatments were used for the duration of the experiment. To ensure that colonies would persist for the duration of the experiment, we provided supplemental feed to all colonies (in all treatment groups) on two occasions: pollen substitute prior to shipping the migratory colonies to California and upon return, 5 lbs. of fondant (sucrose and water stabilized with gelatin). As colonies grew during the duration of the study, additional hive bodies were added as needed to prevent swarming.

We compared bee population size and disease loads in the migratory and isolated stationary group three times: before the migratory group departed for California (Jan. 25), immediately after the migratory group returned to North Carolina from California (Feb. 28), and one month later (March 25). To test for disease spread from the migratory colonies to their stationary counterparts, we monitored the third group of colonies, the

exposed stationary group, which remained in North Carolina but shared a yard with the migratory colonies once they returned from California (34°11'N, 78°46'W). We assessed bee population size and disease loads in the exposed stationary group twice: once before sharing a yard with the migratory group (Feb. 28), and again approximately one month after residing with the migratory colonies in the same yard (March 25). Land cover surrounding each of the North Carolina yarding areas were dominated by crops, mixed forest, and woody wetlands, and we expect that colonies in the two sites had similar access to early spring floral resources. Hives were housed on private land and permission was granted by the owners.

At each sampling event, we inspected all colonies for brood diseases, measured colony size, and collected bees for pathogen analyses. To estimate colony size, we measured frames of bees (FOB) as before (DeGrandi-Hoffman et al., 2016). We also recorded the queen status of each colony (queen-right, queenless, queen cells present, or drone-laying queen). We collected live bees from the brood chamber to detect and quantify the following parasites and pathogens: *Varroa*, *Nosema,* BQCV, DWV, and IAPV. To quantify *Varroa* and *Nosema* spp., we collected approximately 300 bees from the brood chamber and transferred them to ethanol. To quantify virus prevalence and load, we collected an additional 150 bees from the brood chamber. These samples were stored and shipped to Vermont on dry ice and transferred to -80°C for storage prior to analysis.

To examine differences in climate and weather conditions experienced by the migratory and stationary groups, we used publicly available NOAA local climatology

data collected by weather stations nearest to our field sites (NOAA National Centers for Environmental Information).

Varroa mite and *Nosema* spp. quantification

To calculate the number of *Varroa* mites per 100 bees, ethanol samples were agitated for 60 seconds, strained through hardware cloth to separate the mites from the bees, and all mites and bees were counted (Lee et al., 2010). We conducted spore counts to quantify *Nosema* spp. Although our methods did not differentiate between the two species of *Nosema, (N. apis and N. ceranae*) previous work has found *N. ceranae* to be the predominant species in many regions (Klee et al., 2007; Chen et al., 2008; Williams et al., 2008, 2014). To conduct spore counts, we transferred 100 bees from the ethanol sample to a plastic bag and pulverized them using a pestle on the outside of the bag for 90 seconds. We then added 100 mL of distilled water, allowed it to settle for 45 seconds, and transferred 10 µL onto a haemocytometer counting chamber. We counted spores for each sample twice under 40X magnification, averaged them, and converted to spores/bee (Fries et al., 2013).

Virus quantification

To quantify BQCV, DWV and IAPV, we transferred 50 honey bees/sample on liquid nitrogen and homogenized them in an extraction bag with 10 mL of GITC buffer using protocols established by USDA-ARS Bee Research Lab Beltsville, MD (Evans, 2006). We followed EZNA Plant RNA Standard Protocols (Omega Bio-Tek) with 100 µL of the resulting homogenate thereafter. Using a Spectrometer (Nanodrop, Thermo Scientific), we assessed all RNA quantity and quality and diluted all RNA extractions to 20 ng/µL prior to virus assays.

For reverse transcription of RNA and absolute quantification, we performed duplicate reverse transcription quantitative polymerase chain reaction (RT-qPCR) for each sample with a SYBR green one-step RT-qPCR kit in 10 μL reactions using the following thermal cycling program: 10 min at 50°C (RT) followed by 1 min at 95°C, and 40 amplification cycles of 95°C for 15 s, 60°C for 60s. Lastly, we obtained the melt-curve starting at 65-95°C (0.5°C increments, each 2 seconds). We used primers specific to the positive strand of the following RNA virus targets: BQCV, DWV and IAPV, and a housekeeping gene (Actin) as a positive control of RNA extraction efficiency (Appendix A). We calculated quantification using duplicate standard curves of gBlocks Gene Fragments (Integrated DNA Technologies; Appendix B) that were developed using double-stranded, sequence verified genomic blocks consisting of the four targets of interest separated by ten random base pairs. Sequences of random base pairs consisting of at least 50% G and Cs were used at the beginning and terminal ends of the fragment. Efficiencies were 95.21% (BQCV), 91.06% (DWV), 90.27% (IAPV), and 90.12% (Actin), with correlation coefficients (R^2) ranging from 0.993-0.999. To verify RT-PCR analyses, sequences with lengths of 100-130 bps were generated through DNA sequencing performed in the Vermont Integrative Genomics Resource using a 3130xl Genetic Analyzer.

DATA REPORTING

We use "pathogen prevalence" to refer to the percentage of colonies positive for a pathogen (*Varroa, Nosema,* BQCV and DWV). In addition to presence/absence data, we investigated the severity of infection by quantifying each pathogen—we refer to this as "pathogen load". Virus load (BQCV and DWV) results for each colony are presented in

average virus genome copies/bee. We did not detect IAPV in our experimental colonies and it was therefore excluded from further analysis. We report *Varroa* as the number of mites per 100 bees and *Nosema* as average number of spores/bee.

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DATA ANALYSIS AND STATISTICS

Before analyzing, we checked all response variables for normality using Shapiro-Wilk tests. To improve normality, *Varroa* and *Nosema* loads as well as BQCV and DWV loads (genome copies per bee) were log + 1 transformed. To establish that there were no differences between treatment groups at the outset, we analyzed all variables at the initial time step using ANOVAs for continuous variables (FOB, load of *Varroa, Nosema,* BQCV, and DWV) and Chi-Square tests of independence for binary variables (prevalence of *Varroa, Nosema,* BQCV, and DWV*)*.

To test whether the full suite of response variables collectively predicted colony treatment membership, we conducted classification analyses for Experiments 1 (*migratory* vs. *stationary*) and 2 (*exposed* vs. *isolated*) using linear combinations based on all response variables (except BQCV prevalence as it was fixed at 100% prevalence for all groups and as such caused model fitting failures). To examine how groups differed after experimental manipulation, we used data from sampling events two and three for Experiments 1 and 2, respectively. The models were trained using a conservative cross validation approach to reduce over-fitting the model to our data. We tested for differences between groups' centroids in multivariate space for each time point with PERMANOVA, a non-parametric MANOVA, using Euclidian distance-based dissimilarity matrices. To

visualize between-group separation, the centered values from linear discriminate functions (LD1 and LD2) were plotted for each colony.

To test the effect of treatment and time on prevalence, we analyzed all pathogens (*Nosema, Varroa,* BQCV, and DWV) using separate generalized linear mixed effects models (GLMMs) using the binomial (link="logit") distribution family. For measures of pathogen load, and FOB, we used linear mixed effects models (Harrison et al., 2018). All models used the same repeated measures design. Treatment, sampling event, and their interaction were included as fixed effects in order to determine how each dependent variable was affected by our manipulation through time. Colony and bee yard were included as random effects in order to determine the among colony variance within each treatment and account for potential differences between bee yards. To examine how the *Varroa* load of migratory and stationary colonies differed over time with respect to FOB, we conducted a separate linear mixed effects model. We first tested for temporal autocorrelation in the residuals of the model using an ACF plot and no autocorrelation was detected. For this model, we used FOB, treatment, time, and the resultant interactions as fixed effects and colony as a random effect. Significance for all models was determined using Type II Wald Chi-Square tests.

To examine potential differences in climate between California and North Carolina during the 27 days the migratory bees were in California, we used one-way Analysis of Variance (ANOVAs) on average daily temperature, precipitation, and wind speed by state (NOAA National Centers for Environmental Information).

We conducted all statistical analyses using the statistical software "R" (R version 3.3.1). GLMMs were conducted using the lme4 package (v 1.1-13) (Bates et al., 2015).

The corresponding Type II Wald Chi-Square tests were conducted using the Anova function in the car package (v 2.1-4) (Fox & Weisberg, 2011). Temporal autocorrelation was tested using the acf function. Classification analyses were conducted using the lda function in the mass package (v 7.3-45) (Ripley & Venables, 2002). The adonis function was used to perform PERMANOVA in the vegan package (v 2.4-3) (Oksanen et al., 2017).

Results

While in California, migratory colonies experienced similar weather conditions (mean daytime temperature, wind speed, and precipitation) to those experienced by stationary colonies in North Carolina ($F_{1,52} < 3.106$, $P > 0.084$; Table S1). All colonies were absent of IAPV. BQCV was present in all colonies for the duration of the study (Fig. S1).

Experiment 1: Migratory verses Stationary

At the start of the experiment, there was no significant difference between migratory and stationary colonies in prevalence ($\chi_1^2 < 1.143$, $P > 0.285$) or load ($F_{1,30} < 3.01$, $P > 0.093$) of any of the four pathogens. In addition, there was no difference in FOB at the beginning of the experiment (migratory: 7.94 ± 0.57 sd, stationary: 7.44 ± 0.51 sd).

Upon the return of the migratory colonies, our pathogen and hive population measurements collectively predicted whether a colony was migratory or stationary (Fig. 2A). The linear combination (LD1) adequately discriminated between the migratory group and the stationary group and yielded correct classification rates of 87.5% for migratory colonies and 75% for stationary colonies. Also, prior to contact with the migratory colonies, the exposed colonies were similar to the isolated stationary colonies

and essentially formed one large group (Fig 2A). After contact with migratory colonies, there was statistically significant group separation between migratory and stationary treatments ($F_{1,30} = 5.03$, $P = 0.007$).

Migratory colonies returned from California with significantly higher BQCV loads compared to the stationary group ($\chi_1^2 = 16.488$, $P < 0.001$; Fig 3A), and BQCV load increased with time (Fig. 3A and Table 1). The prevalence (Fig. S1) and load of DWV (Fig 3B) did not differ between treatments following return of migratory colonies but both increased with time (Table 1). *Nosema* load and prevalence (Fig. S1) did not differ between treatments following return of migratory colonies and *Nosema* load decreased with time (Table 1). However, for *Varroa*, there was a significant treatment × time interaction (Fig 3C). *Varroa* loads increased steadily for stationary colonies, but decreased in migratory colonies over the month after returning from California ($\chi_1^2 = 6.465$, $P = 0.011$). There was also a significant interaction of treatment × time for FOB, with migratory colonies returning with fewer FOB than their stationary counterparts ($\chi_1^2 = 5.651$, $P = 0.017$). There was a significant interaction of FOB × treatment × time on *Varroa* loads ($\chi_1^2 = 4.045$, $P = 0.044$) indicating that *Varroa* loads were differentially affected by FOB for each treatment group over time. Other interaction terms were not statistically significant (Table 1).

Experiment 2: Exposed verses Isolated

At sampling event two, there was no significant difference between exposed and isolated stationary colonies in pathogen prevalence ($\chi_1^2 < 1.143$, $P < 0.285$) or load ($F_{1,30} < 1.279$, $P > 0.267$). FOB was similar between groups at the beginning of the experiment

($F_{1,29} = 0.858$, $P = 0.362$).

One month after the exposed group foraged alongside the migratory colonies, there was an increase in between-group separation with groups becoming more distinguishable from each other. While all groups separated in this third time step, the exposed and migratory groups were less distinguishable from one another compared to the stationary group (Fig. 2B). The linear combinations (LD1 and LD2) yielded a correct classification rate of 75% for stationary colonies but correct classification rates for migratory and exposed colonies were lower, 43.75% and 56.25%, respectively. PERMANOVA results indicated statistically significant group separation between isolated, migratory and exposed treatments ($F_{2,43} = 4.72$, $P = 0.001$).

We found no effects of treatment (exposed verses isolated) for any of the parasite or disease response variables (Fig 3). However, *Varroa* prevalence and load, *Nosema* prevalence and load, and BQCV significantly increased with time (Table 2). There was a significant treatment × time interaction for both DWV load ($\chi_1^2 = 9.229$, $P = 0.002$; Fig 3B) and DWV prevalence ($\chi_1^2 = 4.94$, $P = 0.026$; Fig. S1) such that DWV in exposed colonies increased at significantly higher rates than the isolated group. There was also a significant treatment × time interaction for FOB ($\chi_1^2 = 9.946$, $P = 0.0016$; Fig 3D) with exposed bees increasing at a significantly higher rate compared to the isolated group. Other interaction terms were not significant (Table 2).

Discussion

Migratory pollination services are an essential component of the US agricultural economy, yet this practice exposes honey bee colonies to a combination of factors that may compromise individual bee and colony health. Although there is widespread concern

that migratory pollination can place honey bee colonies at increased risk to acquire and spread pathogens and parasites, there is a lack of experimental evidence demonstrating this phenomenon. Here, we controlled for management practices and starting conditions as well as the time at which bees were sampled for diseases and parasites. Our results show that while migratory conditions can negatively affect colony health and increase disease load, in some cases these impacts were transient.

With the exception of *Nosema,* honey bee colonies experienced an increase in pathogen prevalence and load over time with the highest levels occurring during the last sampling event in March, following the seasonal trends of other time-course studies (Tentcheva et al., 2004; Runckel et al., 2011). Peak incidences of these viruses occur in warmer months when transmission is more likely to occur as a result of increased brood rearing (Chen & Siede, 2007) and increased foraging (Singh et al., 2010) However, for BQCV and *Varroa*, our results indicate that bees in the migratory conditions were affected differently compared to their stationary counterparts.

The migratory colonies in our study returned from almond pollination with higher BQCV loads compared to the stationary colonies but had converged to similar levels one month later indicating that migratory conditions exacerbated BQCV infection but these effects were transient. Colonies experience stress during transportation (Simone-Finstrom et al., 2016) which impairs immunity (James & Xu, 2012) and promotes elevated levels of virus replication. Pollinators of large monocultures experience a reduction in forage diversity (Decourtye, Mader & Desneux, 2010; Colwell et al., 2017) which increases susceptibility to disease (Di Pasquale et al., 2013). Exposure to agricultural chemicals adversely affects the insect immune response and promotes replication of RNA viruses in

bees (Di Prisco et al., 2013; Doublet et al., 2015). In particular, higher BQCV titers are associated with exposure to organosilicone surfactant adjuvants (OSS), a class of surfactants used to enhance the spread of the active ingredient (Fine, Cox-Foster & Mullin, 2017). OSSs are heavily used in California almonds during the late January to March bloom period when migratory colonies are present (Ciarlo et al., 2012; CDPR (California Department of Pesticide Regulation) CalPIP, 2016; Mullin et al., 2016). We did not measure pesticide exposure in our colonies and are therefore cautious to speculate its role in the increased virus loads in our study. However, in light of our results and previous work, we believe pesticide-pathogen interactions in migratory colonies warrants further study.

Compared to stationary colonies, the migratory colonies had fewer FOB upon return from California. The lower population size observed may be a result of forager die-off after the large pollination event as migratory bees have significantly shorter lifespans when compared to stationary bees as a result of increased oxidative stress (Simone-Finstrom et al., 2016). In addition, foragers could have been displaced during transit. As typical with migratory colonies, our colonies were moved to holding yards before and after pollinating almonds. When colonies are moved, foragers are forced to re-assess and re-learn their surroundings which can cause significant loss and/or drifting of foragers (Nelson & Jay, 1989). Despite migratory colonies returning with fewer numbers and remaining lower in FOB compared to stationary colonies, the two groups experienced similar population growth rates during the month following the large pollination event.

Upon return from California, mite prevalence and load in migratory colonies were similar to their stationary counterparts. However, when sampled one month later, mite prevalence and load in the stationary colonies had significantly increased, while mite prevalence and load in the migratory colonies declined slightly, and was significantly lower than that in stationary colonies. Since female mites must reproduce within the pupal cells of developing honey bees, mite population growth is largely dependent on the availability of bee brood. Although we did not measure brood size, adult bee population size is highly correlated with brood size of the previous time step (Torres, Ricoy & Roybal, 2015) and mite population size (Martin, 1998; DeGrandi-Hoffman et al., 2016) . Thus, the lower mite prevalence and load in migratory bees is likely, in part, a reflection of the lower reproduction of these colonies. Additional unknown factors may be influencing the lower mite loads in migratory colonies, as *Varroa* loads of the migratory and stationary colonies showed different relationships with FOB over time. Results of the US National Honey Bee Disease survey suggested that migratory beekeepers may treat with acaricide more effectively and the mechanical motion of the truck during transportation helps to dislodge mites from bees (Traynor et al., 2016b). Since our colonies returned from California with similar mite prevalence and load as the stationary group, it is unlikely that the motion of the truck had an impact. Additionally, we are confident that the difference in mites we saw during the last sampling event was not due to beekeeper practices as mite treatments were standardized across all groups.

Colonies exposed to migratory bees experienced a significantly greater increase in DWV prevalence and load compared to isolated colonies one month after foraging alongside the migratory colonies. *Varroa* loads could not explain this difference since

exposed and isolated colonies experienced similar *Varroa* loads throughout the study. The greater population size of the exposed colonies in the last sampling event, could have increased dissemination of DWV. However, isolated colonies had higher bee populations than the migratory colonies and we saw no differences in DWV prevalence or load between those two groups. Previous studies found that DWV was a good predictor of weaker colonies (Budge et al., 2015) and thus one would not expect our results to simply be attributed to an increase in numbers and thus exposure. One potential explanation is that the migratory bees returned from pollinating almonds with a more virulent DWV strain that disseminated quickly in the exposed group as a result of their larger colony size and higher *Varroa* population (Martin, 2002; Rosenkranz, Aumeier & Ziegelmann, 2010; Glenny et al., 2017). Using deep sequencing, viruses not previously found in honey bees have been detected in migratory hives (Runckel et al., 2011) and recently, a more virulent recombinant of DWV was found to replicate at high levels when transmitted by *Varroa* mites (Ryabov et al., 2014). Despite this evidence, we remain cautious of speculating transmission of a novel or more virulent strain.

Conclusions

Migratory bees are subjected to a myriad of stressors not experienced by their stationary counterparts including transport, lower diversity of floral resources, exposure to bees from tens of thousands of other colonies that may be diseased, and exposure to large quantities of pesticides. The migratory conditions in our experiment encompassed all these components, and thus we cannot attribute our results to a single or even an exact combination of causes. Furthermore, our study, while novel in scope, was conducted over a relatively short time span using a single set of migratory conditions and focused on a

limited set of bee pathogens. Thus, we are cautious to claim that our results are representative of migratory beekeeping, at large, but do suggest that migratory conditions may exacerbate BQCV infections and lead to slower colony growth. Future studies to examine the underlying mechanisms, individually and in concert, as well as those that encompass colony health and additional pest and pathogens over a longer time span will provide further insight.

A growing body of evidence suggests that pests and pathogens from managed bees are spilling over into wild bee populations (Colla et al., 2006; Spiewok & Neumann, 2006; Hoffmann, Pettis & Neumann, 2008; Otterstatter & Thomson, 2008; Singh et al., 2010; Graystock et al., 2013a; Levitt et al., 2013; Brown, 2017). Sympatric bumble bees and honey bees are infected by the same strains of DWV (Fürst et al., 2014) and virus prevalence in honey bees is a significant predictor of virus prevalence in bumble bees (McMahon et al., 2015). The higher BQCV load we document in migratory bees could thus pose a risk to wild bees. It is also possible that increased disease load as a consequence of migratory pollination could affect honey bees in future years due to disease spillback from infected wild bees (Graystock, Goulson & Hughes, 2015). Therefore, it is important to test whether wild bee populations have higher disease prevalence in proximity to honey bee apiaries, particularly those with migratory management practices.

According to recent forecasts, the US demand for commercial crop pollination services is expected to rise, particularly for almond (USDA National Agricultural Statistics Service, 2017c). Thus, understanding the effects of this current model of crop pollination on bees and identifying where, when, and how to mitigate those effects are

critical to the apiculture industry. Our work suggests that some effects, while important, may be transitory. Thus, honey bees may be resilient to some stressors imposed by migratory conditions and recuperation after a large pollination events is important to maintaining healthy migratory colonies.

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Figure and Table Captions

Figure 1. Schematic of Experimental Design. Three sampling events occurred during the experiment. Three experimental groups (isolated stationary group, migratory group, and exposed group) were located in two separate apiaries in North Carolina throughout the experiment: the stationary yard (where all groups begin and the isolated stationary group remained for the duration of the experiment) and the exposed yard (where the exposed group was exposed to the migratory group). Dotted arrows show movement of colonies throughout the experiment. Between sampling events one and two, the migratory colonies were transported to California for almond pollination and back. Exposed colonies began in the stationary yard and were transferred to the exposed yard at the start of Experiment 2. Geographic distance between yards are specified in kilometers.

Figure 2. Pathogen community and colony health predicts treatment group membership. Linear combinations from discriminant analyses created from all pathogen variables (except BQCV prevalence) and frames of bees for exposed (black), migratory (red) and stationary/isolated (blue) colonies. Axes represent the percentage of between group variance explained. (A) Experiment 1 at sampling event two, migratory and stationary colonies were separated by LD1 while stationary and exposed colonies are clustered. B) Experiment 2 at sampling event three, after the exposed group had been allowed to forage alongside the migratory colonies, exposed and isolated were separated along LD2, while LD1 separated out migratory colonies. The significant PERMANOVA tests for both experiments corroborated the differences between group centroids.

Table 1. Summary statistics for Experiment 1 (A) and 2 (B).

A) Experiment 1, Migratory verses Stationary. B) Experiment 2, Isolated verses Exposed. DWV load, deformed wing virus load; DWV prev., deformed wing virus prevalence; BQCV load, black queen cell virus load; *Varroa* prev., *Varroa* prevalence; *Nosema* prev., *Nosema* prevalence; FOB, frames of bees. Prevalence is the percentage of colonies positive for a pathogen (DWV, *Nosema,* and *Varroa*). Virus load (DWV and BQCV) results for each colony are presented in average virus genome copies/bee. *Nosema* load is reported as average number of spores/bee and *Varroa* is reported as number of mites per 100 bees.

Figure 3. Pathogen and colony population metrics for treatment groups through time. Migratory (solid line) and stationary/isolated (dotted line) colonies were sampled at three time points and exposed (gray) colonies were sampled at two time points. Sampling event (1) occurred before migratory colonies were transported, (2) upon their return, and (3) one month after return. Panels show results for three pathogens and one health metric: (A) black queen cell virus (BQCV) in log genome copies per bee (B) deformed wing virus (DWV) in log genome copies per bee (C) *Varroa* load in mites per 100 bees and (D) Frames of bees (FOB), as a proxy for colony population. In Experiment 1: migratory verses stationary/isolated colonies, there was a significant effect of time for all measures. For BQCV, there was a significant effect of treatment. There was a significant time × treatment interaction for FOB and *Varroa.* In Experiment 2: exposed colonies verses

stationary/isolated, there was a significant effect of time for each measure. For DWV, there was a significant time × treatment interaction. Bars represent standard errors.

Fig. S1. **Pathogen and colony population metrics for treatment groups through time.** Migratory (solid line) and Stationary/Isolated (dotted line) colonies were sampled at three time points and Exposed (gray) colonies were sampled at two time points. Sampling event (1) occurred before migratory colonies were transported, (2) upon their return, and (3) one month after return. Panels show results for five pathogens and one health metric: (A) black queen cell virus (BQCV) prevalence (B) deformed wing virus (DWV) prevalence (C) *Varroa* prevalence (D) *Nosema* prevalence (E) *Nosema* load (spores per bee) times 100,000.

Tables and Figures

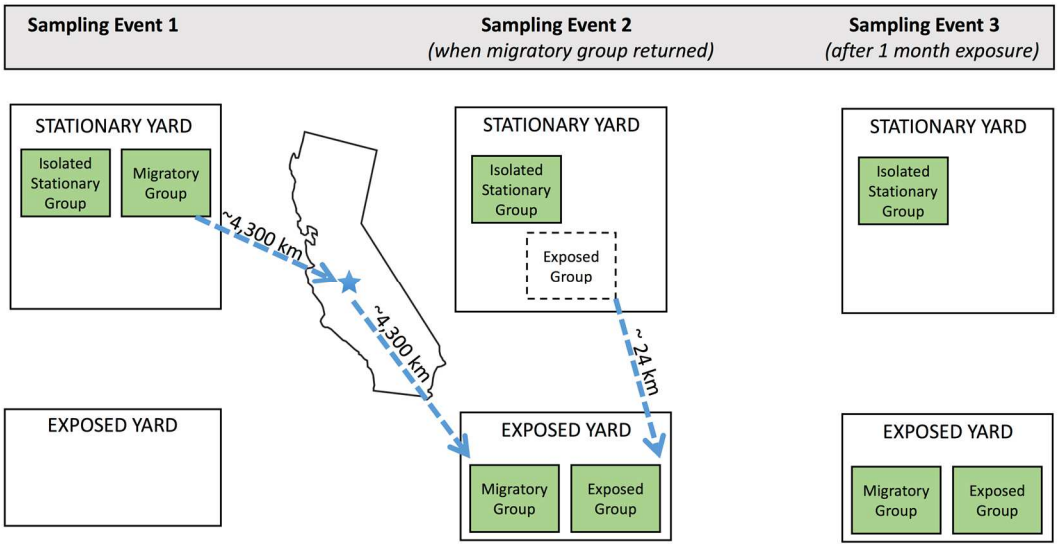

Figure 1. Schematic of Experimental Design.

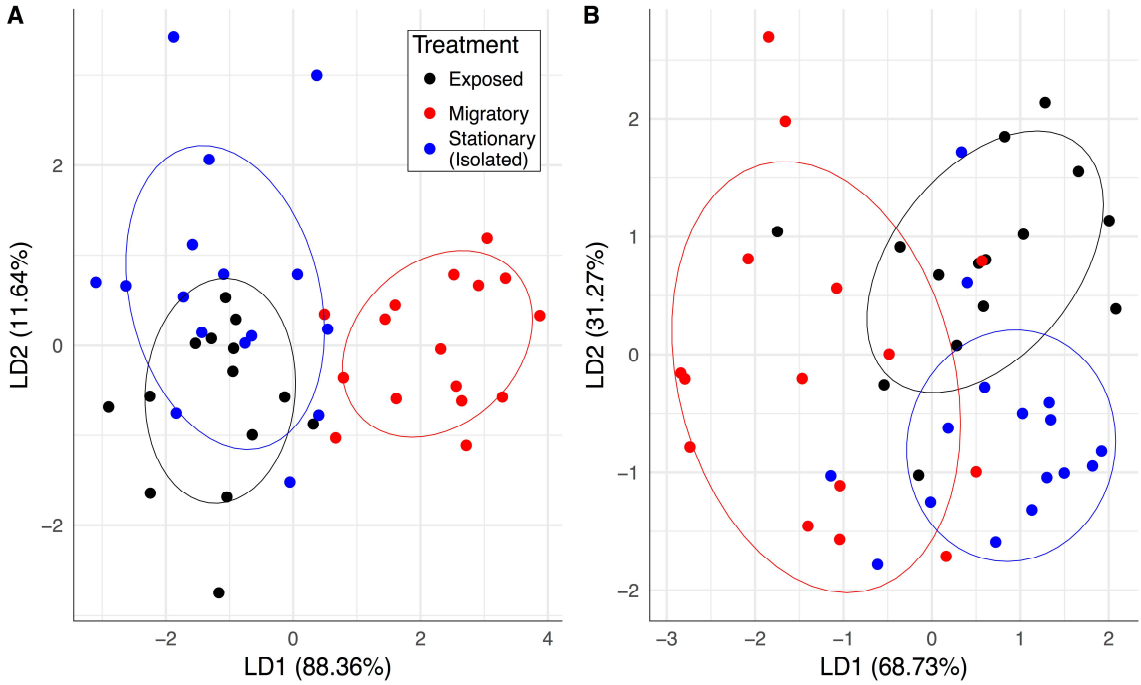

Figure 2. Pathogen community and colony health predicts treatment group membership

Table 1. Summary statistics for Experiment 1 (A) and 2 (B).

Variable	Effect	χ_1^2	P[a]	Sig[b]
DWV load	Treatment	0.004	0.9512	
	Time	39.328	<0.001	***
	Treatment:Time	0.1592	0.690	
DWV prev.	Treatment	0.067	0.796	
	Time	15.805	<0.001	***
	Treatment:Time	0.024	0.878	
BQCV load	Treatment	16.488	<0.001	***
	Time	187.235	<0.001	***
	Treatment:Time	2.229	0.135	
***Varroa* load**	Treatment	0.413	0.520	
	Time	18.391	<0.001	***
	Treatment:Time	6.465	0.011	*
***Varroa* prev.**	Treatment	1.290	0.256	
	Time	4.896	0.0270	*
	Treatment:Time	3.21	0.073	
***Nosema* load**	Treatment	0.645	0.422	
	Time	30.855	<0.001	***
	Treatment:Time	0.280	0.596	
***Nosema* prev.**	Treatment	0.007	0.931	
	Time	3.652	0.056	
	Treatment:Time	3.352	0.067	
FOB	Treatment	3.597	0.058	
	Time	152.838	<0.001	***
	Treatment:Time	5.651	0.0174	*

[a] Significance for all models was determined using Type II Wald Chi-Square tests.
[a]Asterisks represent level of significance.

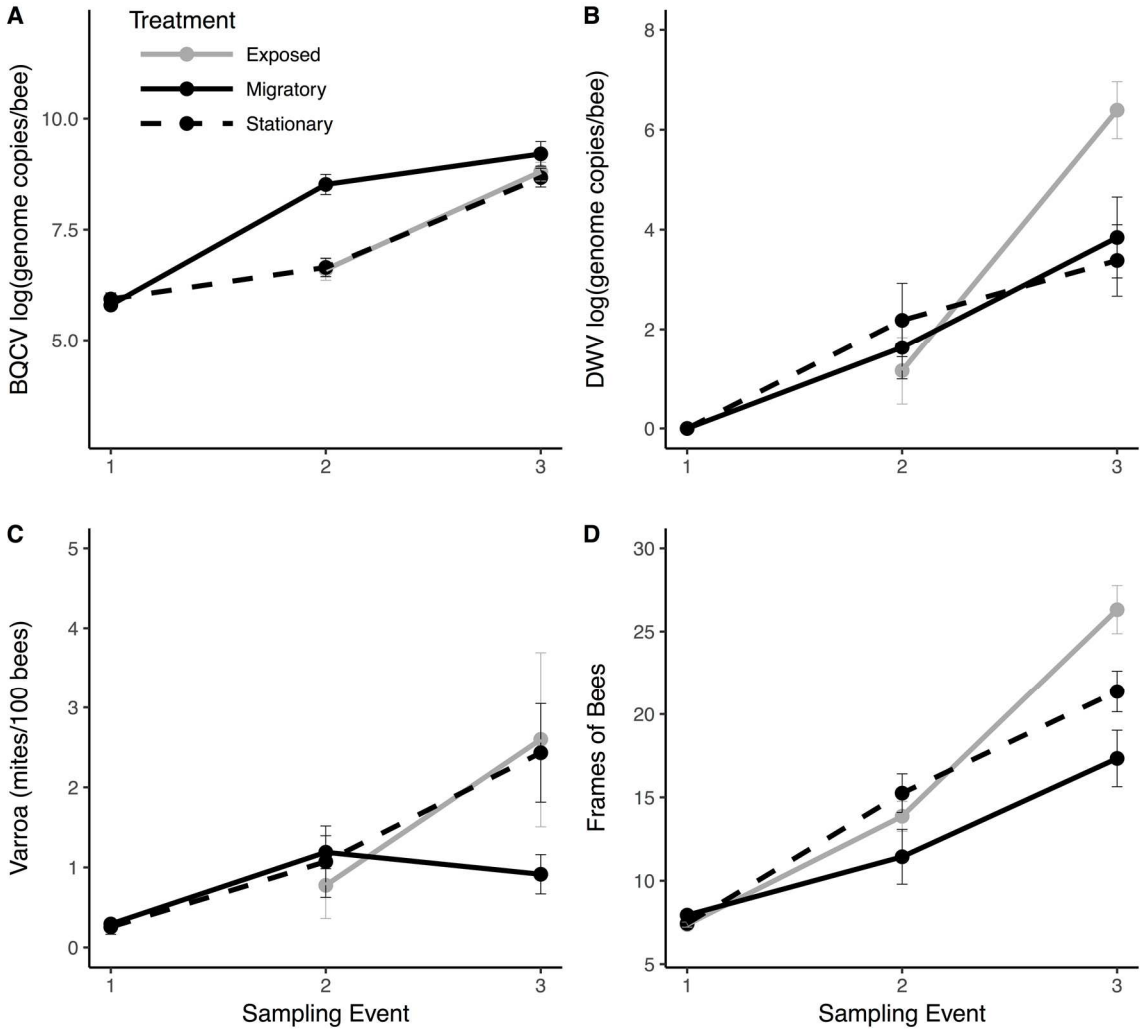

Figure 3. Pathogen and colony population metrics for treatment groups through time.

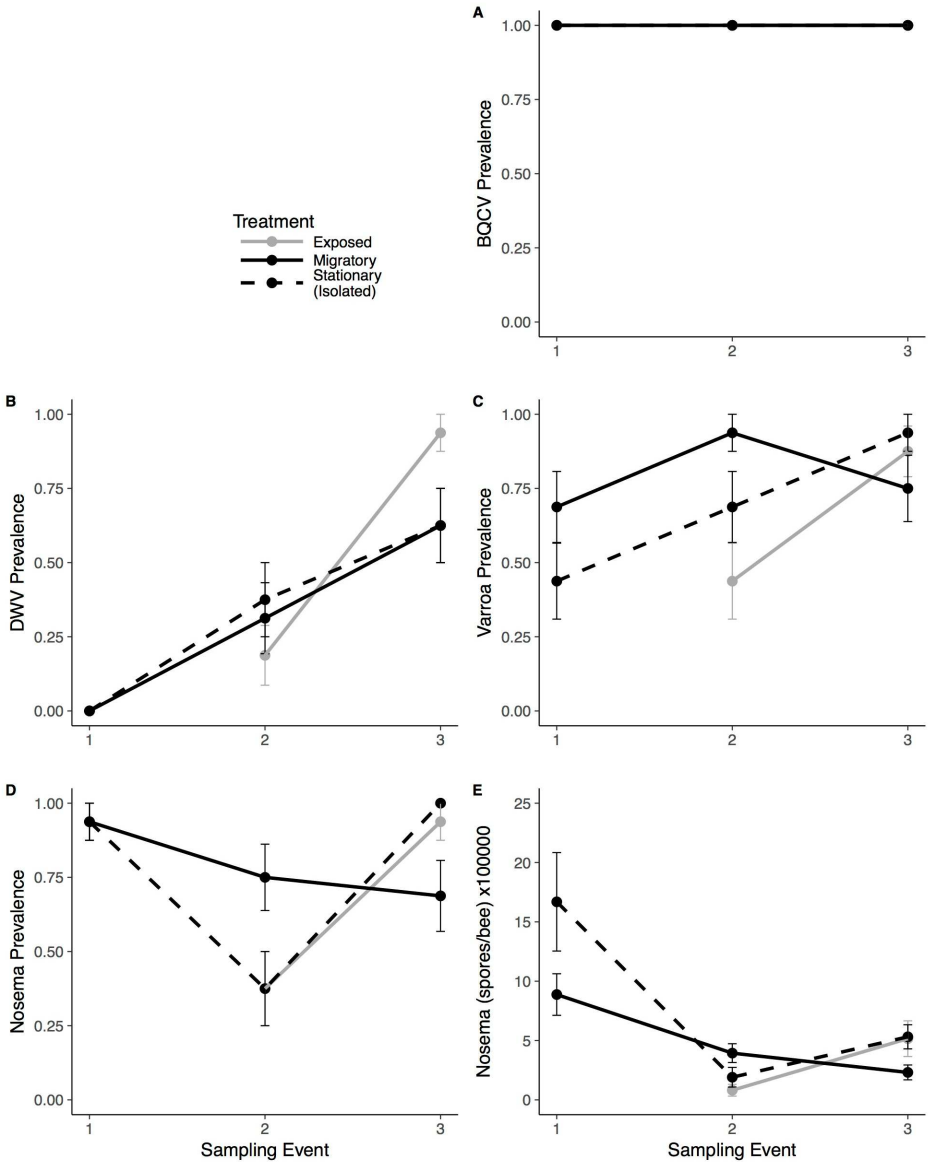

Fig. S1. Pathogen and colony population metrics for treatment groups through time.

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CHAPTER 6: *INSPECTAPP* AND *BEEKAPP*, OPEN-SOURCE TOOLS FOR APIARY INSPECTORS: A CASE STUDY IN VERMONT

Abstract

State apiary inspection programs, while critical to controlling bee disease and maintaining bee health, are often limited by resource constraints. Improving state programs through low cost, low time-commitment methods are a priority for ameliorating bee health. Here, through a public information request, we examined the limitations of state inspection programs and identified common themes. Next, we developed a toolkit built extensively on open-source software aimed at overcoming common limitations of inspection programs. The toolkit consists of data collection tools, as well as two applications, *InspectApp,* an application that allows inspectors to prioritize inspections and *BeekApp*, an online resource that allows users to visualize and explore state apiary data. Using Vermont as a case study, we successfully deployed the toolkit and, using data collected, we made several recommendations to improve bee health in Vermont. Given our success in Vermont, we encourage other apiary inspection programs to adopt our toolkit and offer the opportunity for collaboration. The toolkit allows apiary inspectors to make informed decisions to improve bee health, provides a platform for beekeeper education, and helps to identify priority issues for future bee research.

Introduction.

Honey bees (*Apis mellifera*) are the world's most important managed pollinator and contribute over $170 million annually in global crop pollination services (Garibaldi et al., 2013). Worldwide reports of colony mortality have led to heightened concerns over future crop production and food security. In the US, colony losses have averaged ~33%

since 2006 (Lee et al., 2015a; Traynor et al., 2016b). Despite over two decades of research, the drivers of honey bee losses remain topics of active research. Honey bees are subjected to a number of interacting stressors including pesticide exposure, forage loss, as well as numerous pests and diseases (Evans & Schwarz, 2011; Goulson et al., 2015). Although often overlooked, beekeeper knowledge and management practices also play critical roles in bee health (Jacques et al., 2017). Surveillance and monitoring systems aimed at understanding these stressors and the beekeeper practices that influence them are central to identifying risk factors associated with colony loss and bee health (Lee et al., 2015b).

State apicultural inspection programs serve as local surveillance systems and are the first line of defense for protecting bee health in the US. Their chief aims are to reduce disease spread and improve beekeeping practices through beekeeper education. Through careful monitoring and data collection, state apicultural programs, if properly supported, can serve a critical role in beekeeper education, identifying risk factors at the local scale, and could drive research efforts. State apiary inspection programs are typically managed by each state's Department of Agriculture and vary in robustness (Ellis, 2016). While some state programs are well supported with multiple personnel dedicated to the program, many are under-developed and suffer from a lack of resources and funding. Thus, in order to improve apiary inspection programs, especially in states with resource constraints, the focus should be on the improvement of efficiency through low cost and low time-commitment methods.

We developed an open source toolkit intended for use by apiary inspectors and the beekeepers they service for the collection, utilization, and visualization of state-level

apiary data. To inform the development of our toolkit, we first investigated the current needs and limitations of apiary inspection programs by conducting a nation-wide public information request. In this report, we present the motivation for the development of the toolkit and introduce its components, capabilities, and applications. Using Vermont as a case study, we deployed a toolkit consisting of a census to collect data from beekeepers and two online *Shiny* apps: *InspectApp*, an application to help apiary inspectors prioritize inspections, and *BeekApp*, a public-facing, online resource for the visualization of state apiary data. We highlight the principal findings of Vermont's census and show how data collected through our toolkit can provide promising avenues for future research and inform management recommendations. We believe the toolkits' framework can serve as a template for building low-cost data collection, assessment, and visualization systems for resource-constrained state apiary inspection programs throughout the country. Thus, our secondary purpose of this paper is to provide an open invitation for collaborators. We expect the toolkit to be broadly applicable to other states and also welcome users to adapt and revise the tools as necessary to fit their state's needs.

MOTIVATION

The impetus for this work derived from numerous discussions with state apiary inspectors and officials over the course of several years. Through personal communication, we learned that many states either lacked apiary inspection programs, or were severely resource-constrained. For the relatively few states that had robust apicultural programs, they were typically the result of collaborative efforts with land-grant universities (e.g. Texas, Florida, and Michigan). To formally investigate the needs of state apiary inspection programs, we conducted a public information request by

telephone. We attempted to reach all 50 state's apiary inspection officials and asked a series of questions related to their program to derive standardized data (Table 1). Of the 25 states that responded, we found that 20% lacked an apiary inspection and registration program. For states with programs, 45% had voluntary apiary registration programs and 55% enacted a type of 'mandatory' registration program whereby all or at least certain types of beekeepers (commercial, migratory, etc.) were required to register their apiaries. However, most states recognized that enforcing registrations was difficult and assumed there were more beekeepers than reflected by the apiary registration data. In particular, hobbyist beekeepers were a main concern, as many are unregistered and are more likely to lack education in sound beekeeping practices. Only two states had full-time designated bee inspectors. The majority of states (87.5%) employed inspectors whose duties involved other activities. For these states, the average estimated percentage of time spent on bee-related activities during 'bee season' ranged from 10% - 75% with an average of 57.8%. Funding was identified as a major constraint for states. Only 27.8% of states classified their programs as adequately funded, while 72.2% of states classified their apiary inspection programs as either 'underfunded' or 'severely underfunded'.

In summary, through our public information request, we identified a number of primary challenges shared by state apiary inspection programs (Figure 1) and developed a list of action items addressed by our toolkit:

1. Provide information and education to the large numbers of hobbyist beekeepers;
2. Aid state-level programs in adequately surveying and inspecting apiaries across their states;

3. Enable states to identify and control disease outbreaks when they occur; and
4. Allow programs to face these challenges despite funding constraints.

Our initial public information request results underline the need for a low-cost, low-time commitment approach for apiary inspectors to prioritize apiary inspections, identify opportunities for beekeeper education, investigate state-level patterns, and develop management plans based on the data collected.

OTHER (NATIONAL) MONITORING SYSTEMS

There are several national strategies in place to monitor beekeeper management practices and honey bee health. Since 1986, the United States Department of Agriculture's (USDA) National Agricultural Statistics Service (NASS) has conducted the Bee and Honey Inquiry survey to collect basic statistics on colony numbers and economics (USDA National Agricultural Statistics Service, 2017b,a, 2018). In 2015, NASS began the Colony Loss Survey as a result of the White House Pollinator Task Force's "National Strategy to Promote the Health of Honey Bees and Other Pollinators" (Pollinator Health Task Force, 2015). Bee Informed Partnership, a non-profit housed at the University of Maryland, has collected data on colony losses since 2006 and in 2008 implemented an annual questionnaire on beekeeper management practices (Kulhanek et al., 2017; Bee Informed Partnership, 2018). While national efforts are instrumental in generating state, regional, and national statistics, beekeeper participation is low for some states, and the state level reports do not always provide the necessary resolution for state managers to identify issues of concern within each state. For example, national surveys do not collect or report county-level data or bee vendor purchases, both of which are important for prioritizing site visits to apiaries. Therefore, data collected by state-run

apicultural inspection programs should compliment national surveys to better serve inspectors and managers who work directly with their local beekeepers.

Toolkit Workflow and Components

Built with freely available open-source software, the toolkit is comprised of two major components 1) data collection tools (two online census forms in Google Forms) and 2) data processing and visualization tools (two separate *Shiny* applications) (Figure 2). Written in R, *Shiny* is a simple web application that enables interactive visualizations of data. All code is freely available for download and modification via GitHub.

When deploying the toolkit, data are first collected from beekeepers using two online censuses available as templates on Google Forms. One census collects apiary-level data such as location(s), colony numbers, and losses. The second census collects beekeeper-level data, such as mite treatments used, purchases from bee vendors, suspected reasons for colony loss, and out-of-state activity conducted by migratory operations that transport colonies for agricultural pollination services. Once data are collected, the resultant data sets can be easily downloaded from 'Google Sheets' and uploaded to an R Studio *Shiny* Application that merges the datasets, performs basic data cleaning operations, and exports the data to two separate online *Shiny* user interfaces, *InspectApp* and *BeekApp*. Designed for state apiary inspectors, *InspectApp* allows authorized users to visualize, query, and examine their state's apiary and beekeeper data. In a separate interface designed for public use, *BeekApp* allows users to explore state and county-level data through dynamic, interactive maps and figures.

INSPECTAPP

InspectApp enables access to technology that improves efficiency of routine tasks for apiary inspectors. It offers a simple, easy-to-use interface that may be used for a variety of purposes. *InspectApp* consists of an interactive map, a summary table that displays queried data, and a full table of apiary data that can be queried, sorted, and exported as needed. Here we describe three common scenarios apiary inspectors routinely face and how *InsectApp* could be used in each instance to improve efficiency.

PRIORITIZING INSPECTIONS WITH DISEASE INCIDENCE.

Apiary Inspectors are often limited in the number of inspections they can perform. Thus, it is essential that they have fast and reliable means for prioritizing site visits. For example, in the case of a disease outbreak, inspectors should focus their efforts on apiaries in close geographic proximity to the outbreak. One disease of particular concern is American foulbrood, a highly virulent spore-forming bacterial disease that can be spread by drifting or robbing foragers when they enter a hive other than their own (Ratnieks, 1992). In the case of a foulbrood (or other) outbreak, an inspector would enter the address or latitude and longitude of an infected apiary using the interactive map. Using the 'Distance (Miles)' slide bar, the inspector can identify and select all apiaries within a specified distance from an outbreak (Figure 3), creating a subset of apiaries to prioritize for inspections. The map will display the selected data as points on the map that may be clicked to view information about a particular apiary. A table below the map is automatically populated to display a summary for all queried apiaries. The 'Table' tab contains the selected records of the database with beekeeper contact information and other ancillary data contained in the census data and can be sorted by any attribute,

queried by typing in the search bar, and downloaded as a comma-delimited text (*.csv*) file by clicking the 'download' button (Figure 4).

TRACKING VENDORS AND SALES.

By tracking bee purchases, state inspectors can identify bee supply hubs and prioritize inspections. For example, if a bee vendor is implicated as the source of disease, inspectors should focus on inspecting bee colonies belonging to customers of that particular vendor. Using the search bar in the 'Table' tab, a vendor name can be entered and the resultant table will provide a list of all of the customers that purchased suppliers/materials from that vendor. Inspectors may use the downloadable table as a 'call list' for scheduling upcoming inspections.

IDENTIFYING NEW APIARY LOCATIONS.

Some states mandate that new apiary locations cannot be built within a specified distance of an existing apiary. Therefore, apiary inspectors are routinely asked to verify and approve the building of new apiaries. Using the map, inspectors can enter the geographic coordinates or address of the proposed apiary, and using the "Distance (Miles)" sliding tool, the data can be queried to test for the presence of existing apiaries near the proposed location.

BEEKAPP.

BeekApp provides users with dynamic visualization of state and county-level data. Unlike *InspectApp* where only authorized users are permitted to view data, *BeekApp* is available online for all users to explore and learn about statewide patterns. By granting open access to these data, the goals of *BeekApp* are education and the encouragement of

sound beekeeping practices. End users may include beekeepers, state or county beekeeping clubs, researchers, and apiary inspectors.

BeekApp consists of two main sections, 'Maps' and 'Data'. Under the 'Maps' tab, users may explore interactive maps that display county-level data on topics such as apiary density, colony density, colony loss, as well as *Varroa* mite monitoring, a beekeeping practice considered critical for controlling the potentially devastating parasites (Lee et al., 2010; Honey Bee Health Coalition, 2017) (Figure 5). The 'Data' tab offers a number of interactive figures and graphs and includes sections on registration statistics, colony loss, and pest management (Figure 6).

Applications of *BeekApp* are numerous, with the ability to serve the various needs of multiple stakeholders within each state including beekeepers, researchers, inspectors, and the public. Inspectors can use *BeekApp* to identify geographic areas of concern for either education or inspection purposes if they see spatial patterns in the data such as high colony losses. County beekeeping clubs can view how their constituents compare to other counties and develop informed targeted education programs. Beekeepers may compare their practices and concerns with others within their county and state. Furthermore, by making these data widely accessible, the *BeekApp* provides value to beekeeping communities, helping to justify apiary registration fees. Lastly, *BeekApp* reports could drive future research efforts to examine whether abiotic, biotic, or cultural practices may influence differences in colony loss among counties.

Implementation: Vermont Apiary Inspection Program as a case study

Background

Vermont's Apiary Inspection program began in 1910 with the principal goals of controlling brood diseases such as foulbrood and providing education for beekeepers. All colony inspections were conducted by commercial beekeepers, contracted by the state on modest summer stipends. This tradition continued until 1980 when a full time bee inspector was hired by the Agency of Agriculture, marking the only time in Vermont's history to employ a full time inspector. Ten years later in 1990, the state job became a part-time position as a result of budget constraints (Bill Mares, personal communication). Today, Vermont's apiary inspector (State Apiarist and Food Safety Specialist) estimates spending 60% of the 'bee season' working with bees and is also responsible for state-wide food safety, conducting agricultural practice audits, and law enforcement for maple syrup, eggs, and produce. Apiary registrations were voluntary until 2014, when the state began mandating apiary registrations and a $10 fee for each apiary. The mandatory apiary registration program is a positive step for Vermont beekeepers; it has enabled a summer budget of $12,000 and the hiring of two part-time inspectors during the summer months (each for 1 day/week).

To date, Vermont is home to over 750 registered beekeepers, 1100 apiaries, and 8,500 colonies. There are just over a dozen commercial beekeepers with two participating in out-of-state, large-scale migratory beekeeping operations whereby bees are transported by truck to monocultures for large-scale pollination events. In addition to state apiary inspection operations, Vermont has participated in the National Honey Bee Survey since 2015, and contributed to this nation-wide effort to gather baseline data on pests and

disease. Data collected by the National Honey Bee Survey represent the only standardized data Vermont has on honey bee disease. Vermont is home to at least six local-level beekeeping clubs and one state-level beekeeping organization, the Vermont Beekeepers Association (VBA).

Vermont serves as an ideal candidate for testing our toolkit. The large number of beekeeping organizations and the state's mandatory registration requirement led to high levels of participation from beekeepers across the state. Additionally, Vermont has demonstrated a general interest in bee health and pollinator conservation that greatly aligns with our initiatives. In 2016, a report published by the legislature-appointed Pollinator Protection Committee recommended that the state improve education for beekeepers, take steps to reduce disease spread, and enhance public outreach about pollinator health (Vermont's Pollinator Protection Committee, 2017). By adopting our toolkit, Vermont has made significant strides in addressing these important recommendations.

Methods

Data Collection:

Maintaining an up-to-date database is critical for monitoring bee health and disease outbreaks, identifying suitable locations for new apiaries, and aiding research initiatives aimed at influencing honey bee health. When apiary registrations were mandated in 2014, an opportunity was presented to collect additional data from beekeepers. The Department of Agriculture, Food, and Markets developed and distributed a beekeeper census by mail along with an updated apiary registration form (Supplemental Data 1) in early spring 2017. The census asked about the previous year of

beekeeping (spring 2016- spring 2017). We developed a user-friendly Microsoft Access Database to facilitate data entry and recruited 31 undergraduate students from the University of Vermont's beekeeping club to assist with this task. We distributed census forms by mail, which achieved high levels of participation. In future years, Vermont plans to utilize Google Forms for census data collection in order to reduce data entry time. However, this practice should be evaluated to see if there are trade-offs in beekeeper participation.

DATA ANALYSIS AND VISUALIZATION:

Using our R-based toolbox, data were compiled, cleaned and uploaded to *InspectApp* and *BeekApp*. *BeekApp* was launched and made available for viewing at https://apiarydata.shinyapps.io/BeekApp/. To ensure the privacy of Vermont's beekeepers' personal information as well as apiary locations, *InspectApp* is only available to the Vermont Apiary Inspector.

All data analyses and generation of figures were conducted in R v 3.3.1. To manipulate and analyze geospatial data, we used the packages geosphere, rgdal and rgeos. In *InspectApp*, to develop a tool to query apiaries based on specified distance to a point, we created a function that populated a matrix with the Euclidian distances between each apiary and a specified point using latitude and longitude (sp package). We queried this matrix to retrieve all apiaries within a specified distance from the point. We created *InspectApp* map visualizations using leaflet, with a basemap from ESRI world imagery (sources: Esri, DigitalGlobe, GeoEye, i-cubed, USDA FSA, USGS, AEX, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community). To create reactive tables in *InspectApp* that display selected apiary data, we used functions 'as.datatable_widget'

and ‘datatable’ in the DT package. To create the visualization of figures in *BeekApp*, we used the packages ggplot2 and plotly and tables were generated using the expss package. For maps showing county-level data, we summarized and derived county-level averages for attributes using the ‘ddply’ function in dplyr package. Base maps for Vermont counties were retrieved from the US Census Bureau (Cartographic Boundary Shapefiles) and uploaded using rdal package. We created tables using the ‘datatable’ function in the DT package. To examine spatial clustering of colony losses and mite monitoring efforts, we conducted a Moran’s I test using an inverse Euclidian distance matrix generated by each apiary’s latitude and longitude (ape package). To investigate the factors affecting percent annual colony loss, we conducted a linear mixed model (function ‘lmer’) with a Gaussian distribution with beekeeper type (hobbyist, sideliner, and commercial), mite monitoring (yes/no), miticide use (yes/no), supplemental feed provided (yes/no), and climatological division (northeastern, western, southeastern) as fixed effects while controlling for beekeeper ID. Significance of the model was determined using Type II Wald Chi-Square tests (function ‘anova’, package car. We examined pairwise comparisons using Tukey contrasts (multcomp package, functions ‘glht’ and ‘mcp’).

Results

Vermont had a high level of participation in the beekeeper census. Of the 879 beekeepers who submitted a registration form, 72% (637) also submitted a beekeeper census form. Results of the census data are available online in the VT *BeekApp:* https://apiarydata.shinyapps.io/BeekApp/. Here, we summarize some of the important findings and provide several informed recommendations for future work in Vermont.

There were a total of 743 active registered beekeepers in Vermont and 1091 registered apiaries. We classified beekeepers into three groups based on the number of apiaries they operate: hobbyists (1 apiary), sideliners (2-5 apiaries), and commercial (5+ apiaries.) Hobbyist beekeepers operate the majority of apiaries in the state (56.7%) followed by sideliners (26.2%) and commercial beekeepers (17.2%). The highest densities of apiaries, beekeepers, and colonies were located in the Champlain valley in Chittenden County. The lowest densities were located in Essex and Orleans Counties.

Statewide annual colony loss for 2016-2017 was 38.6%. Colony losses were spatially clustered (Moran's I = 0.034, p = <0.001; Figure 7) with Vermont's climatological divisions (Figure 8) significantly affecting colony losses ($\chi_2^2 = 20.9115$, $p < 0.001$). Colony loss was greatest in the Northeastern division and lowest in the Western division ($p < 0.001$). Mitacide use also affected colony loss ($\chi_1^2 = 8.6137$, $p = 0.003$) with beekeepers who used miticides reporting fewer losses compared to beekeepers who did not use miticides. Colony loss was not affected by beekeeper type, whether beekeepers monitored mite levels, or provided supplemental feed.

Three common explanations given by beekeepers for colony loss included *Varroa*, starvation, and swarming, however most beekeepers reported 'other' reasons. Yet, only 36.1% of Vermont beekeepers reported monitoring *Varroa* mite populations in their colonies. Mite monitoring efforts were spatially clustered (Moran's I = 0.018, p 0.003) with mite-monitoring efforts higher in eastern counties compared to the western counties.

We asked beekeepers which treatments they use to manage pests and diseases in their colonies. 23% of beekeepers reported to using no treatments in their hives. 67.9% of

beekeepers reported to using a miticide treatment. Most beekeepers reported using a single type of miticide treatment (32.1%) followed by two types of treatments (10%), and three types of treatments (0.6%) Of the beekeepers who reported using miticides, 98.6% reported using organic miticides with formic acid (mite away quick strips) being the most commonly used (67.8%). Only 3.46% of beekeepers who used miticides reported using synthetic miticides. We also found that 9% of Vermont beekeepers reported using antibiotics prior to the feed directive prescription requirement, which now mandates a written prescription from a veterinarian.

We asked beekeepers: 'what is the biggest challenge you face as a beekeeper'? We received a wide range of answers with some interesting trends. Common answers included *Varroa* mites, weather and climatic trends, and a general lack of knowledge that may be ameliorated by a beekeeping mentor (Figure 9).

Discussion and Future Directions

We successfully developed, implemented, and assessed an open source toolkit for apiary inspection program to collect, analyze, and display apiary data. Our toolkit addresses the common limitations of state apiary inspection programs. Most state programs found it difficult to adequately inspect apiaries across their state and control disease outbreaks. For resource-constrained programs, the ability to accurately prioritize site visits is critical. *InspectApp* enables apiary inspectors to visualize and query data to locate geographic areas of concern and prioritize apiary inspections. Another common limitation for state programs is the ability to connect with and educate the large number of hobbyist beekeepers in their state. The *BeekApp* interface serves as a platform for apiary inspectors to educate beekeepers on issues specific to their state. *BeekApp* users

are able to interact, learn, and explore patterns related to honey bee health and management. Lastly, funding was identified as a major limitation for state programs. The toolkit is built on freely available, open-source software meaning that states can still access these tools despite funding constraints.

The success of any monitoring program is contingent on the cooperation of participants. By leveraging the state apiary inspection registration process in Vermont, we had a much higher level of participation compared to other national monitoring programs. In our Vermont case study, we had 637 Vermont respondents compared to about 60 who responded the same year for Bee Informed National Management Survey. These results provide strong evidence that apiary inspection programs are well positioned to undertake data collection and our toolkit can provide a low cost means of doing so.

In launching our toolkit for Vermont, we discovered several interesting trends that warrant future study. Colony loss data indicated differences among eastern and western parts of the state along the climatological divisions. In Vermont, varying elevations, terrain, and distance to Lake Champlain and the Atlantic Ocean causes local climate variability, dividing the state into three climatological divisions: the northeastern, western, and southeastern (Vose et al., 2014). Thus, it is likely climate played a role in colony losses. Further research should examine which climatic factors contributed to the higher losses in the Northeastern division. Beekeepers in those regions should be encouraged to use practices that might mediate detrimental conditions, such as wrapping hives during the winter in areas with greater temperature fluctuations. Miticide use was also an important factor with fewer colony losses among beekeepers who used miticides.

Thus, we suggest beekeeping clubs take collaborative effort in education campaigns focused on miticide use.

If medications are misused or overused, pests and pathogens may evolve resistance. For example, widespread use of coumaphos (Elzen & Westervelt, 2002) and fluvalinate (Elzen et al., 1999) has led to resistant *Varroa* mites and the need to develop other treatment options. Beekeepers are advised to rotate different miticide treatments to reduce the risk of mites building resistance to a single miticide (Pettis, 2004). We noted a high proportion of beekeepers in Vermont using miticides use formic acid treatments only (79.1%) and therefore extend a word of caution to reduce the likelihood of the evolution of resistant mites.

At the global scale, the rapid emergence of antibiotic resistant bacteria has become an important problem. Attributed to the misuse and overuse of medications, the prophylactic use of antibiotics in livestock feed was identified as a major contributor. In 2015, the FDA began restricting antibiotics use in livestock by requiring a written prescription from a veterinarian (FDA, 2015). Beekeepers use antibiotics to treat and suppress bacterial infections such as European or the more catastrophic disease, American foulbrood (Ratnieks, 1992). Since honey bees are considered agricultural livestock, beekeepers are now also required to obtain a prescription for antibiotics. This has presented challenges. For example, veterinarians must be trained in beekeeping practices and honey bee diseases. We found a relatively high proportion of beekeepers used antibiotic treatments prior to the Veterinarian feed directive indicating a need for Vermont veterinarians who are trained in beekeeping and bee disease. To better equip Vermont veterinarians, we suggest the state apiary inspection program collaborate with

beekeeping organizations to hold a honey bee training session where veterinarians will be introduced to basic beekeeping practices, inspection protocols, and disease recognition.

Limitations and Future Directions

The toolkit is designed to collect a single year's worth of data. We recognize that more tools and functionality will be required in future years, particularly to assess changes over time. We plan to adapt as necessary and provide upgrades. We hope users will also modify the toolkit to fit their state needs and we welcome inquiries and requests for collaboration.

In Vermont, census forms were distributed by paper, which resulted in the arduous task of data entry. Although we achieved a high level of participation from beekeepers, it is unknown whether we would have the same success through an online census platform. Nevertheless, we believe high participation rates could still be possible if apiary inspection programs announced and endorsed the online census forms by letter or email to beekeepers.

States with voluntary apiary registration programs may have limited levels of participation from beekeepers compared to states with mandatory registration programs. This limitation may be addressed by collaborating with local beekeeping clubs to help increase participation. We also recognize that for some states, particularly those without apiary inspection programs, the toolkit may not be possible as is requires a group to lead the process. In these cases, state universities or private groups could collaborate to start collecting data from beekeepers. By beginning the process, states may be persuaded to take a vested interest in improving honey bee health.

CONCLUSIONS

Our toolkit was instrumental in bringing important and novel data to the state of Vermont. Through our public online platform, *BeekApp*, Vermonters can now view and interact with apiary and beekeeper data specific to their state. As a result of our toolkit and *BeekApp*, we have uncovered several promising avenues for future research. Most notably, the role of local climatological conditions and miticide use on colony losses should be examined. Additionally, *InspectApp* now allows the Vermont apiary inspector to prioritize inspections and perform routine tasks that previously were difficult due to a lack of available technology. Given the toolkit's success in Vermont, we encourage other apiary inspection programs to adopt our toolkit and offer the opportunity for collaboration.

Acknowledgements

We thank the volunteers from the University of Vermont Beekeeping Club for their assistance with data entry, the Vermont Beekeepers Association for their support and helpful suggestions, and the Vermont Department of Agriculture, Food and Markets for developing and administering beekeeper censuses and apiary registration forms. We also thank Bill Mares, coauthor of The History of Vermont Beekeeping, for providing his knowledge on the history of Vermont's apiary inspection program for this manuscript.

Figure and Table Captions

Table 1. Summary of Public Information Request of State Apiary Inspectors.

Figure 1. Word cloud showing common challenges faced by Apiary Inspectors. In our public information request, we asked about the biggest challenges related to their job as apiary inspectors. As depicted in the word cloud, common themes included education for hobbyist beekeepers, controlling pests and disease (such as *Varroa* and foulbrood), funding constraints, and adequately surveying the large number of apiaries in their states (not enough inspectors or enough time).

Figure 2. Schematic showing the flow of information through the toolkit to end users. End users are shown in ovals and tool kit processes are shown in squares. The process begins when beekeepers provide information through two online censuses in Google Forms (1). Data gathered from the censuses populate a Google Sheet which can be uploaded to an R Shiny App (2). This App merges the datasets, prepares it for visualization, and send the data into two user interfaces (*InspectApp* or *BeekApp*) (3) to be viewed by end users. The apiary inspector can use *InspectApp* for routine tasks and data manipulation (4). Beekeepers and other end users including beekeeping organizations, the public, researchers, and the apiary inspector can visualize and explore the dataset through *BeekApp* (5). Information gathered by the apiary inspector is used to educate and inform beekeepers (6).

Figure 3. *InspectApp* Map tab. Screenshot showing the map page of *InspectApp*. Authorized users (apiary inspectors) can use the map to prioritize inspections by querying data by distance or attribute. Yellow dots on the map represent registered apiaries. The search bar can be used to search GPS coordinates or address. The slider bar allows the user to select apiaries only within a specified linear distance from the queried location. The summary table provides a summary of all queried data.

Figure 4. *InspectApp* Table tab. Screenshot showing the table page of *InspectApp*. Once using the 'map' tool to query the data, all queried data records are displayed on the Table tab (with personal beekeeper information redacted for privacy reasons). Records can be sorted by individual attributes or queried using the search bar. The table can be downloaded and exported using the 'download' button at the bottom of the page (not pictured).

Figure 5. *BeekApp* Maps tab. Screenshots showing the Map options of *BeekApp.* Summary statistics are displayed on interactive maps, color coded by county (A). The tab feature allows users to display, sort, or query the datasets (B).

Figure 6. *BeekApp* Data tab. Screenshots showing two examples under 'Data' in *BeekApp.* Registration by beekeeper type shows the number of registered apiaries and colonies within the state by beekeeper type: hobbyist (1 apiary), sideliner (2-5 apiaries),

and commercial (5+ apiaries) (A). The mite monitoring pie chart shows the percentage of Vermont beekeepers who reported to conducting mite counts (B).

Figure 7. Percent annual colony loss for Vermont counties and Climatological divisions. Map shows percent annual colony loss by county in Vermont. The trend follows the pattern of the state's climatological division

Figure 8. Climatological Divisions of Vermont. Varying elevations, terrain, and distance to Lake Champlain and the Atlantic Ocean causes local climate variability, dividing Vermont into three climatological divisions: the northeastern, western, and southeastern. Northeastern division had the highest losses while the Western division had the lowest ($p < 0.001$).

Figure 9. Word cloud showing Vermont beekeeper's biggest challenges. Beekeepers were asked 'What were the biggest challenges you face as a beekeeper?'. This figure depicts the answers we received from Vermont beekeepers.

Supplemental Data 1. Census and Apiary Registration Form. Vermont's apiary inspection registration form and Census administered to Vermont beekeepers by mail in 2017 by the Vermont Department of Agriculture, Food, and Markets.

Figures and Tables

Table 1. Summary of Public Information Request of State Apiary Inspectors.

State	Registration Type¶	Funding Status	% Time during bee season	Other duties required?
Arizona	None, '94	N/A	0%	N/A
Arkansas	Mandatory	Adequately funded		Yes
Colorado	None	Severely underfunded		Yes
Connecticut	Mandatory	Adequately funded		Yes
Delaware	Mandatory	UNK		Yes
Florida	Mandatory	Underfunded	100%	No
Georgia	Mandatory§	Severely underfunded	75%	Yes
Idaho	Mandatory§	Underfunded		Yes
Kansas	None*	N/A		Yes
Louisiana	Mandatory	Adequately funded		Yes
Maryland	Mandatory	Severely underfunded		Yes
Massachusetts	Voluntary	Severely underfunded	100%	No
Michigan	None*, '93	N/A	20%	Yes
Minnesota	None*, '06	N/A		Yes
Mississippi	Voluntary	Adequately funded	15%	Yes
New Hampshire	Voluntary*	Severely underfunded		Yes
New Jersey	Mandatory	Severely underfunded		No
New Mexico	Mandatory§	Underfunded	10%	Yes
New York	Voluntary	UNK		Yes
North Carolina	Voluntary	Underfunded		Yes
Pennsylvania	Mandatory	Underfunded		Yes
Utah	Mandatory	Adequately funded	75%	Yes
Virginia	Voluntary	UNK	65%	Yes
Vermont	Mandatory	Underfunded	60%	Yes
Wisconsin	Voluntary	Underfunded		Yes

¶ If apiary registration was repealed, date is provided

* Inspections are conducted for exports only

§ Registrations mandatory for commercial businesses only

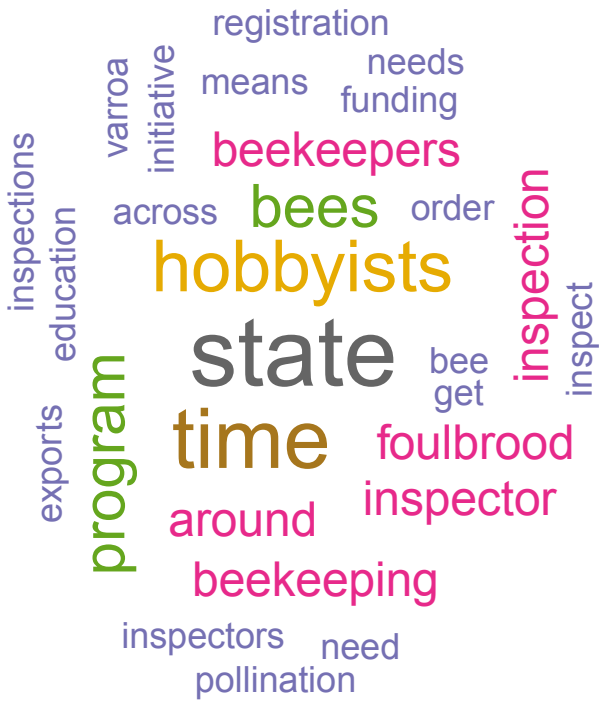


Figure 1. Word cloud showing common challenges faced by Apiary Inspectors.

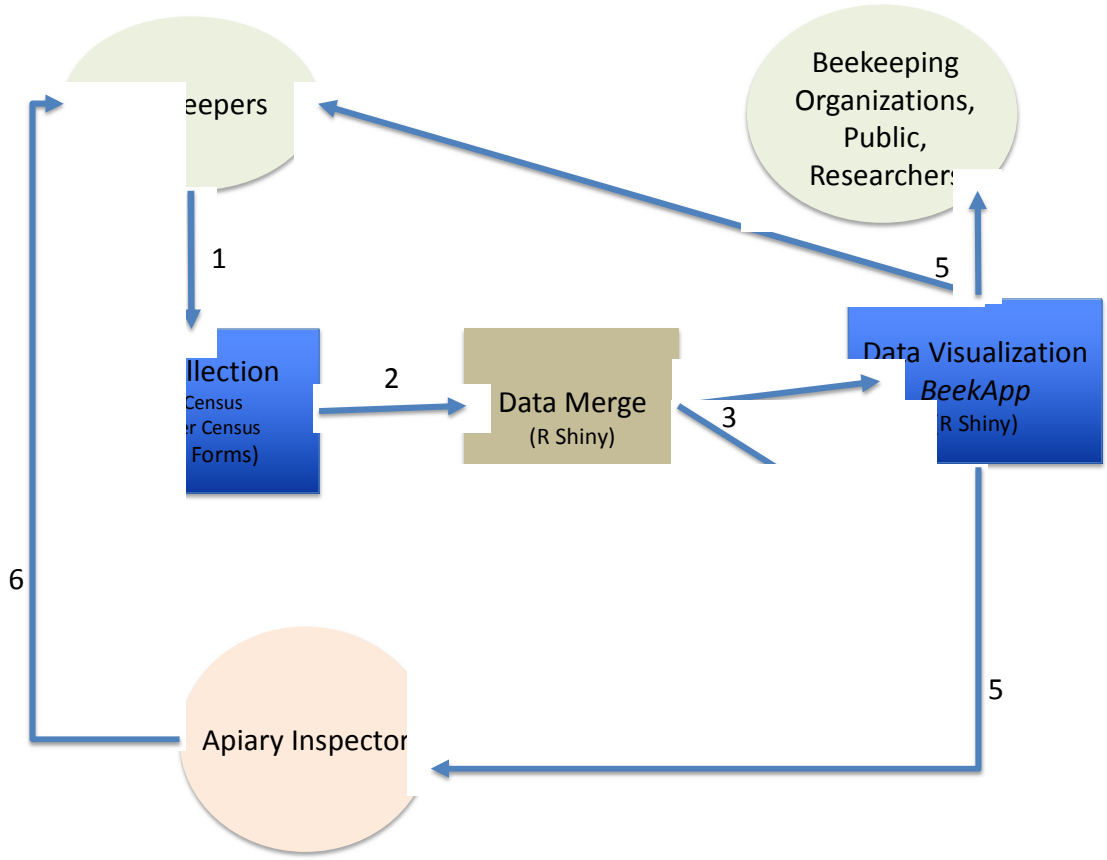
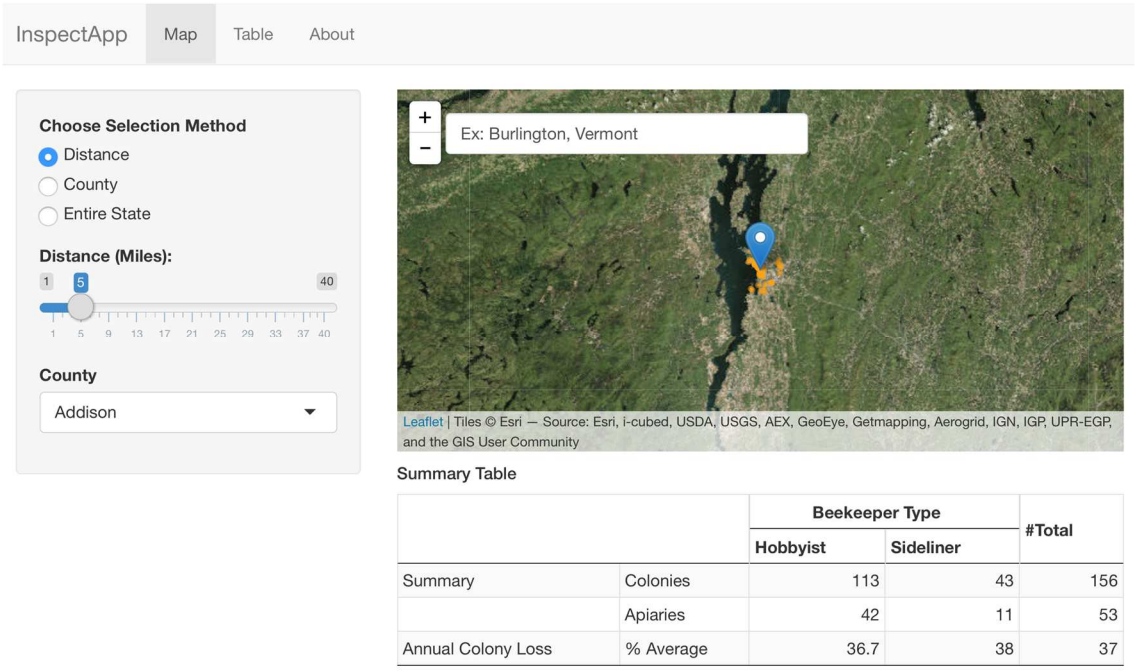


Figure 2. Schematic showing the flow of information through the toolkit to end users.



		Beekeeper Type		#Total
		Hobbyist	Sideliner	
Summary	Colonies	113	43	156
	Apiaries	42	11	53
Annual Colony Loss	% Average	36.7	38	37

Figure 3. ***InspectApp*** **Map tab**

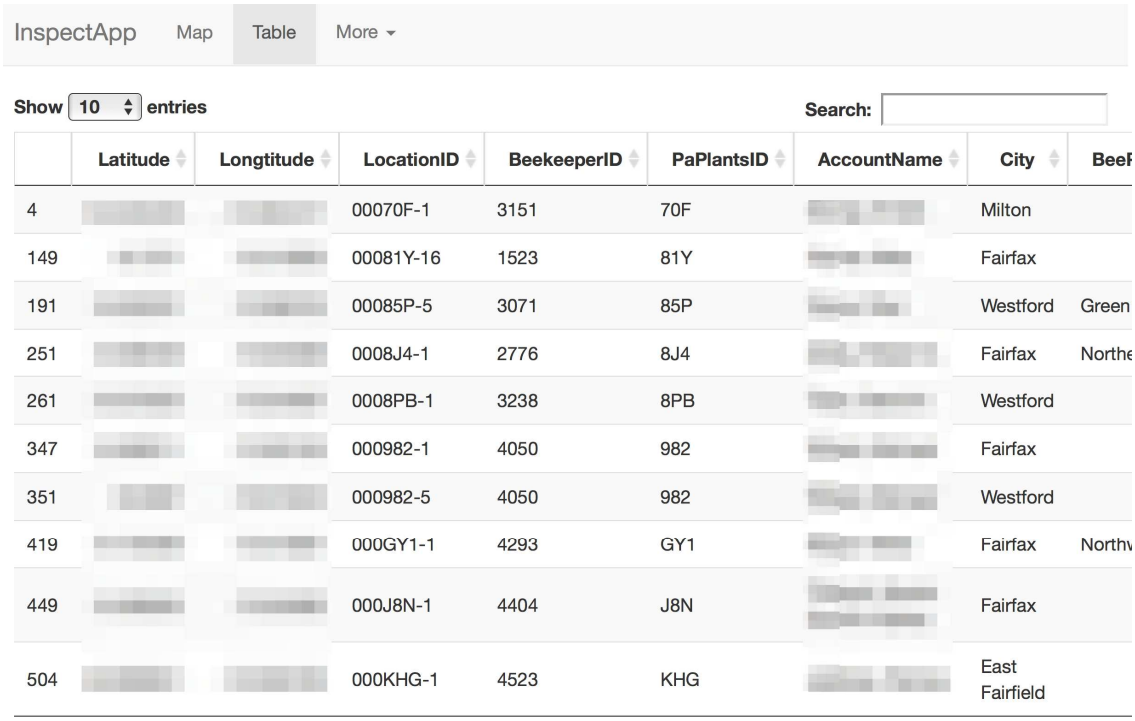
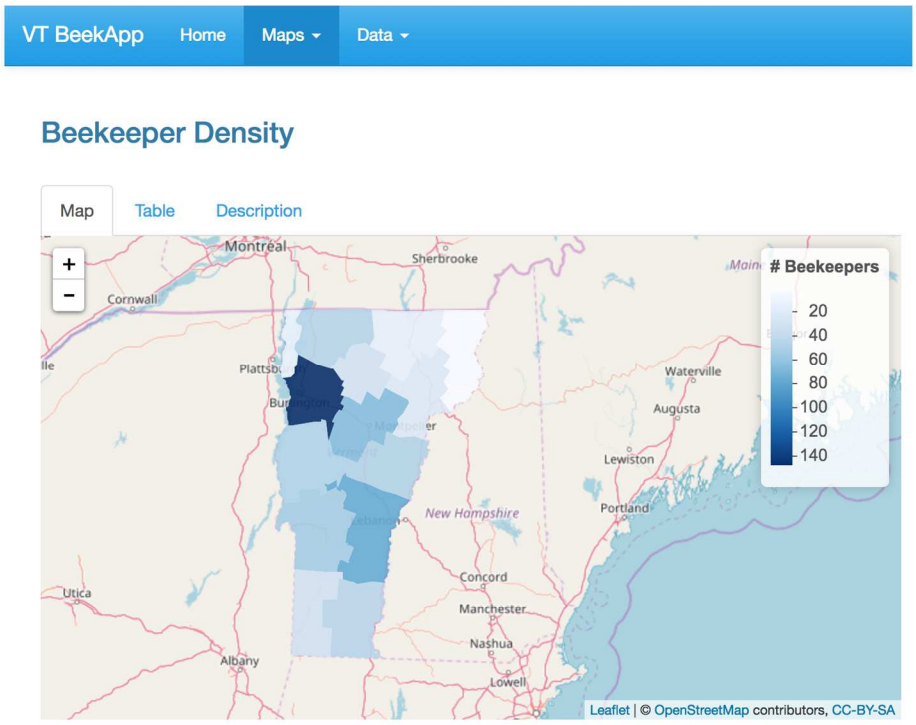


Figure 4. *InspectApp* Table tab

A.



B.

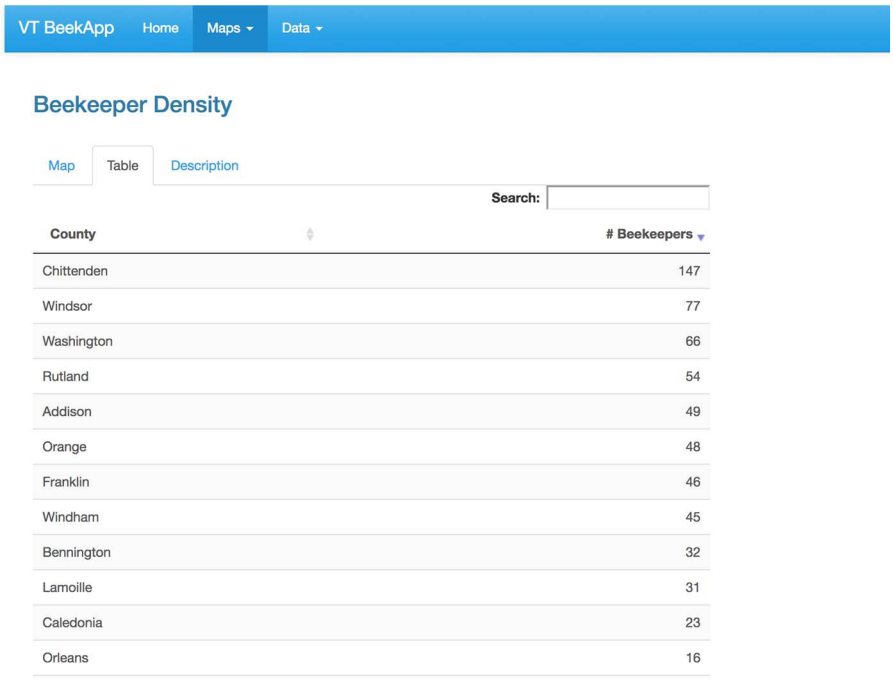
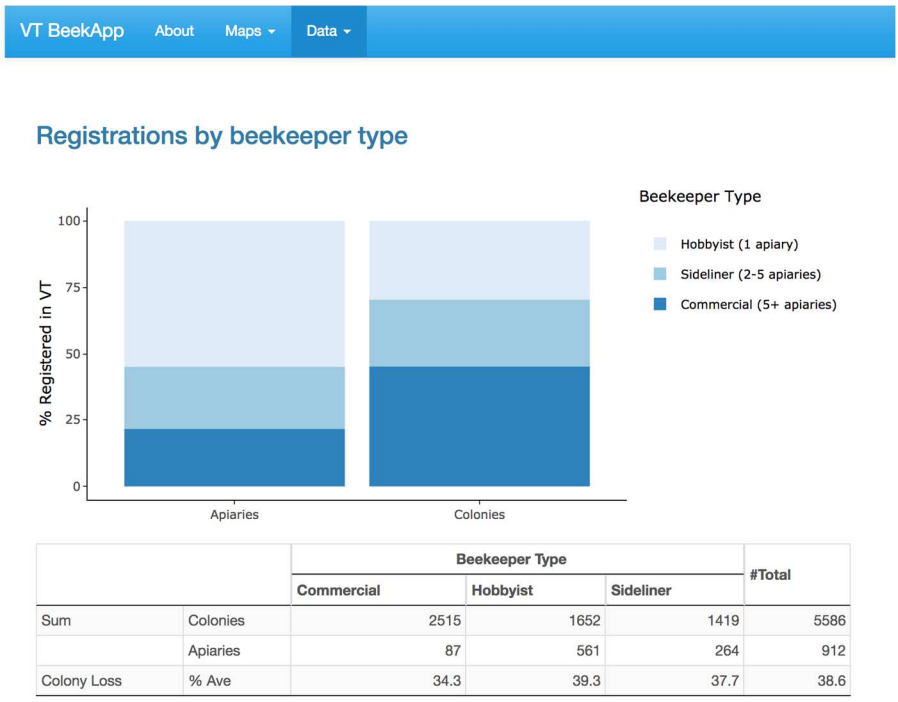


Figure 5. *BeekApp* Maps tab.

A.



		Beekeeper Type			#Total
		Commercial	Hobbyist	Sideliner	
Sum	Colonies	2515	1652	1419	5586
	Apiaries	87	561	264	912
Colony Loss	% Ave	34.3	39.3	37.7	38.6

B.

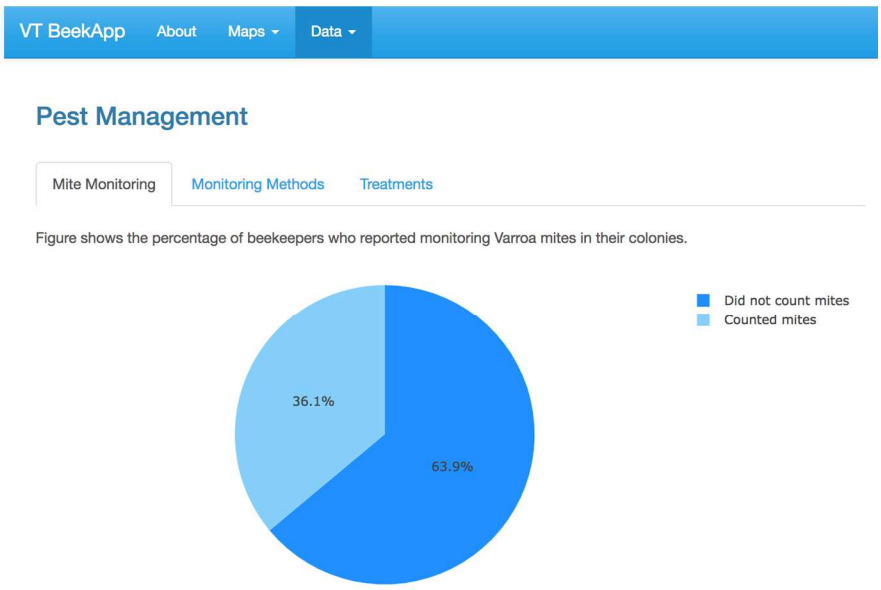


Figure 6. *BeekApp* Data tab

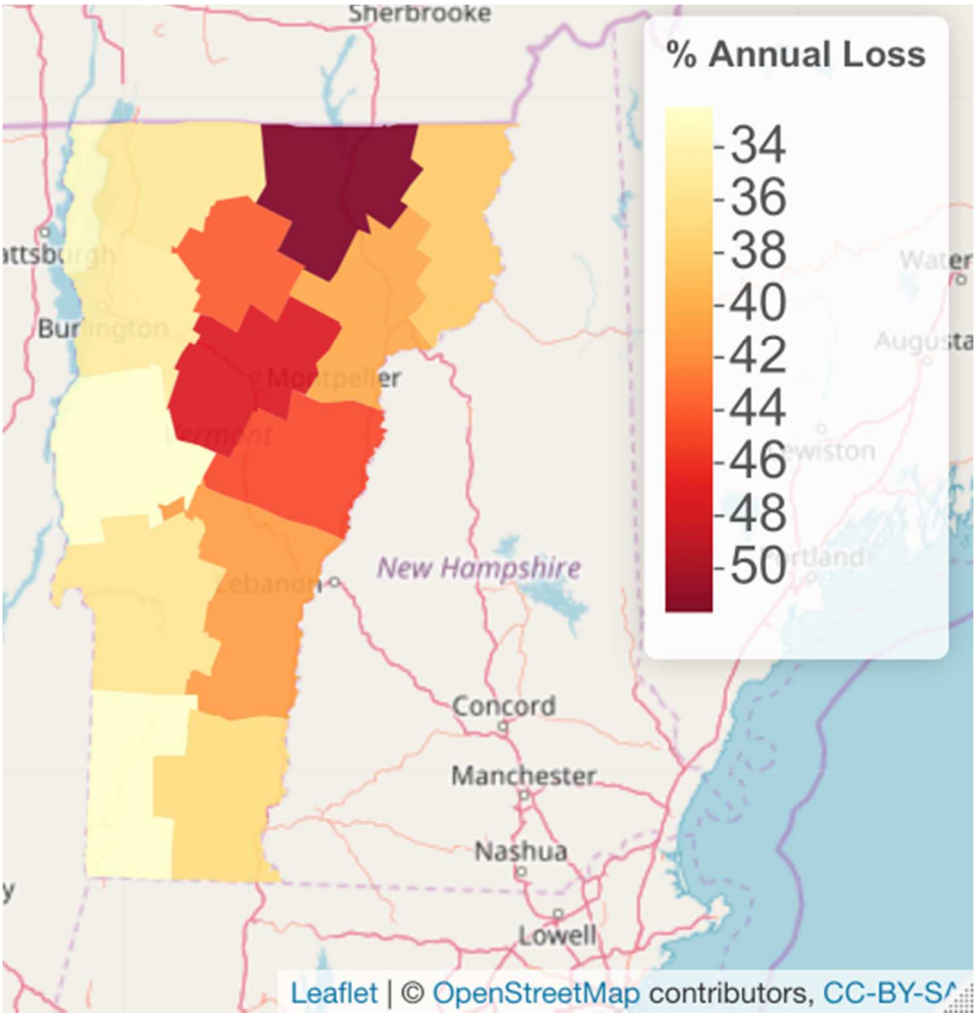


Figure 7. Percent annual colony loss for Vermont counties

Figure 8. Climatological Divisions of Vermont.
Image retrieved from:
http://www.cpc.ncep.noaa.gov/products/analysis_monitoring/regional_monitoring/CLIM_DIVS/states_counties_climate-divisions.shtml

Figure 9. Word cloud showing Vermont beekeeper's biggest challenge.

VERMONT
AGENCY OF AGRICULTURE, FOOD & MARKETS

APIARY REGISTRATION FORM

Applicant Name: _____ **Business Name:** _____

Mailing Address: _____ **Physical Address:** _____

City, State, Zip: _____ **City, State, Zip:** _____

Telephone: _____ **Email:** _____

[ ] I am a new beekeeper [ ] I no longer keep bees I am selling: [ ] queens [ ] nucleus colonies [ ] packages [ ] full colonies

Application for registration of apiaries is hereby filed with the Secretary of Agriculture, Food and Markets for the registration period of July 1 through June 30, inclusive. Registration fees, made payable to the Vermont Agency of Agriculture, Food and Markets are enclosed. In the table below, provide the following information for all apiary locations. If you need more space please copy this form for recording additional locations as needed.
Please also complete the additional enclosed census regarding colony management, disease management, and out-of-state activity.

Apiary # or Name	# of Colonies	County	Town	Land Owner	911 or GPS Address	# colonies lost during this past WINTER only	# colonies lost this past ENTIRE year (7/1-6/30)	Average mite load last Fall (if known)

****TURN OVER AND COMPLETE BOTH SIDES***

APIARY REGISTRATION FORM

VERMONT
AGENCY OF AGRICULTURE, FOOD & MARKETS

Read and initial following statement and sign application. The application will be returned if all areas are not initialed or signed.

Please Initial: _____ I hereby certify that I am in good standing with respect to any obligations for child support and, under the pains and penalties of perjury, that I am in good standing with respect to or in full compliance with, a plan approved by the Commissioner of Taxes to pay any and all taxes due to the state of Vermont as of the date of this application.

Total # Apiaries: __Total # Colonies: _____Date: _____Signature: _____

Please sign and remit payment of $10.00 per apiary location to:

Vermont Agency of Agriculture, Food & Markets
Business Office L&R
116 State Street
Montpelier, VT 05620-2901
(802) 828-2436
www.agriculture.vermont.gov

The State of Vermont is an Equal Opportunity / Affirmative Action Employer and Provider

******TURN OVER AND COMPLETE BOTH SIDES*****

VERMONT BEEKEEPER CENSUS

VERMONT
AGENCY OF AGRICULTURE, FOOD & MARKETS

This census is designed to help the Vermont Apiary Inspection Program better assist Vermont beekeepers. Your participation will help us to prioritize apiary inspections and guide research to improve bee health in Vermont. Please answer each question to the best of your abilities. If you have any questions, please contact state apiarist David Tremblay: (802)-793-2517 david.tremblay@vermont.gov

I. COLONY MANAGEMENT

1. Did you purchase bees within the last year? _____Y____ N

   name(s) of vendor(s):_____

   state(s) of origin: _____

   How many of each did you purchase within the last year?

   _____packages

   _____full colonies

   _____nucleus colonies

   _____queens

   _____other (specify)

2. How do you make up for colony losses? ***Check all that apply***:

   _____Purchase colonies in hives

   _____Purchase nucleus colonies

   _____Purchase packages

   _____Make splits or divides

   _____Other (specify)_____

******TURN OVER AND COMPLETE BOTH SIDES*****

VERMONT BEEKEEPER CENSUS

VERMONT
AGENCY OF AGRICULTURE, FOOD & MARKETS

3. How do you re-queen your colonies? ***Check all that apply***:

_____introduce virgin queens _____queen cells

_____introduce mated queens _____re-queen themselves

4. Did you provide supplemental feed within the past year? __Y __N

What kind(s)? ***Check all that apply:***

____pollen substitutes ____sugar syrup ___Other (specify below)

____pollen ____corn syrup _____

When? ***Check all that apply***:

_____Spring _____Summer _____Fall _____Winter

II. DISEASE MANAGEMENT

5. Did you perform mite counts over the past year? ___ Y___N

For each method you used to count mites, please write the ***number of times*** you performed mite counts over the past year:

Sugar shake _____times per hive

Alcohol wash _____times per hive

Bottom board _____times per hive

Drone survey _____times per hive

Other (specify below) _____times per hive

_____

******TURN OVER AND COMPLETE BOTH SIDES*****

VERMONT BEEKEEPER CENSUS

VERMONT
AGENCY OF AGRICULTURE, FOOD & MARKETS

6. Prior to the feed directive prescription requirement, did you use antibiotic treatments on your bees? ___Y ____ N

7. What treatments did you use within the past year for mitigating colony health problems? ***Check all that apply***:

____ NONE

_____ fluvalinate

_____ coumaphos

_____ amitraz

_____ Apiguard

_____ Api-life VAR

_____ Sucrocide

_____ powdered sugar

_____ oxalic acid

_____ formic acid (Mite Away Quick Strips)

_____ menthol

_____ Hopguard

_____ Honey B Healthy

_____ Fumagillin-B

_____ tylosin (Tylan, Tylosin, Tylovet)

_____Lincomycin (Lincomix)

_____ Oxytetracycline - (TM, OXTC, Pennox. Terramycin)

_____Herbal antibiotics

_____Other (specify)_____

******TURN OVER AND COMPLETE BOTH SIDES*****

VERMONT BEEKEEPER CENSUS

VERMONT
AGENCY OF AGRICULTURE, FOOD & MARKETS

8. Provide an ***estimated number of total colonies lost*** within the past year to the following causes:

_____*Varroa* mites
_____starvation
_____bears
_____American foulbrood disease
_____swarming
_____pesticides
_____mitacides
_____ other (specify)
_____

III. OUT-OF-STATE ACTIVITY

9. Within the past year, did any of your hives travel outside Vermont? ___ Y ___ N

If so, ***list all locations*** (cities and states) where your hives were located within the past year:

_____

Why were the hives brought out of state? ***Check all that apply***:

____Pollination, ***list crops***:_____

___Overwintering, ***give city and state***:_____

___ Other (specify):_____

IV. OTHER

10. In your opinion, what is the most important problem you face as a beekeeper?_____

_____

_____

_____

******TURN OVER AND COMPLETE BOTH SIDES*****

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Appendix A: Primers Used for Amplification of Virus and Actin Amplicons

Primer	5' to 3' Sequence	Product Size (bp)	Annealing Temp (°C)	Reference
DWV-F	TTCATTAAAGCCACCTGGAACATC	136	53	(Traynor et al., 2016b)
DWV-R	TTTCCTCATTAACTGTGTCGTTGA			
BQCV-F	TTTAGAGCGAATTCGGAAACA	140	51	(Traynor et al., 2016b)
BQCV-R	GGCGTACCGATAAAGATGGA			
IAPV-F	CCATGCCTGGCGATTCAC	203	47	(Traynor et al., 2016b)
IAPV-R	CTGAATAATACTGTGCGTATC			
Actin-F	CGTGCCGATAGTATTCTTGC	138	56	
Actin-R	CCATTGTCAACTACGAGTGC			

Appendix B: gBlocks Gene Fragments Sequence

gBlocks gene fragments were developed from Integrated DNA Technologies. Virus and actin amplicons are colored for visualization: Green = DWV, Blue = IAPV, Red = Actin, Yellow = IAPV. Ten random base pairs (uncolored) flank each target of interest.

GGACGGACAGTTCATTAAAGCCACCTGGAACATCAGGTAAGCGATGGTTGTT
TGACATTGAGCTACAAGACTCGGGATGTTATCTCTTGCGTGGAATGCGTCCCG
AACTTGAGATTCAATTATCAACGACACAGTTAATGAGGAAAACCATGTACGC
CATGCCTGGCGATTCACAACAAGAAAGCAATACTCCCAATGTACACAACACG
GAACTCGCTTCGTCAACTAGTGAAAACTCGGTTGAGACCCAAGAAATCACAA
CCTTTCATGATGTGGAAACTCCAAATAGGATCGATACCCCCATGGCTCAGGA
TACTTCATCGGCTAGGAACATGGATGATACGCACAGTATTATTCAGCTTCCCT
GCTCGTGCCGATAGTATTCTTGCGGTGTCTCTTTGCCGATCAACGATCGTGTA
CTTTGTTGGTTACCTTCGATTCTAAAAGATAACTCAATAAACCAAACATGTGT
GACGAAGAAGTTGCTGCACTCGTAGTTGACAATGGCGTCCACCTGTTTAGAG
CGAATTCGGAAACATTTTACTATAGTTCAGGTCGGAATAATCTCGATATAGCC
ACTTCACCTCCTTCCATCAATCGCTACTATGCGGTAGGTGCGGGAGATGATAT
GGACTTTTCCATCTTTATCGGTACGCC ATGAGCGCCA

Appendix C: Field Experiment To Examine Spillover

To experimentally test for pathogen spillover from honey bees to bumble bees, I conducted a field experiment using lab-reared bumble bee colonies placed either *near* (300 m) or *far* (1 km) from a known infected honey bee apiary for one month (mid July to mid August) and measured weight, worker number, and virus loads of the bumble bee colonies. I reared 18 colonies from wild caught *Bombus impatiens* queens. I placed queens in individual plastic containers and provisioned each with a pollen ball dipped in wax and 30% sucrose *ad libitum*. Queens were kept in a climate controlled room at 26 °C and 52-55%. Once established in July, I transferred the colonies to wooden boxes and randomly assigned each to a treatment: either near or far from a known infected honey bee apiary. During weekly nighttime checks, I measured the weights and counted workers of each colony. After one month, I brought the colonies back to the lab and preserved them at -80° C. Using RT-qPCR, I tested each queen and up to 10 individuals from each colony for DWV, BQCV, and IAPV.

No IAPV was detected among bumble bee colonies. Prevalence of DWV was 67.7% and BQCV was 100% prevalent among bumble bee colonies. DWV prevalence was not statistically different between near/far groups ($\chi_1^2 = 1.542$, P = 0.214), yet I observed a trend with higher prevalence in colonies in the near group (Fig. 1). Caste had a significant effect on DWV prevalence with workers having significantly higher DWV prevalence compared to queens ($\chi_1^2 = 5.378$, P = 0.020). For both DWV and BQCV, there was no significant difference in virus load between near/far treatment groups (DWV: $\chi_1^2 = 1.89$, P = 0.169; BQCV: $\chi_1^2 = 1.854$, P = 0.173) or caste (DWV: $\chi_1^2 = 0.209$, P = 0.648; BQCV: $\chi_1^2 = 0.977$, P = 0.323). Profile analyses yielded no statistically

significant differences in weight or worker number between treatments. However, trends showed bumble bee colonies near the apiary gained weight while colonies far from the apiary lost weight (Fig. 2).

Although I did not find a statistically significant difference in virus results among our near/far groups, the trends I observed suggest that increased replication is important for future studies. To increase the likelihood of detecting differences, future studies should use greater distance intervals between treatment groups. In this experiment, I observed honey bees foraging at the near and far location, indicating that bumble bees in both treatment groups had significant opportunities for exposure to honey bees. I found DWV prevalence was higher in workers compared to queens, indicating that virulence may differ among castes and/or that DWV is more likely to be contacted and transmitted outside the nest while foraging, rather than within the nest, where queens reside. The ubiquitous detection of BQCV among all castes and colonies indicates that this virus may be vertically transmitted by queens or highly virulent among nest mates. It was unclear whether wild-caught queens used to rear colonies were already infected with RNA viruses. Testing feces at the onset of the experiment could help to confirm this fact. In contrast to a previous study (Elbgami et al., 2014) in which bumble bee colonies placed near a honey bee apiary gained less weight than colonies placed 1 km away, I found no differences in colony weight or worker number. Variation of forage availability may explain differences in results. While the forage between my two treatment locations appeared homogeneous at the start of the experiment, forage quality may have differed between my treatment sites over time. Conducting more frequent flowering plant surveys could help explain the observed results.

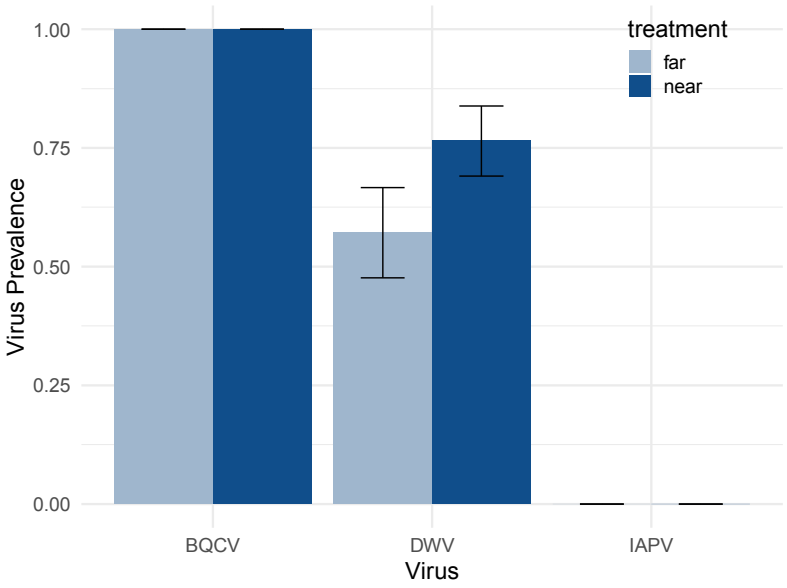

Figure 1. Virus prevalence among bumble bee colonies placed either near or far (1 km) from known infected honey bee apiary. There was no significant difference between treatment groups for virus prevalence, yet a trend showed higher virus prevalence among 'near' colonies compared to 'far'. Bars indicate confidence intervals.

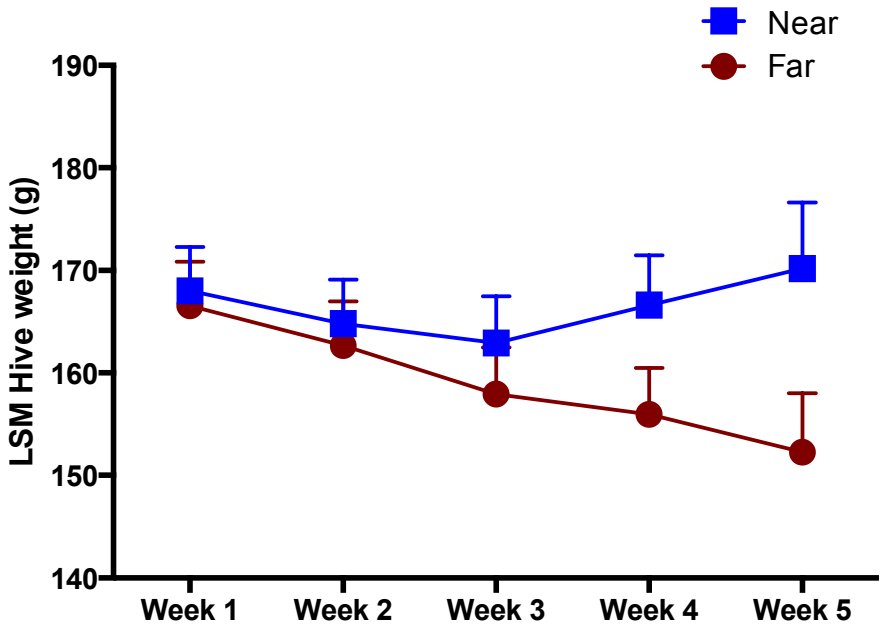

Figure 2. Lab reared bumble bee colonies were placed either near or far (1 km away) from honey bee apiary. Hive weight (g) is given in least square means (LSM). In a profile analysis, no significant differences were found between the two treatments. Bars indicate mean error.

Appendix D: Viruses in Gamma-Irradiated Pollen and Infectivity

Honey bee collected pollen is used as a feed for commercially reared bumble bee colonies and it may serve as a source for RNA viruses to bumble bees. Previous reports suggest that gamma irradiation will inactivate virus particles (Meeus et al., 2014). However, sensitive molecular techniques may still detect inactive virus particles on gamma-irradiated pollen ingested by bees, resulting in false positives. We conducted an experiment to test the infectivity of gamma-irradiated pollen and developed protocols for reducing false positives during laboratory experiments using captive bumble bees.

We received gamma-irradiated pollen from a commercial supplier. Upon arrival, we tested a 0.65 gram sample of this pollen for DWV, BQCV, and IAPV using qRT-PCR. The pollen sample was positive for DWV and BQCV but negative for IAPV. To test whether this pollen was infective, we created 14 bumble bee microcolonies from the 7 original commercial bumble bee (*Bombus impatiens*) colonies each consisting of 12 worker bees. The microcolonies were maintained in a growth chamber at 26 °C and 52-55% relative humidity and provided gamma-irradiated pollen and 30% sucrose *ad libitum*. After one week, we transferred the bumble bees from each microcolony into new clean containers and pollen starved each colony for 72 hours, providing only 30% sucrose *ad libitum*. After 72 hours, we tested each individual bee for DWV and BQCV. All samples were negative for both viruses indicating that the gamma-irradiated pollen was not infective to the bumble bees after one week of consumption.

Appendix E: Viruses Detected Throughout Honey Bee Anatomy

In previous experiments examining virus deposition from honey bees to flowers, I found that RNA viruses were not equally distributed across plant species, suggesting that different viruses are deposited by different methods from honey bees to flowers (fecally vs. orally) and may be mediated by floral traits that alter how honey bees contact the flowers while foraging. I hypothesized that black queen cell virus (BQCV) was deposited both fecally and orally while deformed wing virus (DWV) was deposited through feces only. Thus, I predicted that I would detect BQCV in both the salivary glands and guts of honey bees but DWV would only be detected in honey bee guts. To test this prediction, I conducted a laboratory experiment where I dissected and tested various honey bee tissue for DWV and BQCV using qRT-PCR. I collected 15 honey bees from a honey bee colony I confirmed to be positive for both BQCV and DWV. From each specimen, I dissected the salivary glands (from the head and thorax), hypopharyngeal glands (from the head) and the gut (from the abdomen) and made composite samples. Once dissected, each tissue sample was rinsed once in PBS buffer, twice in nuclease free water, and stored on liquid nitrogen (Chen et al., 2014). I extracted RNA from each of the three composite tissue samples using Qiagen protocols and used qRT-PCR to quantify virus loads.

I detected both DWV and BQCV in all bee tissues suggesting that both feces and salivary secretions may deposit DWV and BQCV on flowers. Future experiments should test feces directly rather than the entire gut. To investigate how floral morphology mediates virus deposition, future controlled experiments should test individual flower parts (nectary vs. petals) after honey bee visitation.

Appendix F: Commercial Bumble Bee Colonies Host High Virus Prevalence

Commercial bumble bee colonies, primarily used for the pollination of greenhouse crops, are implicated as sources of disease spread to wild bumble bee populations (Colla et al., 2006; Otterstatter & Thomson, 2008). We examined the prevalence of RNA viruses in commercially available bumble bee colonies. We obtained nine *Bombus impatiens* colonies from a commercial supplier. Upon arrival, we collected five workers from each of the colonies, extracted RNA using Qiagen protocols and tested for RNA viruses, DWV, BQCV, and IAPV using qRT-PCR (Fig. 1). All colonies were positive for BQCV and DWV and one colony had IAPV. For five of the colonies, I detected BQCV and DWV at 100% prevalence.

These results are alarming as commercially available bumble bee colonies may be contributing to the spread of RNA viruses and other pathogens. We suggest that all lab reared commercial colonies receive gamma-irradiated pollen, to reduce the risk of virus spread.

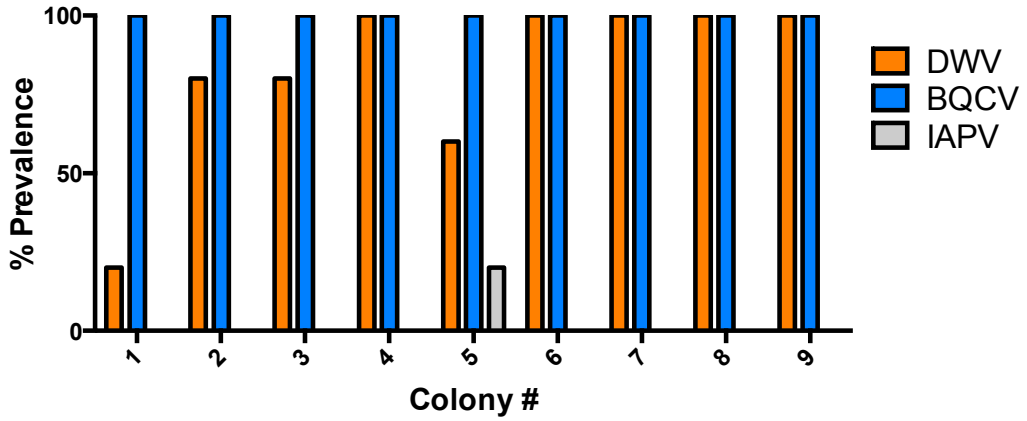

Figure 1. Virus prevalence upon arrival for 9 different commercial bumble bee colonies.