**How do macro-level disease dynamics influence patterns of coinfection and resultant pathogen-pathogen interactions?**

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**Introduction:**

In the study and prevention of human disease, the practice of modeling diseases outbreaks using various agent-based and ordinary differential equation-based strategies has been used to great effect for many years. Computational recreation of a disease system affords the scientific community with the opportunity to better understand disease dynamics and increases our ability to predict how an outbreak will behave in a future population, respond to a treatment or be affected by some other abiotic or biotic factor. While these practices have been used to great effect in the epidemiological approach to infectious human disease, disease in other systems could greatly benefit from this under-utilized approach. In addition to enabling us to better understand the dynamics of a multitude of other disease systems in important non-human hosts, modeling non-human disease systems allows the scientific community to parameterize models using relatively more attainable empirical data. An ideal system would be easily manipulated and exhibit diverse pathogen and parasite types. It would have analogues for human spillover events, coinfections, transmission routes and share the dynamical complexity and population structure found in human disease.

One such system is that of the bumble bee (*Bombus Spp.)* and its many taxonomically distinct pathogens and parasites. The documented decline of important pollinators has garnered much attention and concern in recent years. Bumble bees in particular are important native pollinators whose decline has been understudied in light of managed honeybee losses (van Engelsdorp et al., 2008). Around 50% of bumble bee species have decreased in abundance since the 1960s (Colla et al., 2012) and as of 2016, *B. affinis* was the first bumble bee to be federally listed as endangered. In addition to their agricultural value, their eusocial behavior and population structure in conjunction with the rich diversity of pathogens and parasites they host make them an ideal candidate system for studying population level and spatial transmission and dissemination dynamics.

Pathogens and parasites 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 behavior, 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 as 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.

The bee-pathogen system affords epidemiologists with a model system capable of extrapolating model parameters to other complex dynamical disease systems. Bumble bees have been used as a model organism in behavioral ecology and data from bumble bees in the field have informed many of the models used in optimal forging theory. The hierarchical levels of interaction space in the bumble bee system mirror those of humans, making bumble bees an ideal model organism for parametrizing robust general models for disease transmission and dissemination. The individual bee is a stand in for an individual human, the colony as a city populated by individuals, and a field of colonies as a network of interconnected cities. Using this approach, human disease outbreaks as well as emerging infectious disease in other animal systems can be modeled by adjusting parameters accordingly.

Studying spatial dissemination, coinfection and resultant pathogen-pathogen interactions in this bumble bee system, replete with diverse pathogen types, spillover events and human-analogue transmission routes, affords the scientific community with an opportunity to better understand this complex dynamical network helping to push the fields of disease ecology and epidemiology forward. In order to address the above mentioned knowledge gaps, I aim to use a combination of empirical data and epidemiological models to examine **1)** the prevalence of *Nosema* in bumblebee populations **2)** how pathogens spillover from one population into another and disseminate throughout the environment at the individual, colony and colony network levels, **3)** what common coinfections occur innature andwhat are the main drivers behind co-infection in the bumble bee **4)** and finally, testhow pathogens interact with each other and their host environment once coinfected.

**Chapter 1**

***Objective:***

***Methods:***

*Completed Work:* 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.

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. 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, “deSolve”. 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.

*Remaining Work:*

***Preliminary Findings:*** 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.

**Chapter 2**

***Objective:***

***Methods:***

*Completed Work:* To determine the amount of purified DWV virus isolate to infect our experimental flowers with, an absolute quantification qPCR experiment was conducted on composite samples of wild flowers collected near honey bee apiaries. In addition, a single flowers that were positive for BQCV, a similar RNA viruses were also assayed. Viral loads in genome copies per flower were calculated by diluting extracted RNA to a constant concentration and normalizing by flower mass. It was determined that the composite samples carried a maximum DWV load of 700,000 genome copies per gram of flower material and the single flowers had a maximum BQCV load of 200,000 genome copies per flower. An acceptable realistic range was then determined to be between 100,000 and 1,000,000 genome copies per flower. In order to test our inoculation protocol on real flowers, 10uL of 100,000 copies/uL active virus solution were placed inside the nectaries of a 4 flowers. We conducted absolute quantification qPCR experiment on the flowers as described for the collected wild flowers with a mean of ~1,000,000 copies per flower detected.

*Remaining Work:*

***Preliminary Findings***

**Chapter 3**

***Objective:***

***Methods:***

*Completed Work:* 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.

*Remaining Work:*

***Preliminary Findings***

**Chapter 4**

***Objective:***

***Methods:***

***Preliminary Findings***

Chapter 1 Figures:

Chapter 2 Figures:

Chapter 3 Figures:

Chapter 4 Figures: