

**RNA VIRUSES: PREVALENCE, EFFECT, AND TRANSMISSION AMONG NATIVE  
BUMBLE BEES IN VERMONT**

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## **RNA VIRUSES: PREVALENCE, EFFECT, AND TRANSMISSION AMONG NATIVE BUMBLE BEES IN VERMONT**

**SUMMARY.** Emerging infectious diseases (EIDs) threaten global biodiversity, ecosystem function, and human welfare. Historically, research has focused on EIDs among domestic animals. More recently, EIDs contributing to devastating declines in wildlife species are gaining attention with examples such as chytridiomycosis in amphibians and white nose syndrome in bats. RNA viruses, once considered to be specific to European honeybees, are among the suspected threats to native bumble bees. However, most studies on RNA viruses have focused on the effect and prevalence among European honeybees and little is known of the prevalence and level of infection (viral load) among native pollinators (McMahon *et al.* 2015). Additionally, detailed knowledge of the mode of transmission between species and the conditions that promote viral replication is still lacking (Mcart *et al.*, 2014; Manley *et al.*, 2015). **My goal is to examine the propensity of RNA viruses in bumble bees, their means of transmission, and their effects on bee fitness and colony health.** Twenty-four viruses are known to affect bees (de Miranda *et al.* 2013). I will focus on two common viruses (deformed wing virus and black queen cell virus) and a third (Israeli acute paralysis virus), which has been recently described from managed honeybees and thought to contribute to colony collapse disorder. I will address five main questions: (1) What is the prevalence of these viruses in bumble bee species? (2) What factors influence viral prevalence in bumble bees? (2) What is the role of plants in viral transmission? (3) How do viruses affect bumble bees and what conditions are necessary for viral replication? (4) How do landscape level factors influence both managed and wild bee health and/or colony success? Filling these gaps in present knowledge is an important step in understanding the dynamics of emerging disease and is critical to maintaining healthy pollinator communities.

**INTELLECTUAL MERIT.** Emerging infectious diseases (EIDs) pose a substantial threat to global biodiversity, ecosystem function, food security, and human health. Complex interactions between host species, pathogens, and the environment create challenges to predicting and managing disease outbreak and spread. The complexity of these interactions is driven by variation of host species' life history, behavior, and immunity, differences in pathogen virulence and specificity, and environmental variation across a landscape. By providing a framework for studying host-pathogen systems, classical ecology theory has led to the development of disease ecology (Anderson & May, 1979; May & Anderson, 1979) and a burgeoning of theoretical concepts relating to transmission dynamics, pathogen evolution, host or pathogen community effects on transmission, and spatial or biological heterogeneity in disease dynamics (Joseph *et al.*, 2013).

Disease ecology is rich in theory yet lacking in empirical studies. According to theory, variation in community composition (Dobson & Foufopoulos, 2001) and transmission mechanisms (Joh *et al.*, 2009) may influence disease risk. However, especially among wild animal systems, few empirical studies have considered the strengths and interactions between biotic and abiotic mechanisms that affect disease transmission (Vander Wal *et al.*, 2014). Furthermore, since most hosts are infected by multiple pathogens capable of infecting multiple host species, theory highlights the importance of

studying multihost-multi pathogen interactions (Pedersen & Fenton, 2007; Tompkins *et al.*, 2011; Joseph *et al.*, 2013). However, empirical studies often focus on single host-single pathogen interactions. The importance of considering heterogeneity among multiple hosts in a variable landscape is underlined in studies of the baculoviruses that infect larvae of various species of Lepidoptera. In baculoviruses, transmission and infection is influenced by host behavior, variation in the distribution of the virus or hosts, host susceptibility, and variation in transmission mechanisms (D'Amico *et al.*, 2005; Parker *et al.*, 2010). While our understanding of EID's has undoubtedly been furthered by ecological theory, expanding the library of empirical evidence through field and laboratory studies should be a key focus.

The practical application of disease ecology to wildlife management may be limited by the unique needs and constraints of academics and wildlife managers. For example, models and experiments developed by academics to test theory may lack the system-specific detail necessary for managers to develop effective and practical management strategies. The goal of my Ph. D. dissertation is to bridge this gap across academics and managers by conducting empirical research that will help to inform both disease ecology theory and management recommendations. Specifically, I will examine the ecological processes governing the prevalence, transmission and effects of RNA viruses among bumble bees and provide practical disease management recommendations that will lessen transmission and disease risk to pollinators.

**INTRODUCTION.** Pollinating insects are important for ecosystem function and food security, providing over \$200 billion annually in pollination services (Jha & Kremen, 2013). Recent honeybee declines have underlined the importance of native pollinators and their ability to provide effective pollination services. However, many of the threats to honeybees are also affecting native bees (Naug, 2009; Potts *et al.*, 2010; González-Varo *et al.*, 2013; Hopwood *et al.*, 2013). In particular, the spread of viral pathogens is emerging as a serious threat. Once considered to be specific to European honeybees, RNA viral infections have been detected in bumble bees as well (Singh *et al.*, 2010; Fürst *et al.*, 2014; McMahon *et al.*, 2015).

With 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; J.L. Li *et al.*, 2014). Indeed, although virological research is biased towards honeybees, many of the 24 RNA viruses detected in honeybees have a broad host range including bumble 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). Three of the most common in the US are deformed wing virus (DWV), black queen cell virus (BQCV), and Israeli acute paralysis virus (IAPV), a more recently described virus associated with managed honeybee hives affected by colony collapse disorder (Cox-Foster *et al.*, 2007). These obligate intracellular pathogens have a positive-sense single stranded RNA genome. Viruses may persist in the host as subclinical infections but are capable of replicating quickly under certain conditions. High viral titers result in symptoms such as deformed wings, paralysis and mortality (Chen & Siede, 2007; de Miranda *et al.*, 2013). Although RNA viruses are known to infect wild bumble bees (Otterstatter & Thomson, 2008; Singh *et al.*, 2010; Fürst *et al.*, 2014; Manley *et al.*, 2015; McMahon *et al.*, 2015), their effects on bumble bee behavior, physiology and colony fitness

are almost entirely unknown. This may, in part, be due to sampling biases if bees are less likely to forage when suffering from symptoms and are therefore rare to encounter. To my knowledge, only three published studies cite the effects of viruses in bumble bees and are limited to only two Old World species. Genersch *et al.* (2006) observed wing deformities in both wild and commercially reared *B. terrestris* and *B. pascuorum* naturally infected with deformed wing virus, Fürst *et al.* (2014) demonstrated reduced survival in *B. terrestris* after oral inoculation of DWV, and Meeus *et al.* (2014) found worker production was reduced in *B. terrestris* micro-colonies infected with Kashmir bee virus and Israeli acute paralysis virus. More studies elucidating the individual and colony level effects across additional bee species is critical to understanding the dynamics and epidemiology of these emerging pathogens.

In honey bees, observable symptoms result from viral replication. External stress agents that affect bee immunity and health may promote viral replication through immunosuppression (Nazzi & Pennacchio, 2014). In honeybees, accelerated viral replication has been demonstrated after exposure to Varroa mites (Rosenkranz *et al.*, 2010), pesticides (Di Prisco *et al.*, 2013), and *Nosema ceranae* (Zheng *et al.*, 2015). While viral replication has been demonstrated in 7 bumble bee species, the conditions necessary for replication within these hosts is unknown (Manley *et al.*, 2015). Examining the external factors influencing viral replication would help to disentangle the multiple threats to wild pollinators.

Although poorly understood, there is some evidence of RNA viral spillover from managed honeybees (*Apis mellifera*) to wild bees. In the UK, sympatric bumble bees and honeybees are infected by the same viral strains (Fürst *et al.*, 2014) and viral prevalence in honeybees is a significant predictor of virus prevalence in bumble bees (McMahon *et al.*, 2015). Outside the UK, little is known about the prevalence of viruses among wild bees. Although it has been assumed that the virus moves from infected honey bees to bumble bees, the principal direction of viral transmission has yet to be confirmed. Both relatedness and shared foraging habits undoubtedly present a great risk of disease transfer among managed bees and native bumble bees (Goulson, 2003; Peng *et al.*, 2011). Indeed, evidence for spillover from managed bees to wild bees has been found for several other pathogens including microsporidian parasites *Nosema ceranae* and *N. bombi*, a trypanosome *Crithidia bombi*, and a parasitic protozoan *Apicytis bombi* (Colla *et al.*, 2006; Otterstatter & Thomson, 2008; Graystock *et al.*, 2014; McMahon *et al.*, 2015). More comprehensive surveys conducted outside the UK in areas of both high and low honeybee abundance would help to elucidate the prevalence of these viruses among wild bees and could help identify the directionality of viral transmission.

Transmission of viruses can occur both vertically from infected queens and drones to offspring. (Chen *et al.*, 2006; Chen & Siede, 2007). Horizontal transmission routes between species are currently unknown. 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 bees as well as in pollen loads carried by bees (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 co-mingling on flowers or indirectly through infected pollen. Interestingly, viral infections differ among individual bee specimens and the pollen loads they carry (Singh *et al.*, 2010); suggesting differences in viral ecology, and/or differences in

pollinator contact with contaminated pollen. Despite the *suggestion* for viral transmission through floral resources, this route remains untested (Mcart *et al.*, 2014). Furthermore, no published study has attempted to detect viruses directly on flowers. If flowers act as “bridges” in viral transmission, this presents several intriguing questions regarding the role of plants in viral transmission: How are pollinators transferring viruses onto plants? Do floral traits influence transmission and do plant species differ in their propensity to harbor viruses? In a previous study examining transmission of *Critidilia bombyi* among bumble bees, floral morphology was found to influence the likelihood of bees becoming infected (Durrer & Schmid-Hempel, 1994). Bees were more likely to become infected at simple, linear inflorescences than at complex spiral ones of *Echium vulgare*. When comparing the transmission success of two different plant species, bees were more likely to become infected after visiting flat and accessible flowers of *Rubus caesius* rather than the complex and narrow corollas of *E. vulgare*, suggesting that other floral traits besides morphology affect transmission rate. More research is needed to understand the specific underlying mechanisms of transmission of these viruses.

**OBJECTIVES AND HYPOTHESES.** My goal is to examine the prevalence, means of transmission, and effects of RNA viruses on bumble bees in Vermont. Twenty-four viruses are known to affect bees (de Miranda *et al.* 2013). I will focus on two of the most common—deformed wing virus, and black queen cell virus—as well as Israeli acute paralysis virus, a recently described virus of managed honeybees affected by colony collapse disorder.

**OBJECTIVES:** My research has 5 main objectives: (1) Conduct a comprehensive field survey to elucidate the prevalence and level of infection (viral load) of RNA viruses among native bumble bee species to (a) examine the extent to which viral load in bumble bees can be explained by honeybee proximity, abundance and honeybee viral load; (b) examine if the diversity and abundance of shared flowering resources affects bumble bee viral prevalence and/or load. (2) Using greenhouse experiments, examine the role of flowering resources in viral transmission (3) Using laboratory experiments, examine the effect of viruses on bumble bees and test the extent to which pesticide exposure affects viral replication. (4) Using GIS and modeling, determine how landscape level analyses can be used to predict honeybee and bumble bee health. (5) Provide practical management recommendations to lessen pathogen spillover and maintain healthy pollinator communities.

**HYPOTHESIS I:** I predict bumble bee viral loads to be highest in areas with the highest concentrations of honeybee hives, and I hypothesize that *viruses are spilling over from managed honeybee hives into wild bumble bees through the use of shared floral resources*. I also predict that sympatric bees will share the same viral strains, and viral prevalence and load will be negatively correlated with the diversity and abundance of floral resources.

**HYPOTHESIS II:** *Differences in floral morphology influences the likelihood of a flower becoming infected as well as the transmission of viruses between and within foraging bee species.*

**HYPOTHESIS III:** *In bumble bees, pesticide exposure promotes viral replication, leading to reduced fitness.*

**HYPOTHESIS IV:** *Forage quality, distance to forage, competition from neighboring apiaries, and pesticide exposure affects bee pathogen load and honeybee production.*

## OBJECTIVE 1

**Conduct a comprehensive field survey to elucidate the prevalence and level of infection (viral load) of RNA viruses among native bumble bee species to (a) examine the extent to which viral load in bumble bees can be explained by honeybee proximity, abundance and honeybee viral load; (b) examine if the diversity and abundance of shared flowering resources affects bumble viral prevalence and/or load.**

**HYPOTHESIS 1:** I predict bumble bee viral loads to be highest in areas with the highest concentrations of honeybee hives and I hypothesize that *viruses are spilling over from managed honeybee hives into wild bumble bees through the use of shared floral resources*. I also predict that sympatric bees will share the same viral strains, and viral prevalence and load will be negatively correlated with the diversity and abundance of floral resources.

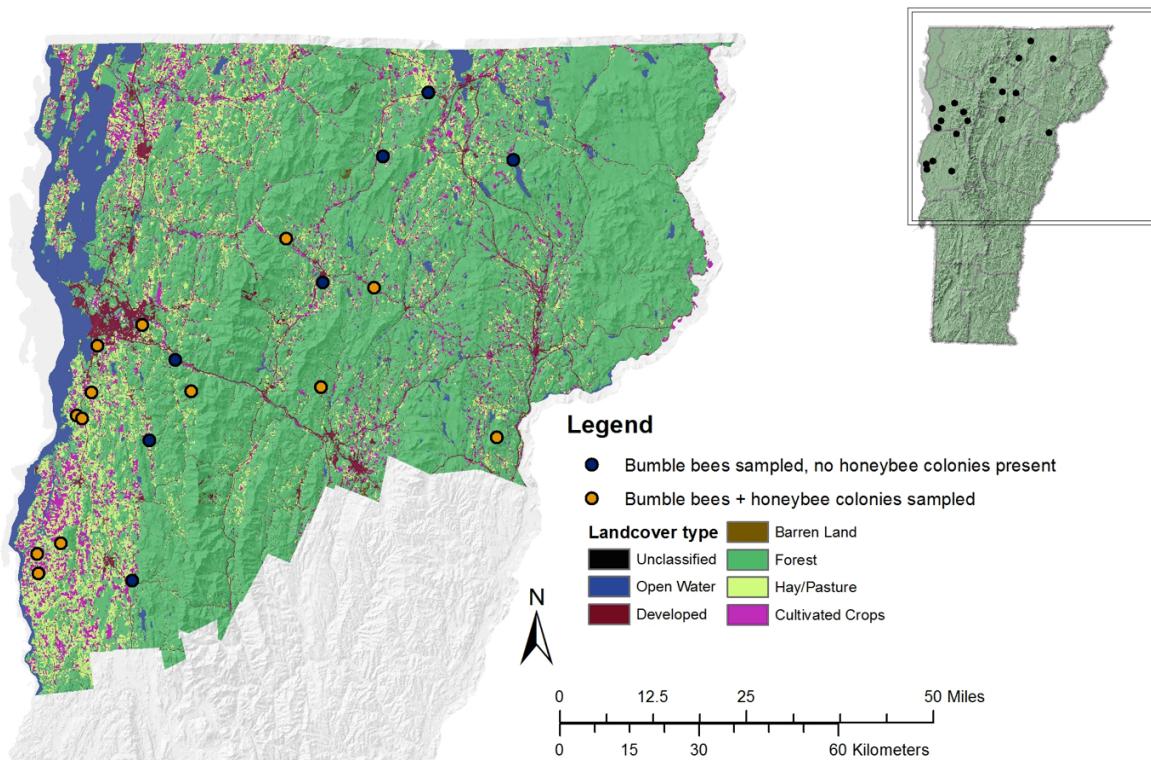
**BACKGROUND.** Viruses are suspected of spilling over from managed hives into wild bee populations (Singh *et al.*, 2010; Fürst *et al.*, 2014). Consequently, previous surveys of viruses in wild bees have primarily focused on areas near honeybee apiaries (Evison *et al.*, 2012). More information is needed on the prevalence of these viruses across a variable landscape in areas of both high and low honeybee abundance to help explain patterns of prevalence and the direction of viral spread between pollinator genera. Most previous surveys of viruses among wild pollinators have focused solely on detection using molecular techniques that provide presence/absence results. While these data are important for identifying viral host range, they provide little information regarding the factors influencing pathogenicity. Measuring the level of infection (viral load) rather than the presence/absence of viruses would allow for higher resolution comparisons of viral infection across host species and populations. Measuring viral load is particularly important for RNA viruses, due to their ability to persist as latent infections until conditions permit replication. Currently, the conditions that promote viral replication are unknown. A quantitative assessment of viral load across a variable landscape could provide insight into the potential factors influencing viral replication. To my knowledge, the survey I propose will be the first comprehensive field survey to **quantitatively** assess viral load in New World bumble bee species.

The shared use of flowers by pollinators is a likely route of horizontal transmission between bee species (Mcart *et al.*, 2014). Therefore, local flower diversity and abundance may play a role in patterns of viral prevalence. If flower diversity and abundance is limited, greater foraging overlap and competition for the available flowers by pollinators may result in 'hot-spots' for disease transmission. In areas with a high diversity and abundance of floral resources, foraging overlap among bees may be reduced. This may result in a "dilution effect" whereby the risk of infection is lessened for individual foragers. However, greater local floral diversity and abundance may instead act as a "pollinator

magnet”, attracting more foragers and increasing the probability of contact with a contaminated individual or flower.

To address *Hypothesis 1*, I will conduct a comprehensive field survey and field experiment. Using field survey data, I will examine if viral load in bumble bees and prevalence in bumble bees can be explained by honeybee abundance, honeybee viral load or flower density/diversity. I will conduct a field experiment to look for evidence of directionality of viral transmission from honeybees to bumble bees. I will determine if distance to an infected honey bee colony predicts viral infection in bumble bee colonies.

Bumblebee and Honey Bee Sampling Locations, 2014



**Figure 1. Bumble bee and Honey Bee Sampling Locations. Note: Map depicts 6 additional sites not included in my original 14 chosen field sites.**

**APPROACH. Field Survey.** To begin to examine the prevalence of viruses in bumble bees in Vermont, I selected 14 field sites in 2014 (>20 km apart) across northern Vermont (**figure 1**) and included sites with both high and low honeybee abundance. I visited field sites in early summer and collected newly emerged queens and in late summer and collected workers. For each netted bee, I identified the species, identified and recorded the plant on which they were collected, and placed the bee specimens on dry ice. In total, I collected 8 species of bumble bees consisting of ~10 queens from each of 9 sites and ~20 workers from each of 14 sites. I also collected honeybees if they were present. Because shared flowers are suspected bridges for spillover of viruses from honeybees to wild bumble bees

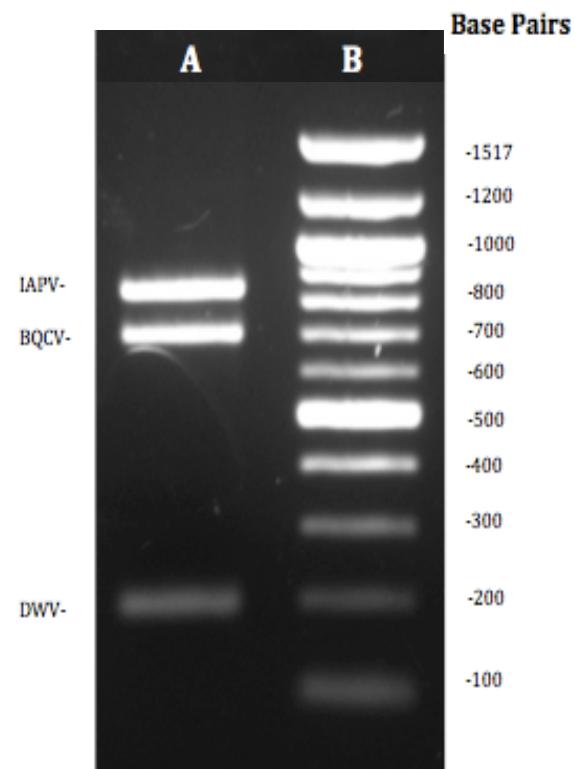
(Singh *et al.*, 2010; Mcart *et al.*, 2014), I surveyed the flowering plants and honeybees at each field site. To measure plant diversity and abundance, I counted and identified all flowering plants within a 1m x 1m quadrat placed 10 times along a 100 m transect at each site. I also collected several samples of highly visited pollinator host plants at each site to later be tested for viruses. To measure bee abundance and diversity, I conducted a 10 minute survey along a 100 m transect. All bees within 5 m of either side of the transect were identified to morphospecies and the plant on which they were foraging was recorded.

Results from 2014 will inform my 2015 surveys; I plan to collect bees and census plants in at least 30 field sites across VT, including areas of both high and low honeybee abundance. I was recently invited to help conduct the National Honey Bee Survey (NHBS), and I will be sampling 24 managed honeybee yards as part of the survey in 2015. These samples will be processed at the USDA lab in MD and used in analyses comparing viral loads of honeybees and sympatric bumble bees.

**RNA Extraction and Pathogen Detection.** In 2014, I used RT-PCR to successfully amplify and detect three bee viruses: DWV, BQCV, and IAPV (**figure 2**). In spring 2015, I began learning the molecular protocols necessary for RT-qPCR. Primers for virus targets were obtained from the USDA bee lab in MD. I developed primers for Actin to be used as a reference gene. So far, I have detected both DWV and BQCV in bumble bee samples using RT-qPCR. I am currently working to develop standardized serial dilutions to run alongside bee samples to obtain accurate viral load measurements.

**RT-qPCR protocol:** All bees are stored in -80°C until processed. Whole bees are flash frozen using liquid nitrogen and homogenized with a buffer using a pestle. Total RNA is extracted manually using RNeasy Mini kit (Qiagen) following manufacturers protocols. Specific primers are used for the following viruses: BQCV, DWV and IAPV. Actin is used as a reference gene. Total RNA is transcribed to cDNA and viral load in each sample is tested using RT-qPCR (iTaq™ Universal SYBR® Green One-Step Kit) using the following program: 10 minutes at 50°C, (reverse transcription reaction), 1 minute at 95°C (polymerase activation and DNA denaturation), followed by 40 cycles of 15 sec at 95°C (denaturation), and 60 Sec at 60°C (annealing/extension + plate read).

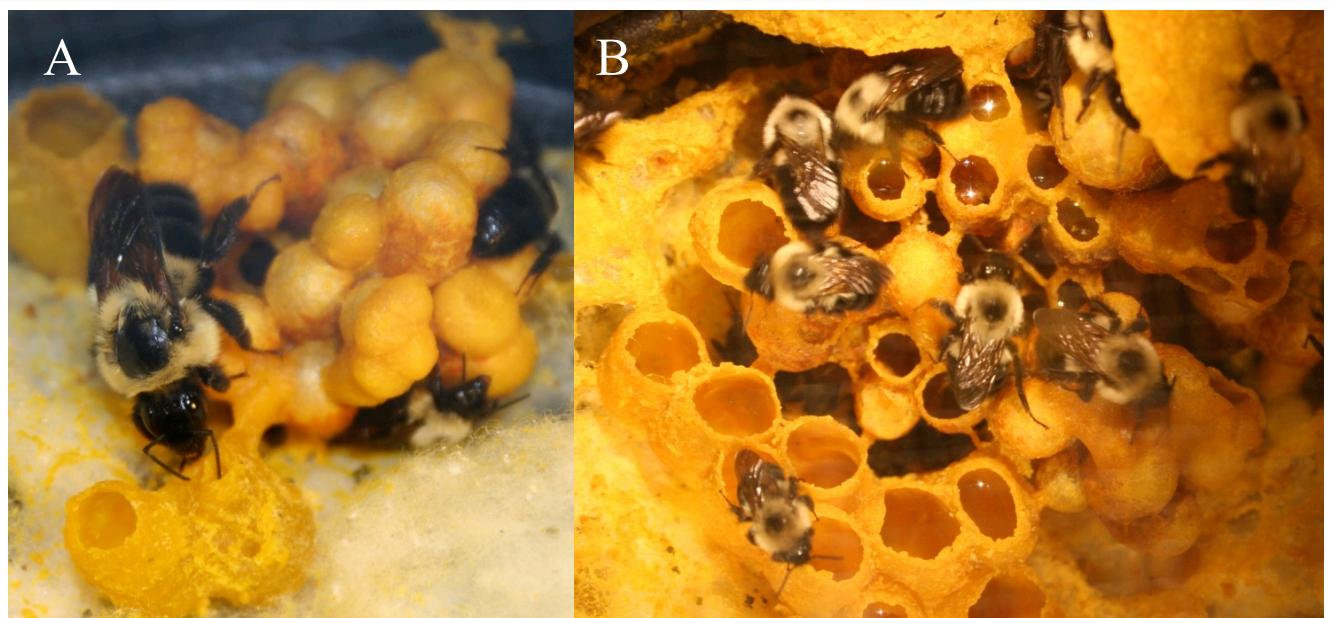
**Spillover Field Experiment.** In 2014, I conducted a pilot experiment to test captive bee rearing techniques, identify methods for measuring colony fitness and ultimately test



**Figure 2. Agarose gel electrophoresis. A:** Honeybee positive for Israeli acute paralysis virus (IAPV), black queen cell virus (BQCV), and deformed wing virus (DWV). **B:** 100 base pair ladder.

disease transmission between honeybees and bumble bees. I reared seven *Bombus bimaculatus* colonies from wild-caught queens in the lab (**figure 3**). I later transferred each colony to field boxes and placed them on the UVM horticultural farm either 'near' (3 colonies) or 'far' (4 colonies) from resident honeybee hives (0.5 km apart) (**figure 4**). To monitor the viral status of all colonies, I collected individuals from both the *Bombus* colonies and honeybee colonies several times throughout the field season. To estimate fitness and colony growth, I conducted weekly nighttime counts of *Bombus* workers, queens, and males for each colony. I measured the weight of the *Bombus* hives at the beginning and end of the experiment. To test differences in viral load between colonies and their pollen loads, I collected several pollen samples from *Bombus* and honeybee individuals.

In 2015, I will repeat the spillover experiment, using a larger sample size and measuring more dependent variables. Beginning in late April, I will use modified techniques to rear ~18 *Bombus bimaculatus* colonies in the lab. Prior to the start of the experiment, I will identify three honeybee yards that test positive for at least one RNA virus. I will test captive reared bumble bee colonies for RNA viruses prior to the start of the experiment. At each of the three sites, I will place bumble bee colonies either 'near' or 'far' from honeybee hives (0.5 km apart). To elucidate the effects of infection on bumble bees, I will measure several fitness variables throughout the field season and compare these data to viral load a posteriori. To measure colony fitness, I will weigh hives and count individuals on a weekly basis. I will measure queen production by fitting each hive entrance with a reducer once colonies begin producing sexuals to limit new queens from escaping. To monitor viral load, I will collect and test individuals from both honeybee colonies and bumble bee colonies using RT-qPCR.



**Figure 3. Images of a bumble bee colony (*Bombus bimaculatus*) reared in the lab from wild-caught queens. A: Queen bee incubating brood at 3 weeks since first eggs laid. B: Workers tending the nest at 7 weeks since first eggs were laid.**

*Data analysis.* To test the drivers of bumble bee viral load, I will analyze the field survey data using generalized linear models (GLM). I will test if bumble bee viral load (number of viral genome copies per individuals) is dependent on honeybee abundance, viral load, and flower density/diversity. I will use separate GLMs for each of the three target viruses (DWV, BQCV, and IAPV). For the field experiment, I will use an ANCOVA to test how much of the variance in bumble bee viral load can be explained by distance from honeybees (near/far) with honeybee viral load as a covariate.



**Figure 4. Field box at UVM horticultural farm containing lab reared bumble bee colony.**

**OBJECTIVE 2: Using field and greenhouse experiments, examine the role of floral traits in viral transmission.**

**HYPOTHESIS II:** *Differences in floral morphology influence the likelihood of a flower becoming infected as well as the probability of viral transmission of viruses within and among bee species.*

**BACKGROUND.** As bees forage on flowers, RNA viruses may be left behind on flowers through infected oral secretions or feces (Chen *et al.*, 2006; Yue *et al.*, 2007). If viruses are transmitted through contact with feces, floral morphology that encourages 'hovering' behavior, thus only coming in contact with the anterior part of a pollinator, may reduce the likelihood of a flower becoming infected through fecal deposition (Mcart *et al.*, 2014). If viruses are transmitted through oral secretions, floral morphology that excludes some bee species from accessing floral nectaries may reduce the likelihood of interspecific viral transmission among bee species. If a plants' propensity to harbor viruses is a function of pollinator visitation rates, one would expect to find more viruses on plants with highly attractive flowers that stay open longer. Although the above assumptions are intuitive,

virtually nothing is known regarding the role of plants and whether floral traits can affect viral transmission. More research is needed to fill these knowledge gaps in viral transmission routes (Mcart *et al.*, 2014; Manley *et al.*, 2015).

RNA viruses can sometimes cross species, and even kingdom boundaries, as with the tobacco ring spot virus—an RNA plant virus recently found replicating within honeybee hosts (Li *et al.*, 2014). If bee viruses are capable of making a similar phylogenetic leap, they could persist systemically within plants and be expressed in nectar and pollen. RNA viruses have been detected within pollen grains, providing evidence for a more intimate relationship between the virus and plant (Singh *et al.*, 2010).

To test *hypothesis II*, I will conduct two greenhouse experiments to investigate the role of plants in viral transmission. The experiments will answer the following questions: 1) In what plant tissues can I detect viruses? 2) Does floral morphology and/or visitation rate influence the viral load on flowers left behind by foragers? And 3) Does floral morphology and/or number of flowers a bee visits influence the transmission success of viruses to bumble bees and/or honeybees?

**APPROACH.** *Pilot experiment.* In 2014, a pilot experiment was conducted to test if bee viruses can be detected on flowers. In the field, I allowed infected bumble bee colonies to forage on ‘clean’ flowers within a screen enclosure. Flowers were collected at 1, 2, 4, and 6 hours after exposure to infected forages and will be tested using RT-qPCR.

In future greenhouse experiments, I will use multiple flowering plant species to determine the role of plants and floral traits in viral transmission. As the first known study examining the role of flowers in viral transmission, results will help fill the knowledge gap in bee viral transmission routes.

*Study organisms.* *Bombus impatiens* and honeybee colonies will be purchased from a commercial supplier and tested for viral infection. Purchasing commercial bees allows me to conduct this experiment with a large sample size. I will choose a plant species that has cultivars differing in floral morphology such as highbush blueberry, *Vaccinium corymbosum*. Floral morphology across cultivars of *V. corymbosum* varies greatly in terms of corolla width, depth, and throat width. Because bees differ in tongue length and their ability to probe floral nectaries, visitation rates of bee species differ across cultivars (Cane & Payne, 1993; Courcelles *et al.*, 2013). If bees are inefficient at reaching nectaries, they may be less likely to become infected by inoculated floral nectaries. I will test for differences across both bumble bees and honeybees.

*Experiment I: In what plant tissues can I detect viruses? Does floral morphology and/or visitation rate influence the viral load on flowers left behind by foragers?* Using flowering plants and captive bee colonies, I will conduct a greenhouse experiment to test if viral loads detected on flowers is a function of floral morphology and/or number of visits it receives from an infected bee. I will expose the plants to infected honey bees and record visitations to each flower. I will test remaining nectar, pollen, flower surfaces, leaves and stems for viruses to determine viral loads on the plants and if viruses are persisting systemically within the different plant tissue.

*Data analysis* To test if floral traits and/or number of visits each flower receives influences the viral load detected on flowers, I will use floral morphology (corolla depth x width) and visitation rate as predictor variables in a multiple regression analysis with viral

load detected on flowers as a dependent variable.

*Experiment II. Does floral morphology and/or number of flowers a bee visits influence the transmission success of viruses among bumble bees and/or honeybees?* In a greenhouse, I will establish two treatments with flowers with either 'open' or 'closed' morphology. 'Open' morphology includes flowers with easily accessible nectaries while 'closed' morphology includes flowers with nectaries more difficult for pollinators to access due to a narrow or deep corolla. I will infect each flower in the treatment groups by inoculating nectaries with viral isolate. Control groups will consist of un-inoculated flowers with either 'closed' or 'open' morphology. To test for differences in transmission success across floral traits, I will expose uninfected bumble bees to either a treatment or control. The number of flowers each bee visits will be recorded. After exposure, I will test each bee for viruses. To test for differences in transmission success across bee species, I will repeat the experiment using honeybees.

*Data analysis.* To test if floral morphology and/or the number of flowers a bee visits predicts the success of viral transmission I will conduct an ANCOVA. My response variable will be infection status (Yes/No) for each bee. Predictor variables include: number of flowers visited, flower type (open/closed), and bee species (either honeybee or bumble bee).

### **OBJECTIVE 3: Using laboratory experiments, examine the effect of viruses on bumble bees and test the extent to which pesticide exposure affects viral replication.**

#### **HYPOTHESIS III:** *In bumble bees, pesticide exposure promotes viral replication, leading to reduced fitness.*

**BACKGROUND.** Despite the burgeoning interest in viruses among wild bees, the effects of viruses on non-*Apis* species physiology and fitness are almost completely unknown. Only three published studies have cited fitness consequences of viruses on bumble bees (Genersch *et al.*, 2006; Fürst *et al.*, 2014; Meeus *et al.*, 2014). More studies need to be conducted across other species to examine additional fitness consequences on bumble bees. 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.

High viral titers are associated with symptomatic bees but the conditions necessary for viral replication are unclear. Stressors that adversely affect insect immunity such as poor nutrition and pesticide exposure may induce viral replication (Brown *et al.*, 2000; Pettis *et al.*, 2013). Neonicotinoids, a class of neurotoxic pesticide, was found to promote DWV replication in honeybees (Di Prisco *et al.*, 2013). I hypothesize that pesticide exposure suppresses immunity and promotes viral replication in bumble bees. Understanding how stressors affect immunocompetence and increase susceptibility to pathogens is epidemiologically important and a critical step in disentangling the factors influencing bee decline. To address *hypothesis III*, I will conduct a laboratory experiment that tests the effects of deformed wing virus on bumble bees and if pesticide exposure increases viral load - a proxy of viral replication.

**APPROACH.** Using a 2x2 factorial design, I will examine the effects of deformed wing virus (DWV) on bumble bees and the interactive effects of viral infection and pesticide exposure. I will also test if pesticide exposure causes viral replication in bumble bees. Treatments will include DWV (Yes/No) and pesticide challenged (Yes/No). I will measure weight, drone production, and viral load as response variables. To obtain a large sample size, I will use microcolonies consisting of 5 workers, one of which becomes a pseudo-queen, producing unfertilized (drone) eggs. Ten microcolonies will be assigned to each treatment. DWV+ colonies will receive sugar water inoculated with DWV. Pesticide challenged treatments will receive sugar water inoculated with field realistic doses of imidacloprid, a commonly used neonicotinoid pesticide. Viral load will be tested at 2, 4, and 6 weeks after exposure to imidacloprid. I will replace workers that I remove with marked individuals to maintain 5 workers per treatment. After 6 weeks, I will discontinue the imidacloprid treatment and again test viral titers at 8 and 10 weeks.

*Data analysis.* To test the effects of viruses on bumble bees, I will use an ANCOVA to test how much of the variation in fitness (drone production, weight, foraging behavior) can be explained by viral load. To test if pesticide exposure promotes viral replication, I will use an analysis of variance to test how much of the variation in viral load can be explained by pesticide exposure.

#### **OBJECTIVE 4: Using GIS and modeling, determine how landscape level analyses can be used to predict honeybee and bumble bee health.**

**HYPOTHESIS IV:** *Forage quality, distance to forage, competition from neighboring apiaries, and pesticide exposure affects bee pathogen load and in honeybees, honey production.*

**BACKGROUND.** Previous lab-based studies provide evidence that diet directly affects bee health. In honeybees, both pollen quality and diversity affects immunocompetence and resistance to pathogens (Alaux *et al.*, 2010; Di Pasquale *et al.*, 2013). Bees may travel up to several kilometers to forage on rich floral patches that are scattered widely across a landscape (Jha & Kremen, 2013). Thus, landscapes that provide a high quality diet through an abundance and diversity of floral resources could help bees better withstand disease (Goulson *et al.*, 2015). Already, landscape level analyses have identified habitat quality, distance to natural areas, and anthropogenic disturbance as drivers of wild bee abundance and richness (Ricketts *et al.*, 2006; Lonsdorf *et al.*, 2009; Winfree *et al.*, 2011; Kennedy *et al.*, 2013). However, I am unaware of any published studies quantitatively linking landscape composition to bee health. Identifying the landscape level factors that contribute to observed patterns in honeybee and native bee pathogen load would help management decisions for apiculturists, farmers, and conservationists.

To address *hypothesis IV*, I will combine GIS landscape level analyses and bee pathogen and fitness measurements to test landscape level factors that influence bee health (Table 1). I will test a series of models based on four *a priori* hypotheses: I hypothesize that bee health (as measured by pathogen loads in both bumble bees and honeybees) and honeybee colony fitness (as measured by honey, wax, and brood production) will be influenced by 1) forage quality, 2) distance to forage, 3) apiary overlap, and 4) pesticide

use.

**APPROACH.** *Field data collection.* In summer 2015, I will visit and sample 24 commercial honeybee apiaries throughout VT as part of the National Honeybee Survey. At each apiary, I will collect honeybees from 8-10 hives. These will be tested at the University of Maryland for: *Nosema* spp. spore count, Tropilaelaps mites, Varroa mite loads, *Apis cerena*, and viral load of 8 different RNA viruses. I will also conduct a frame-by-frame inspection to determine quantity of honey/nectar, pollen, brood and wax.

To examine landscape level effects and honeybee abundance/viral load on wild bee health, sympatric bumble bees will be collected at each honeybee apiary and later tested for viral load of DWV, BQCV, and IAPV.

*GIS analysis.* Using Arc GIS, I will use National Land Cover Data to analyze habitat type, habitat arrangement, and apiary overlap within the flight zones of each apiary to predict bee health and honey production. Land cover data for Vermont will be obtained from the National Land Cover Database (NLCD). County and town boundary data will be obtained from the Vermont Center for Geographic Information. Data collected from Google earth (WGS 84) will be converted to the projection system of the project (NAD 83). All spatial data analyses will be conducted in ArcGIS and R.

*Forage quality.* Forage quality within each apiary flight zone (AFZ) will be calculated by combining land cover data and expert opinion on floral quality. Land cover will be reclassified into five levels: cultivated crops, hay/pasture, forest (consisting of deciduous, evergreen, mixed, woody wetland, and shrub/scrub), urban (open space, low intensity, medium intensity, and high intensity), and inhospitable (water/barren land). Each reclassified land cover type will be assigned a Forage Quality Value (FQV). Both expert opinion and a literature review will inform FQV assignments. Using the FQVs and the proportions of each land cover type within an apiary flight zone, a final forage quality score will be calculated for each AFZ (see **figure 5** for preliminary map).

*Forage distance.* Bees that travel shorter distances from their hive to high quality forage should maintain higher energy input than bees that travel longer distances for the same forage (Rands & Whitney, 2010). Distance to each habitat type will be used in a model that tests how distance to surrounding forage predicts bee health/colony success. For each apiary, forage quality scores will be calculated within a series of nested buffers (0.5, 1, 2, 3, 4, and 5 km) around each AFZ. Using nearest neighbor, a score will be assigned that is inversely proportional to the distance.

*Apiary overlap.* Competition for flowering resources within a flight zone can also affect energy input to a hive. In the commercial beekeeping industry, apiaries can contain over a thousand hives. While commercial apiaries in VT are much smaller, apiary size and apiary overlap may result in resource competition for both honeybees and sympatric bumble bees. Apiary overlap may also result in higher pathogen loads due to the high density of bees and increased opportunities for transmission. Using the known locations of apiaries, I will use GIS to determine the amount of apiary overlap (measured in pixels) for each of the 24 study site apiaries.

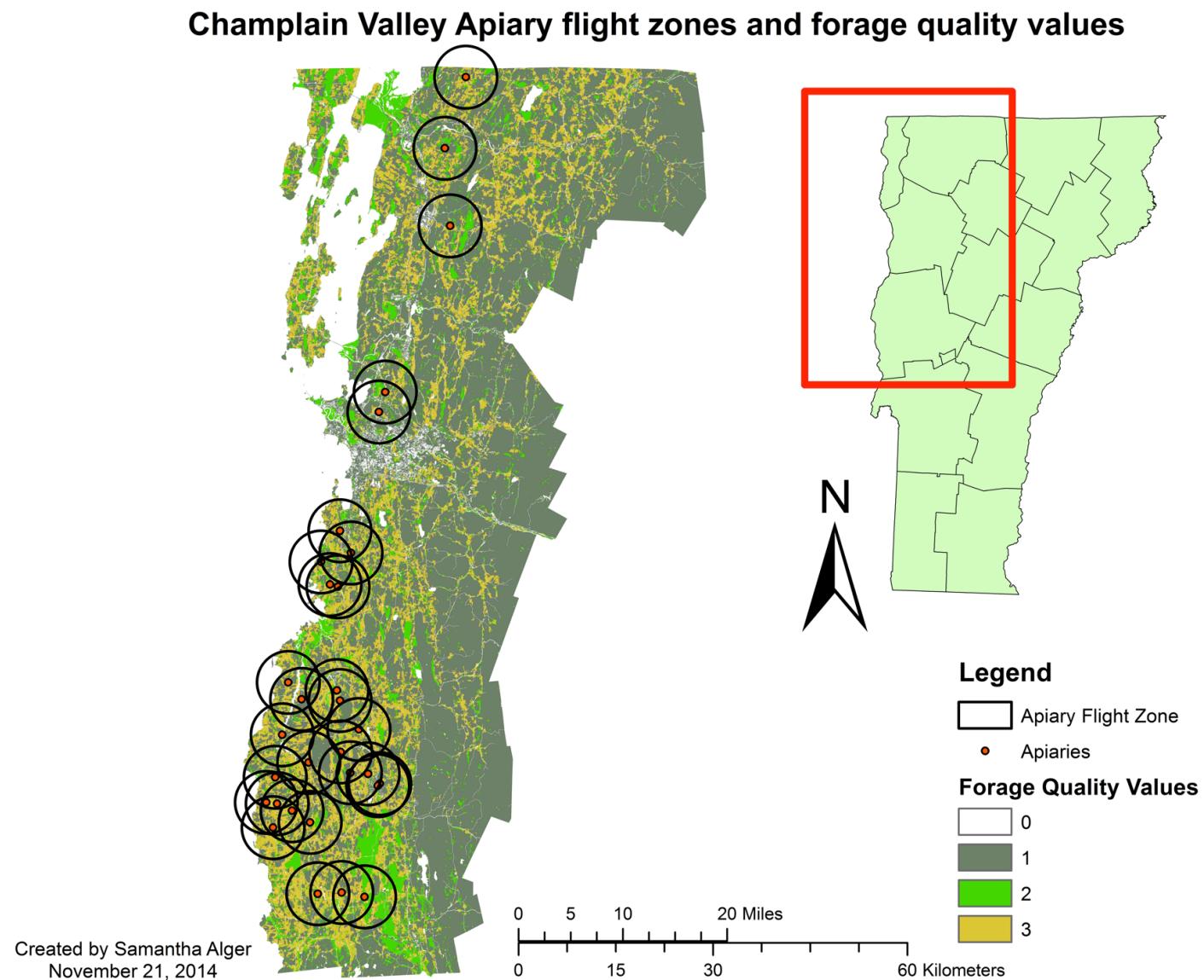
*Pesticide exposure:* I will attempt to collect data of pesticide use within and around my study sites. These data will be used to create a pesticide exposure index for each study site.

*Modeling and data analysis.* Using multiple linear regression analyses, I will test if landscape level factors predict honeybee pathogen loads, bumble bee viral loads, and honeybee colony success. Model quality will be tested using a stepwise comparison and Akaike information criterion (AIC).

**BROADER IMPACTS.** In discovering how flowers might act as reservoirs for viruses, and how multiple insults affect bee fitness and colony health, my work will lead directly to management recommendations for pollinator conservation. I am currently spearheading Vermont's involvement with the National Honey Bee Survey (NHBS), an ongoing national effort by the Bee Informed Partnership (BIP) and USDA Animal Plant Health Inspection Service, to collect data on bee disease to better inform research and management decisions. Maintaining healthy honeybee hives through this monitoring program will benefit commercial beekeepers as well as help to lessen pathogen spillover into wild bee populations. In January 2015, I presented a formal proposal to the state apiculturist and the Vermont Beekeepers Association outlining the plan for VT's involvement with the survey. The proposal was approved and I am currently working with USDA and BIP to plan the upcoming field season. With support from the Vermont Beekeeper's Association, I will hold workshops and give presentations on NHBS results and progress in VT in order to spread awareness about bee pathogens and disease. Results of my research will be published in scientific journals. Lastly, I will mentor at least two undergraduate assistants each summer and academic year, giving them opportunities to develop independent research projects in the field or in the laboratory

**Table 1. Description of four model hypotheses for three parameters of interest: honeybee and bumble bee pathogen load and honeybee colony success (as measured in brood, honey, and wax production). Hypotheses 1 and 2 will be evaluated at 6 spatial scales, resulting in a total of 14 models that will be fit to the observed field data.**

Parameters of interest	Hypotheses	Spatial Scales Evaluated	Variables
Honeybee pathogen load	1. Forage quality	0.5, 1, 2, 3, 4, 5 km	% Cultivated crops % Hay/pasture % Forest (deciduous, evergreen, mixed, woody wetland, shrub/scrub) % Urban (open space, low intensity, medium intensity, high intensity) % Inhospitable (water/barren land)
Bumble bee pathogen load			
Honeybee colony success	2. Distance to forage	0.5, 1, 2, 3, 4, 5 km	Distance of each pixel of habitat type to apiary center
	3. Apiary overlap	Stationary	# of pixels within each AFZ that overlap 1, 2...n apiaries
	4. Pesticide exposure	5 km	Pounds of pesticides used



**Figure 5.** Preliminary map showing each apiary of *Champlain Valley Apiaries*. Each apiary is surrounded by a 3 km. buffer zone (Apiary Flight Zone- APZ). Land cover data has been reclassified and assigned a Forage Quality value. Values were assigned based on a literature review. Overlapping AFZs depict apiary overlap.

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