**How do landscape level disease dynamics play a role in coinfection resulting in pathogen-pathogen interactions?**

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**Introduction:**

*Brief description: In this section, I aim to introduce the bee system, the importance of addressing their many threat (especially pathogens- related threats) and frame the usefulness of this study in for advancing our knowledge of coinfection in the fields of disease ecology and epidemiology. I would like to include work showing how similarities in population hierarchies (bee = individual, colony = city, apiary = network of cities) can be useful in modeling transmission, spillover and outbreak in other systems.*

*My main questions:*

1. *How do these pathogens disseminate throughout the environment at the individual, colony and apiary levels?*
2. *What are the main drivers behind co-infection in honey bee and bumble bee systems?*
3. *How do pathogens interact with each other (organismal interactions) and their host (environment) when the host is coinfected?*

**Figures included: diagram of bee -> human parallels. Data slide of decreasing *Bombus* and HB pops.**

**Current relevant material:**

As we gather evidence for disease spillover from honey bees into bumble bee populations, it is important that we understand how these shared pathogens fluctuate through time, interact within the host, and affect bumble bee health. In honey bees,pathogen prevalence and load fluctuate throughout the growing season (Runckel *et al.* 2011). However, this information is lacking in the bumble bee literature. Knowledge of this relationship will aid in pinpointing times of the year where disease levels increase in both populations and help identify the biotic and abiotic factors influencing pathogen fluctuations in bumble bees. In honey bees, *N. ceranae* outcompetes *A. mellifera*’s unique species of *Nosema, N. apis* (Bourgeois *et al.* 2010; Natsopoulou *et al.* 2014). While both *N. bombi* and *N. ceranae* infect bumble bees, no studies have yet examined how these pathogens interact within the bumble bee host and whether coinfection of both species denotes higher mortality. In honey bees, coinfection of multiple pathogens results in higher mortality and colony losses (Cox-Foster *et al.* 2007). Although *Nosema* and RNA viruses have been detected in bumble bees, relatively few studies have examined their combined effects on bumble bee health.

Building on previous field work I conducted, I will use molecular techniques to examine **1)** evidence for the emerging infectious *Nosema* species(*N. ceranae*) to outcompete the naturalized species (*N. bombi*) in bumble bees, **2)** patterns of coinfection of *Nosema* spp*.* and RNA viruses in wild bumble bee populations and **3)** temporal variation in pathogen load of these four infectious agents. To my knowledge, this study will be the first to address these knowledge gaps in bumble bee species. Considering coinfection in native pollinators is of vital importance as multiple infection is common and the repercussions are poorly understood.

**INTRODUCTION:** The documented decline of important pollinators has garnered much attention and concern in recent years. Bumble bees (*Bombus* spp*.*) in particular are important native pollinators whose decline has been relatively understudied compared to managed honey bee losses (van Engelsdorp et al., 2008). Certain plants, most notably of the genus *Solanum* (tomatoes, eggplant etc.), primarily rely on pollination provided by bumble bees as honey bees are incapable of the buzz pollination required by these plants (Strange, 2015; Thornsbury and Jerardo, 2012). Bumble bee declines in recent years have the potential to drastically disrupt the pollination services they provide and the industries that rely on them. Species such as *B. affinis, B. borealis, B. ashtoni, B. fervidus, B. pensylvanicus, and B. sandersoni* have decreased in abundance since the 1960s (Colla et al., 2012). In 2015, the state of Vermont listed two species of bumble bees as endangered (*B. affinis and B. ashtoni*) and one as threatened (*B. terricola*) (Vermont Fish and Wildlife Department, 2015). In 2017, *B. affinis* was the first bumble bee to be listed as federally endangered.

Pathogens, pests, and diseases are among the top threats to bumble bee species. Of particular interest, are *Nosema* spp. and several RNA viruses because the prevalence of these pathogens are linked to spillover events from commercial bees (Otterstatter et al., 2005; Fürst et al., 2014). The prevalence of *N. bombi* in declining bumble bee species increased dramatically between 1995 and 2010, coinciding with the importation of commercial bumble bees from Europe. More recently, *N. ceranae*, a pathogen of honey bees (*Apis mellifera*), may be emerging into bumble bee populations from honey bees (reviewed in Brown, 2017). In honey bees, *N. ceranae* outcompetes the honey bee’sunique species of *Nosema*, *N. apis* (A. Bourgeois et al., 2010; M. Natsopoulou et al., 2014). While both *N. bombi* and *N. ceranae* infect bumble bees, no studies have yet examined how these pathogens interact within the bumble bee host and whether coinfection of both species denotes higher mortality. In addition to *Nosema*, RNA viruses, once considered specific to honey bees, have been detected in bumble bees and evidence is accumulating that these viruses are spilling over from managed honey bees into wild bees (Fürst et al., 2014; Alger & Burnham, unpub. data). Symptoms of RNA viruses include behavioral abnormalities, inefficient foraging, deformities, abnormal queen cells and death (Chen, 2007; Schroeder and Martin, 2012; Graystock et al., 2015).

In honey bees, coinfection of multiple pathogens results in higher mortality and colony losses (Cox-Foster et al., 2007). However, similar studies in bumble bees are severely lacking. Although *Nosema* and RNA viruses have been detected in bumble bees, no studies have examined interactions between these pathogens within the bumble bee host. Furthermore, while many studies have examined how pathogens fluctuate through time in honey bees, very few studies have examined this in bumble bees (Rigaud et al., 2010). Filling these knowledge gaps is important because varying fluctuations in disease loads between pathogens might result in particular pathogens peaking in abundance simultaneously, increasing the probability of coinfection and synergistically exacerbating their effects (Burnham et al., unpub. data). As coinfections (multiple pathogens in one host) play an important role in honey bee losses (Cox-Foster et al., 2007), documenting these mechanisms in bumble bees is vitally important in understanding and mitigating population declines.

**OBJECTIVES:** Building on previous field work I conducted, I will use molecular techniques to examine: **1)** evidence for the emerging infectious *Nosema* species(*N. ceranae*) to outcompete the naturalized species (*N. bombi*) in bumble bees **2)** patterns of coinfection of *Nosema* spp. and RNA viruses in wild bumble bee populations and **3)** temporal variation in pathogen load of these four infectious agents. To my knowledge, this study will be the first to address these knowledge gaps in bumble bee species.

**IMPLICATIONS & BROADER IMPACTS:**

**Intellectual Impacts:** There has been a recent call for experts in infectious disease to apply a community ecology approach to studying host-parasite and multi-pathogen interactions (Johnson et. al., 2016). Considering coinfection in native pollinators is of vital importance as multiple infection is common and the repercussions are poorly understood (Rigaud et al., 2010). The study of infectious disease in bumble bees has been primarily concerned with the transmission, dissemination, and clinical signs of a pathogen. With this work, I aim to bridge disease ecology and conservation biology to ask how multiple pathogens interact, and how those interactions affect host health. This work will add to the growing body of peer-reviewed literature on both native bee conservation and the basic science of disease ecology.

**Impacts on Conservation:** In general, native bee declines are difficult to document due to the lack of historic records. However, Vermont is unique in that it has one of the best historic collections of bumble bee species dating back to 1915 (Vermont Center for Ecostudies, unpub. data). Surveys conducted 2012-2014 by the Vermont Center of Ecostudies confirmed that over half of our state's native bumble bee species are in decline. Among the many threats to our native bees, parasites and pathogens are serious concerns. Many have recognized a gap in our knowledge of the interactions of multiple pathogens within a host (Rigaud et al., 2010). Increased mortality due to coinfection poses a risk to the native bee community as well as managed honey bees. These pathogens, especially *Nosema* and the RNA viruses are spilling over into wild bee communities from managed bees (Fürst et al, 2014; Alger & Burnham, unpub. data). Understanding how pathogens interact and fluctuate with time in bumble bees will allow us to make better recommendations for honey bee management options, potentially decreasing honey bee disease instance and thereby reducing native pollinator declines due to spillover. The study I propose will contribute to our knowledge on the multiple threats affecting our native pollinators benefitting beekeepers, growers, bee communities and consumers alike.

2). **My goal is to advance our understanding of the mechanisms and outcomes related to coinfection by applying fundamental concepts of community ecology (i.e. competition theory) to the honeybee disease system.** I will focus on four honeybee pathogens known to adversely affect bee health: *Nosema ceranae* (a microsporidian parasite), *Varroa destructor* (a parasite mite) and two RNA viruses, deformed wing virus (DWV) and black queen cell virus (BQCV). **Using a combination of field surveys, laboratory experiments, and epidemiological modeling, I will examine: 1)** The importance of temporal variation in disease prevalence, disease load, and patterns of coinfection; **2)** How pathogens interact with each other once coinfection has occurred; **3)** Synergistic effects due to multi-pathogen interactions that may influence host mortality at both the individual and colony levels. In addition to furthering the study of disease ecology, my proposed research will enhance native and managed pollinator conservation efforts by improving our understanding of high-risk pathogen combinations that increase bee mortality.

**1) Temporal Variation and Patterns of Coinfection:** I will determine how four common honeybee pathogens fluctuate in prevalence and load through time, and how these fluctuations influence patterns of coinfection. ***Approach:*** In 2017, I sampled 32 individual colonies spread across three comparable field sites. I inspected and sampled each colony three times at four week intervals and analyzed each for the four previously mention pathogens (**Fig. 1**) using microscopy for *Nosema*, RT-PCR for RNA viruses, and determined *Varroa* mite loads using established methods (6). In addition, I measured colony population size and queen health (brood pattern) at each time point. ***Preliminary findings*** indicate that during times of increased pathogen prevalence, the probability of certain coinfections occurring increase significantly. Most notably, when *Varroa* is highly prevalent, colonies are more likely to have high BQCV and DWV loads.

**2) Pathogen Interactions:** I will determine how pathogens interact with each other in a coinfected host. ***Approach:*** Three of the four pathogens (*N. ceranae, Varroa* and DWV) have already been isolated. With the help of a collaborating virologist, I will isolate BQCV. I will conduct inoculation studies in the lab using field-realistic pathogen combinations identified from the field study in Obj.1. By measuring disease loads after coinfection, and comparing to known competition models, I will characterize how the primary infection reacts to secondary infection.

**3) Synergistic Effects and Host Mortality:** I will determine how multi-pathogen interactions affect host health and mortality at both an individual and colony level. ***Approach:*** I will select a subset of pathogen combinations from Obj. 2 and conduct inoculation experiments using small experimental colonies. Individual mortality and colony loss will be recorded. Bees will be assayed for final pathogen loads and sub-lethal health effects such as learning and activity.

**Chapter 1: Survey of *Nosema* in Vermont Bumble bees to parameterize an epidemiological model of how prevalence of *Nosema* changes through time based on fecal deposition.**

*Brief description: In this chapter I aim to collect bumble bees from multiple sites in Vermont and look at patterns of Nosema infection in order to parametrize a model that shows prevalence of infection through time based on parasite deposition and an oral-fecal transmission route.*

**Figures included: Panel of Data from survey, Model diagram, Model Equations, Model figure**

**Current relevant material:**

**METHODS: Data Collection and Analysis:**

Over 350 bumble bees were collected in northern Vermont from 13 different field sites during the summer of 2014. The bees were netted randomly while foraging on flowers. Queens and males were caught as well as workers. The bees were put on dry ice in the field and were transferred to a -80oC freezer within 12 hours of being captured. At each site, bee abundance and vegetation surveys were performed on 100m transects. In addition, forging honeybees were netted and pollinator friendly flowers collected at each site. GPS coordinates, elevation, weather conditions, and nearest town were also logged at each location.

In order to assay each bee for *Nosema*, the ventriculus was dissected from the bee by pulling on the last segment (terga) of the abdomen. The ventriculus for each bee was then homogenized in 500uL of GITC buffer with a polypropylene pestle for one minute. These were then vortexed and 10uL of the homogenized bee gut were put into each chamber of a hemocytometer. Counts were made of the *Nosema* spores present using a traditional Neubauer® counting grid and the two chambers were averaged together resulting in a total count.

These data were analyzed in R using a contingency table and a Pearson’s Chi-squared test. Tables of the independent variables (species, caste and honeybee proximity) by the presences/absences Nosema data (binary) were created. Infected and uninfected vectors were created and bound into a matrix using the function, “rbind()”. The tests were performed using the “chi.sq()” function and mosaic plots were created using the “mosaicplot()” function.

**Epidemiological Modeling:**

Figure 1: The beta and gamma terms were derived from the empirical data of the survey. Beta is the conversion rate from susceptible to infected. Gamma is the conversion rate from infected to critically infected (diseased state). Beta=(#infected/total#), gamma=(#critically infected/#infected). The death rates (muA and muB), fecal deposition rates (alpha1 and alpha2) were estimated, as was the initial rate of infection (I1).

This model describes the dynamics in this parasite system. Parameters were calculated or estimated depending on availability of data to describe the dynamics of the infection rate (Fig. 1). Terms were developed to describe the system. The system of equations that makes up the model is shown below:

dS =−SPβ−SμA dt

dI1 =SPβ−I1μA−I1γ dt

dI2 =I1Pγ−I2μB dt

dP =I1α1+I2α2−Pθ dt

The equations were solved using an R package called “deSolve”. This eliminated the need for for-loops. Vectors for initial values and parameters were created and a time sequence of 150 days was set up to replicate the period that bumble bees are active in a temperate climate. The infected and critically infected vectors were subtracted from 1 to represent the susceptible population. These vectors were bound in a matrix and plotted using “matplot()”.

**Data Collection and Analysis**

The prevalence of *Nosema* was found to be 20.2% across all species and castes. The critically infected bees comprised of 5% of all infected bees. There was found to be variability among species. *B. vagans* showed the lowest susceptibility to *Nosema* while *B. borealis* and *B. ternarius* showed the highest (Fig 2). However, this variability was found to be insignificant using a contingency table with a Chi-squared test (p=0.299) and we failed to reject the null hypothesis that prevalence across species is the same. When looking at prevalence by caste, we found that the percent infected was highest in males and lowest in workers contrary to our hypothesis. However, the sample size of males was significantly lower than the other two castes (Fig. 3). The

differences were also found to be insignificant. The results of a Chi-squared test yielded a p-value of 0.468. Proximity to honeybees was also found to be statistically insignificant. Though prevalence was higher in bumble bees caught near honeybee apiaries, the Chi-squared test showed that the difference was minimal (p=0.481) (Fig 4). As these three factors appeared to show little effect upon the prevalence of *Nosema*, a more simplified model of this system (ignoring caste and species effects on infection) could be created using the total prevalence of the infected bees and total prevalence of critically infected bees as rates in an elementary SIR-style model.

Figure 2: The prevalence of *Nosema* (binary variable) plotted against the five most common *Bombus spp.* in this data set (*B. bimaculatus, B. borealis, B. impatiens, B. ternarius and B. vagans*). This mosaic plot shows the infected and uninfected proportions of each species of bumble bee. A Chi-squared test yielded and insignificant p-value (p=0.299) indicating no difference between species.

Figure 3: The prevalence of *Nosema* (binary variable) plotted against the three castes (males, queens and workers). This mosaic plot shows the infected and uninfected proportions of bees in each caste. A Chi-squared test yielded and insignificant p-value (p=0.468) indicating no difference between castes.

Figure 4: The prevalence of *Nosema* (binary variable) plotted against the honeybee proximity (near an apiary/far from an apiary). mosaic plot shows the infected and uninfected proportions of bees near and far from an apiary. A Chi-squared test yielded and insignificant p-value (p=0.481) indicating no difference in parasite prevalence due to honeybee proximity.

**Epidemiological Modeling**

The model recreated the dynamics at play within this system. The relationship between infected and critically infected bees as a result of shedding on the landscape in the form of fecal deposition on flowers was shown as a function of time (early spring to fall). The model recreated an early spring outbreak with the infected prevalence peaking at around 20%. The critically infected curve showed a tendency to occur later in the season after the infected curve had reached its apex (Fig. 5). The initial prevalence of the parasite (time step 0) was estimated at 5%. However, changing this value had significant effects on how the course of the outbreak proceeded. If less parasites survived the winter inside hibernating bumble bee queens and the initial rate of infection was lower the following year (1%), the infected curve occurred much later in the year peaking at only around 0.17. The critically infected bees also were shown to exhibit a lower prevalence in this case and were even further shifted to the right (Fig 6). If the initial rate of infection were raised to 10% to indicate a higher parasite survival at the last time step of the preceding year, both rates of infection followed similar patterns however the curves peaked at higher values and the outbreak occurred earlier in the year.

Figure 5: The infection rate through time beginning in the early spring and ending in the early fall. The susceptible population is shown in green and the infection rate in blue. The critically infected (diseased state) rate is shown in red. The parameters for this model are: beta=0.202 (data), gamma=0.05 (data), I1=0.05

(estimated).

Figure 6: The infection rate through time beginning in the early spring and ending in the early fall. The susceptible population is shown in green and the infection rate in blue. The critically infected (diseased state) rate is shown in red. The parameters are set to: beta=0.202 (data), gamma=0.05 (data). I1 (initial infection rate) is being changed from lower value (0.01) on the right to a higher value (0.10) on the left. The higher value results in a higher rate of infection that occurs earlier in the year.

**DISCUSSION:**

This survey gives valuable insight into a relatively understudied parasite in a very important host organism. The data seem to suggest that though there is some variation in *Nosema* prevalence across species, caste and proximity to honeybees, the parasite is ubiquitous and seems to be equally prevalent regardless of these variables. The 20.2% prevalence that we found also appears to be relatively high for this parasite, which was found in much lower abundance in a 2006-2007 survey (Kissinger et al., 2011). It is possible the limited sample sizes for male bees might have lowered the sensitivity of the test and perhaps a power analysis might be prudent to attempt to discover an optimal sample size for future surveys. It is also true that while our study didn’t show a significant difference in prevalence in terms of these three variables, it is possible that

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future analysis of the actual count data might yield significant results. However, this study does provide useful pilot data that gives us a general idea of how to approach these questions and allows us to parameterize a model. Modeling improves our understanding of the system dynamics and helps us to look at new questions posed by the model.

The model describes a *Nosema* outbreak during a 150 day period ranging from early spring to mid fall. The infection peaks in early spring as described by the literature. The way that the critically infected population grows after the infected population peaks seems to describe the latency period where certain infected bees might become critically infected (Fig. 5). The part that initial infection rate at time step 0 plays seems to be of critical importance to the system. Fluctuations in that value change the temporal pattern and intensity that the outbreak follows (Fig. 6). Though the initial infected population is estimated, it is a good example of a question posed by the model that might be important to the overall system. The number of infected bees that survive the winter and begin the infection anew the following spring could be determined experimentally and would help add to our understanding of the way *Nosema* persists on the landscape on a year to year bases.

This study both helps us on the path to understanding some important question about this parasite, but also asks many others. Since *Nosema* seems to be so prevalent on the landscape and commonly found across northern Vermont, we might consider the increased probability of co-infection. Since the probability of being infected with *Nosema* is higher than previously thought, the chances of an infected bee coming into contact with a different pathogen such as an RNA virus might also increase. Viruses like Deformed Wing Virus (DWV) and Black Queen Cell Virus (BQCV) cause behavior abnormalities, inefficient foraging, wing deformities, abnormal queen cells and death (D. Schroeder and S. Martin, 2012; P. Graystock et al., 2015). As there are also two species of *Nosema* that infect bumble bees, and competition has been shown to exist between *Nosema spp.* in honeybees (A. Bourgeois et al., 2010), a similar interaction might exist in bumble bees. In the future we will examine if co-infection between *Nosema spp.* and RNA viruses (DWV and BQCV) leads to synergistic effects that further compromise bee health. We will also examine if the introduction of the invasive parasite *N. ceranae* has led to interspecific competition with *N. bombi*.

**Chapter 2: Field survey of *Apis* and *Bombus* pathogens through time to understand what field-realistic pathogen combinations are and how these patterns of co-infection change through time.**

*Brief description: This study examines how pathogens operate in a natural setting in order to parametrize models of coinfection and inform controlled lab inoculation experiments in chapter 3. Are pathogens in this system static or dynamic? What are common coninfections for Bombus and Apis pathogens and how do those patterns change through time. Does temporal variation have an effect on the order or level of coinfection?*

**Figures included: BQCV figure, Coinfection Figure, table of Bombus collected**

**Current relevant material:**

***Methods:*** In 2018, I will conduct assays to examine pathogen load and prevalence in 400 bumble bee specimens I collected in my field survey. I have already developed all laboratory protocols and primers necessary for the proposed work. For *Nosema* spp., I will dissect a portion of the gut and use microscopy to examine each specimen for *Nosema* spores. On samples I confirm to be positive, I will extract DNA from the sample and use quantitative polymerase chain reaction (qPCR) to differentiate between the *Nosema* species. Results will provide evidence of whether the newly emerging species, *N. ceranae*, has begun to outcompete *N. bombi*. For RNA viruses,I will extract RNA from all specimens and use qPCR to detect and quantify RNA viruses: Lake Sinai Virus (LSV) and Black Queen Cell Virus (BQCV). I chose these two viruses because both are present in Vermont and previous research in honey bees suggests a tight association with *Nosema* spp. (Bailey et al., 1983; Traynor et al., 2016). Results will inform future laboratory experiments where I plan to experimentally test pathogen interactions and effects in captive bumble bee colonies. By using statistical analyses (repeated measures ANOVA and generalized linear models), I will examine patterns in viral and parasite load between these four pathogens across time. Using these data as well as data from 2015, I will quantify fluctuations in the prevalence and pathogen loads throughout the course of the season. Lastly, I will use my skills as a computational biologist to mathematical model my results in bumble bees and patterns previously observed in honey bees to examine differences in this multi-pathogen, multi-host system.

**Chapter 3: Lab inoculation experiments for RNA viruses and *Nosema spp*. in Bumble bees.**

*Brief description: When a host is coinfected, how do the pathogens respond to each other and how does the host respond. Does the order, load and type of pathogen matter in terms of influencing pathogen reproduction and host health.*

*Experiments Planned (Nosema – Nosema, Nosema-Virus, Virus-Virus):*

1. *N. ceranae vs. N. bombus in bumble bees*
2. *N. ceranae vs. Deformed Wing Virus*
3. *Deformed Wing Virus vs. Kashmir Paralysis Virus*

**Figures included: experimental design figure, figure of potential outcomes and explanation**

**Current relevant material:**

**3) Examine interactions and effects of multiple pathogens on bumble bee hosts.** As we start to gather more information on spillover from managed honey bees to native bumble bee populations examining how combinations of these shared pathogens is vitally important (Fürst *et al.* 2014). Synergistic effects resulting from pathogen-pathogen interactions may arise if stress from one pathogen results in immune suppression allowing for higher pathogen loads and increased probability of secondary infection (Toplak *et al.* 2013). This may influence host mortality at both the individual and colony levels. In addition, the presence of both *N. ceranae* and *N. bombi* in the population could lead to competition, as seen in honey bees, resulting in increased prevalence of the more virulent pathogen (Natsopoulou *et al.* 2014).

**Approach.** To measure the effects of pathogen(s) on bee health, we will select a subset of *Nosema*/RNA virus combinations identified from our field study and conduct inoculation experiments at the colony level using micro-colonies and measure mortality and brood production. By measuring disease loads after coinfection, we will examine how pathogens interact within a host and characterize how the primary infection reacts to secondary infection. In particular, we will conduct competition experiments between the two species of *Nosema* in order to examine the potential for *N. ceranae* to outcompete *N. bombi* within a bumble bee host*.*