PREVALENCE AND CROSS INFECTION OF EUKARYOTIC AND RNA PATHOGENS OF HONEY BEES, BUMBLE BEES, AND MASON BEES.

by

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Bachelor of Science
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DEDICATION

This is dedicated to my father, who passed away in late 2018. He is responsible for the love of science that I have today.

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ABSTRACT

PREVALENCE AND CROSS INFECTION OF EUKARYOTIC AND RNA PATHOGENS OF HONEY BEES, BUMBLE BEES, AND MASON BEES.

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George Mason University, 2020

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Pollinators worldwide are in decline, and honey bees (*Apis mellifera*), bumble bees (*Bombus* spp.), and mason bees (*Osmia* spp.) are no exception. Research implicates pollinator pathogens as one of the main reasons for decline, and studies suggest shared floral resources and spillover from commercially managed bees as mechanisms for the spread of infection. The goal of my research was to document the prevalence of viral infection and potential for cross-infection of bee pathogens in local populations of honey bees, bumble bees, and mason bees in the Northern Virginia and Northern Shenandoah area. I sought to observe the presence or absence of two groups of eukaryotic pathogens (*Nosema* spp. and Trypanosomatids) and the levels of infection of three RNA viruses (acute bee paralysis virus (ABPV), black queen cell virus (BQCV), and deformed wing virus (DWV).

Overall, 166 bees were sampled for DNA analysis. Sixty three percent of bees collected for DNA analysis were infected with at least one pathogen. *Nosema* spp. were found in 12.7% and trypanosomatids in 60.2% of samples. Mason bees are poorly studied compared to *Apis* and *Bombus*; this research is the first instance of recorded *Crithidia bombi* infections in mason bees. I sampled 136 bees for RNA analysis. Results indicated that 84.9% of bees collected for RNA analysis were infected with at least one virus, 39.7% of bees were infected with two viruses, and 19.9% were infected with all three viruses tested. BQCV was the most prevalent of the three viruses with 75% of bees infected.

In my study, high levels of prevalence were found in all samples. Infection was widespread across all sites tested, and a higher percentage of bees were infected with at least two viruses than bees infected with just one virus. This is one of the first studies to example the prevalence and incidence of multiple eukaryotic and RNA pathogens in Northern Virginia and the northern Shenandoah region.

CHAPTER 1 | INTRODUCTION

The Importance of Pollinators

The European honey bee (*Apis mellifera),* bumble bees (*Bombus* spp.), and mason bees (*Osmia* spp.) are important worldwide pollinators. An estimated 87% percent of flowering wild plants require pollination by insects (Ollerton *et al.* 2011). Insect pollination is required for 75% of crops, whereas other crops significantly benefit from pollination (Klein *et al.* 2007). Honey bees pollinate large numbers of flowers due to their large colony sizes and numbers. Melittologists attribute a large part of the importance of honey bees to the perennial nature of colonies and the transfer of information on floral resources among foraging workers. Yet, their perennial nature provides ample opportunity for persistence and transmission of pathogens. As social species', bumble bees are also excellent pollinators, and they exceed honey bees in various pollination performance categories such as pollen disposition and purity of the pollen load (Bushmann *et al.* 2012). Yet, the two genera show large differences in colony size and persistence. Honey bee colonies reach sizes of >20,000 individuals, workers may overwinter, and queens may live up to four years but do not forage. Bumble bee colonies, on the other hand, have fewer than 1,800 workers (del Castillo *et al.* 2015), but queens do forage early in spring and live less than a year. Nonetheless, many bumble bee species appear to be experiencing declines like those attributed to colony collapse disorder in honey bees (Potts *et al.* 2010).

Mason bees exhibit different behavior than the social behavior exhibited by honey bees and bumble bees. Mason bees do not live in hives but in solitary nests. Female mason bees lay their eggs, along with collected pollen, in narrow spaces and separate each egg with a loam wall (Keller *et al.* 2013). Until hatching, and chewing through the nest material, offspring do not contact the external environment, apart from the collected pollen. The microbiome of mason bee larvae was found to be quite diverse, yet most of the sequences found happened to be incidental distribution of environmental samples occurring the during the nest building and egg laying process (Keller *et al.* 2013). However, many different bacterial families were found in the microbiome, and several known pollinator bacterial threats were sequenced. These included *Bacillus Cereus, Bacillus thuringiebsis,* and two *Paenibacillus larvae* subsp. (Keller *et al.* 2013). Though a handful of studies look for other pathogens in mason bees, they remain understudied in terms of infection by pathogen similar implicated in losses of *Apis* and *Bombus* populations.

In the United States, honey bees have declined from around 6 million colonies in 1947 to 2.5 million colonies in 2015 (Torres *et al.* 2015). Several bumble bee species in the United States are experiencing sharp declines as well, such as *B. occidentalis, B. pensylvanicus, B. affinis, and B. terricola* (Cameron *et al.* 2011). Over the winter of 2007-2008, approximately 19% of the United States' colonies were observed, and 35.8% of those observed colonies were lost (vanEngelsdorp *et al.* 2008). Honey bees are declining not only in the U.S. but also worldwide. Out of an approximate 2.44 million honey bee colonies in the United States, an estimated 0.75 to 1 million colonies of honey bees were lost during the winter months of 2007-2008. Colony Collapse Disorder (CCD) is a term that is used to

describe the large-scale loss of honey bee hives (vanEngelsdorp *et al.* 2008). CCD occurs when the loss of honey bee adults surpasses the rate of reproduction. Yet, these CCD colonies show no dead bees within the hives as well as an abundance of food stores (Cox-Foster *et al.* 2007). This food is not stolen or used by other bees but are abandoned (Tantillo *et al.* 2015).

Drivers of Pollinator Decline

It is important to understand the various patterns of prevalence that these pathogens may have in pollinators. Pathogens are considered drivers of decline in pollinators: however, there are numerous factors that contribute to the spread of disease in managed and wild bees. Although this paper focuses primarily on the prevalence and incidence of pathogens, three potential drivers of pathogen spread leading to pollinator decline, are discussed below.

Transmission of pathogens through Shared Resources

In the United States, honey bee commercial colonies are transported from the Northern Great Plains region and are moved across the United States to pollinate various food plants, such as almonds, apples, and cherries (Otto *et al.* 2016). Honey bees interact with and visit the same flowers as native bees, but this competition does not appear to affect the foraging rate or success of either taxon (Steffan-Dewenter and Tschantke 2000). Nonetheless, the presence of both bees in the same area can lead to cross infection of pathogens (Durrer and Schmid-Hempel 1994, Singh *et al.* 2010).

Wild flowers have been proposed as possible vectors of pollinator pathogens. Uninfected bumble bees were shown to become infected with *C. bombi,* after visiting

flowers which had been previously visited by infected bumble bees. The transmission of *C. bombi* occurred at the flowers, through *C. bombi* infected feces (Durrer and Schmid-Hempel 1994). In a field study, honey bees deposited DWV and BQCV across three different plant species. It was found that flowers were more likely to host viruses when the honey bees visited for relatively longer periods of time. However, in their experiment bumble bees did not become infected after visiting flowers previously visited by honey bees or after contact with the honey bees themselves (Alger *et al.* 2019).

Spillover from managed bee populations

Like honey bees, commercialized *Bombus impatiens* are widely used in North America to help offset the loss of wild bees. Bumble bees are mass produced and used in large scale greenhouses. However, these bees often escape and collect most of their pollen from outside the greenhouse (Colla 2006). Commercially grown bumble bees have more pathogens than wild populations of bees. These pathogens include the parasite *Crithidia bombi* and the mite *Locustacarus buchneri* (Colla 2006). Pathogen spillover from contact between wild bees and commercialized bees has been shown to spread Emerging Infectious Diseases (EIDs). It was found that *C. bombi* had infected bumble bees that forage near commercial greenhouses, but the parasite was not present in bumble bees that do not forage near greenhouses (Colla 2006). Another pathogen, *Nosema bombi*, had triple the prevalence in bumble bees found near greenhouses (Colla 2006). Managed honey bees also can influence wild bumble bees. McMahon *et al.* (2015) found that managed honey bees had a positive correlation on the prevalence of BQCV, ABPV, and DWV in wild bumble bees. In the United Kingdom, *B. hypnorum*, an invasive bumble bee species, was found to

have higher rates of infection of high impact diseases than the native species (Jones and Brown 2014).

Mason bees are also affected by the common honey bee viruses. Ravoet *et al.* (2014) tested the presence of several viruses in honey bees and solitary bees including two species of mason bees, *O. bicornis* and *O. cornuta*. They found DWV, BQCV, and three other viruses in solitary bees. *Nosema ceranae* was found in both mason bee species (Ravoet *et al.* 2014). *Crithidia bombi* has also been found in *O. cornuta* (Schoonvaere *et al.* 2016) and *O. bicornis* (Ravoet *et al.* 2014). Müller *et al.* (2019) also found *N. ceranae* can infect *O. bicornis* but found the survival rate of the mason bees was not negatively affected. In a laboratory setting, *O. bicornis* were inoculated with *N. ceranae* and while larvae treated with *N. ceranae* spores did experience high rates of mortality, viable spores were detected in only low amounts, suggesting *N. ceranae* did not easily establish itself in the mason bees (Bramke *et al.* 2019). Honey bees colonies are large and live in enclosed conditions, which may support the spread of pathogens. Because of their large size, it is possible that they are the source of many pollinator pathogens.

Parasitic Mites

Varroa destructor (*Varroa* mite), a parasitic mite, is a well-known vector of honey bees diseases. *Varroa* mites infect larvae by entering the cell before it sealed and spend their whole life with the bee. They emerge with the bee (Shen *et al.* 2005) and may travel with the bee when it flies to flowers or other colonies. *Varroa* mites are established vectors of a multitude of RNA viruses (Tentcheva *et al.* 2004, Shen *et al.* 2005, Chen *et al.* 2015). Overall, *Varroa* mites have been found to act as a vector for acute brood paralysis virus

(ABPV), black queen cell virus (BQCV), deformed wing virus (DWV), and sacbrood virus (SBV) (Chen and Sieve 2007, Mondet *et al.* 2014). It is not exactly clear how *Varroa* mites transmit viruses to bees. *Varroa* may be a natural reservoir in which DWV constantly lives and reproduces, as viruses have been found in mite saliva (Zheng and Han 2018) and in mites themselves (Tentcheva *et al.* 2004). High *Varroa* loads lead to high viral loads but infection can spread throughout a colony with low numbers of mites (Francis *et al.* 2013). *Varroa* mites carrying ABPV and DWV are more dangerous for honey bee colonies in the fall seasons (Sumpter and Martin 2004). DWV is the virus most strongly associated with *Varroa*. In New Zealand, five viruses were tested to determine if the prevalence of these viruses increased when *Varroa* was present (Mondet *et al.* 2014). These viruses were also detected in the mites themselves. Of the five viruses tested, all five were found to have increased prevalence with the presence of *Varroa* mites. The difference was significant for DWV, SBV, and Kashmir bee virus (KBV) but not for BQCV and chronic bee paralysis virus (CBPV). Apart from a single colony, DWV was not found in any area where *Varroa* was not found (Mondet *et al.* 2014).

Study System and Research Goals

Due to the complexity of the interacting factors outlined above, many questions remain about the potential for spillover of diseases from commercial or introduced species. *Varroa* mites only infect honey bees. However, these mites, along with other factors, facilitate or exacerbate viral spread within and across species. The goal of my research was to answer the following questions: What is the prevalence of pollinator pathogens across the Northern Virginia region? Is the region as a whole experiencing widespread infection

by these pathogens? I hypothesized that eukaryotic pathogens known to infect honey bees will not be found in bumble bees and mason bees, and vice versa. While some research has found *N. ceranae* (originally described in the eastern honey bee, *Apis cerana*) in bumble bees, other *Nosema* spp. have not been found to infect other bees outside of their typical hosts. Next, because previous research found correlated infections between sets of pathogens, including correlations between BQCV and *Nosema spp.* and between DWV and ABPV, I asked if eukaryotic and RNA pathogens, that were found to occur in multiple species, will exhibit these patterns at a site wide level, similar to those previously reported. I hypothesized that these correlations will be found in the Northern Virginia samples. I also hypothesized high rates of co-infection of RNA viruses and widespread prevalence of pathogens will occur.

To that end, I collected and analyzed over 300 individual species from 10 different Northern Virginia pollinator communities. Northern Virginia was an ideal location to examine disease spread for several reasons: it is a diverse area with 13 known native species of bumble bees (Malfi and Roulston 2014), there is severe decline in two species of *Bombus* that were once common throughout Eastern United States, *B. pensylvanicus* and *B. affinis* (Cameron *et al.* 2011), and prior research in the Northern Virginia area, gives estimates of 7.3% and 17.4% of captured bumble bees (n = 835) being infected with *Nosema* and *Crithidia* respectively (Malfi and Roulston 2014).

In the summer of 2018, honey bee, bumble bee, and mason bee individuals were collected from 10 sites in the Northern Virginia region. Five of the visited sited were privately owned homes where the honey bees were managed by local beekeepers. The other

five locations were Blandy Experimental Farm, The Clifton Institute, The Piedmont Environmental Council, St. Benedicts Monastery, and George Mason University - Fairfax campus. In 2019, three sites (Blandy Experimental Farm, The Clifton Institute, and The Piedmont Environmental Council) were visited again for more samples for eukaryotic analysis. Given the differences in methodology for finding and sequencing eukaryotic pathogens (storage in -20°C freezer, DNA extraction, PCR, and DNA sequencing) versus RNA viruses (storage in liquid nitrogen tank and -80°C freezer, RNA extraction, and RT-qPCR), the methods, results, and discussions are addressed separately for eukaryotic and viral analysis. Sampling locations are provided in Figure 1 and sample sizes are provided in Table 1. Specific breakdowns of species caught per site for eukaryotic and RNA analysis can be found in their respective method and material sections.

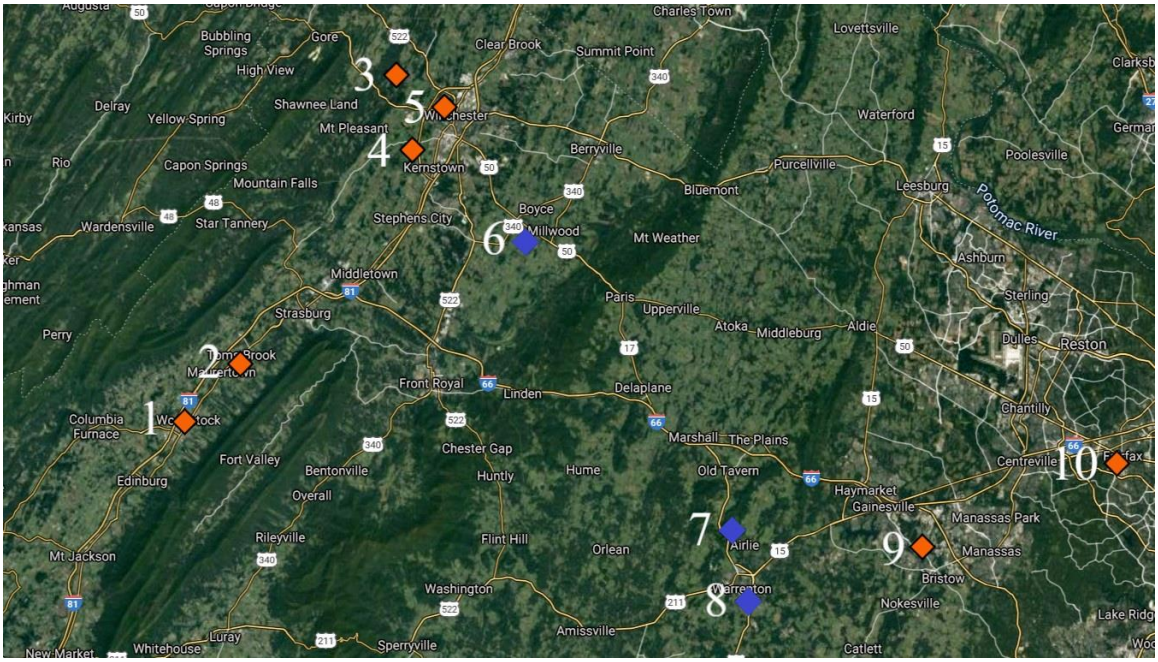

Figure 1 | Map of sampling sites in Northern Virginia. Orange diamonds are sites where bees were collected in 2018. Blue diamonds represent sites visited in both 2018 and 2019. Sites are numbered from west to east, and numbers correspond to descriptions in Table 1.

Table 1 | Numbers of specimens collected per site. Numbers in parentheses represent samples collected in 2019.

Site Number	Location Name	Latitude/ Longitude	Number of individuals collected for eukaryotic analysis	Number of individuals collected for RNA analysis
Site 1	Private Residence – Woodstock, VA	38.87, -78.50	1	12
Site 2	Private Residence – Maurertown, VA	38.92, -78.42	3	13
Site 3	Private Residence – Winchester, VA	39.22, -78.23	15	17
Site 4	Private Residence – Winchester, VA	39.14, -78.22	1	11
Site 5	Private Residence, Winchester, VA	39.19, -78.16	0	6
Site 6	Blandy Experimental Farm	39.06, -78.06	27 (12)	38
Site 7	The Clifton Institute	38.77, -77.80	25 (21)	21
Site 8	The Piedmont Environmental Council	38.71, -77.79	0 (13)	8
Site 9	St. Benedictine Monastery	38.75, -77.56	36	10
Site 10	George Mason University – Fairfax campus	38.83, -77.31	12	0

CHAPTER 2 | EUKARYOTIC PATHOGENS

Introduction

Numerous pathogens infect pollinators: parasites, bacteria, fungi, and viruses can severely infect and kill pollinators. Some of these pathogens, such as *Nosema* and Trypanosomatids, have been studied more often than others. *Nosema* is genus of obligate, intracellular microsporidians that has several different species that infect bees. *Nosema apis* and *N. ceranae* primarily infect honey bees (Klee *et al.* 2007) whereas *N. bombi* primarily affects bumble bees (McIvor and Malone 1995). *Nosema* is thought to be easily transmitted between bees, with transmission primarily through the oral-fecal route (Graystock *et al.* 2016, Chen *et al.* 2007). However, *N. ceranae* and *N. apis* found on pollen remain infectious (Graystock *et al.* 2013b). A recent study found infectious *Nosema* spores from feces can be carried by wind and infect other bees (Sulborska *et al.* 2019). *Nosema ceranae* and *N. apis* are closely related species, but *N. ceranae* is thought to have more deleterious effects on honey bee colonies (Paxton *et al.* 2007, Higes *et al.* 2009), although not all studies have shown similar findings (Forsgren and Fries 2010).

Trypanosomatids are flagellated parasites, some of which are known to infect humans. One such example is the causative agent of Chagas disease, *Trypanosoma cruzi*. Trypanosomatids that are known to affect bees are all in the Leishmaniinae subfamily (Kostygov and Yurchenko 2017). *Crithidia mellificae* primarily infects honey bees (Langridge and McGhee 1967) while *C. bombi* and *C. expoeki* primarily infect bumble bees (Tripodi *et al.* 2018). Trypanosomatid infection is widespread throughout the world

and wide ranges of infection levels are common. For example, in a two-year study in Massachusetts, *Crithidia* rates jumped from 24% in the first year to 51% in the second year, with some individual sites reporting infection rates has high as 80% (Gillespie 2010).

These pathogens are already suspected to be worldwide but there is little record of the presence of these pathogens in the Northern Virginia region. Increased knowledge of the prevalence of *Nosema* and *Crithidia* provides information for not only the locations that were sampled, but also helps other nearby apiaries. These nearby apiaries can use these data to make judgments about what possible pathogens may be infecting their hives and appropriate remediation efforts. Outlined below are the specifics of each type of pathogen.

Fungi

Nosema apis

Nosema apis, a parasitic microsporidian, is one of the most common diseases found to infect *A. mellifera* (Paxton 2009). *Nosema apis* was first isolated in honey bees by a German scientist (Zander 1909), although *N. apis* has now been detected worldwide. The microsporidian is transmitted horizontally, via spore ingestion (Fries *et al.* 1996), and causes nosemosis, a disease-causing diarrhea-like symptoms and a general weakening of the bee. *Nosema apis* has only been found in honey bees (Graystock *et al.* 2016).

Nosema bombi

Nosema bombi is closely related to *N. apis* and for years was thought to be the same species (McIvor and Malone 1995). Electron microscopy and structural analysis revealed that the two were separate species (McIvor and Malone 1995). However, *N. bombi* mostly

infects bumble bees, not honey bees (McIvor and Malone 1995), and primarily infects the bumble bee queen, although it does not cause queen death or seriously affect queen health (Fisher and Pomeroy 1989). For bumble bee workers, however, *N. bombi* is more virulent than *N. apis.* In a laboratory setting, worker bumble bees infected with *N. bombi* showed significantly higher mortality than uninfected bees, with far fewer infected bees surviving past 21 days (Otti and Schmid Hempel 2007). Infected males had decreased fitness in part due to lower sperm count and lower survival (Otti and Schmid-Hempel 2007). A year later, the same authors performed a field experiment testing the effects on *N. bombi* on *B. terrestris* and found overall colony fitness was lowered (Otti and Schmid-Hempel 2008). *Nosema bombi* and *N. ceranae* infected pollen can transmit *Nosema* to other bumble bees and from honey bees to bumble bees (Graystock *et al.* 2013b). In two declining bumble bee species in the United States, *B. occidentalis* and *B. pensylvanicus*, significantly higher levels of *N. bombi* were found when compared to more stable species (Cameron *et al.* 2011). In a separate study, *B. pennsylvanicus* and *B. fervidus* had significantly higher infection rates of *N. bombi* compared to more common bee species (Gillespie 2010), suggesting a role for this fungus in their decline.

Nosema ceranae

Fries *et al.* (1996) first discovered the existence of *Nosema ceranae* in *A. mellifera*, in China. *Nosema ceranae* typically infects *Apis cerana*, the eastern honey bee. *Nosema ceranae* is also closely related to *N. apis* (Fries *et al.* 2006). With little data showing previous infections of *N. ceranae* in western honey bees, *N. ceranae* can be considered an EID (Paxton 2009). *Nosema ceranae* is usually spread via the fecal-oral route (Chen *et al.*

2007) but has also been found to pass through the oral-oral route, via feeding (Smith 2012). *Nosema ceranae* infection peaks in late summer/early fall (Runckel *et al.* 2011).

Nosema ceranae was an original infector of the eastern honey bee, *Apis cerana*, and was first found to infect *A. mellifera* only relatively recently in Taiwan (Huang *et al*. 2007). After *N. ceranae* was uncovered in *A. mellifera, N. ceranae* was retrospectively detected in samples collected in the United States in 1995 to 2007 (Chen *et al.* 2008). This indicates *N. ceranae* had jumped host species long before it was reported in *A. mellifera*. A comprehensive study of *A. mellifera* across the United States found *N. ceranae* in all 12 states studied, representing all geographic regions of the United States (Chen *et al.* 2008). The first detection of *N. ceranae* in England and Wales occurred recently (Budge *et al*. 2015). Western honey bees can host both *N. apis* and *N. ceranae* at the same time (Klee *et al.* 2007).

Nosema ceranae is much more virulent that *N. apis.* A pathogen with high virality that leads to a high death rate and kills bee quickly may be more beneficial in the long-term to population persistence (Betti *et al.* 2014). When the sick colony members die quickly, it lessens the overall transmission of the disease and a colony can overcome the disease. Slow acting pathogens that reduce the fitness of a colony may not outright be the cause of the death of a colony, but they do contribute to losses.

Impact and remediation

Fürst *et al.* (2014) found levels of *N. ceranae* in honey bees negatively correlated to levels of *N. ceranae* in bumble bees at the level of sampling sites. This correlation occurred when there was low prevalence of *N. ceranae* in honey bees; this low prevalence

in honey bees led to high prevalence in bumble bees. Higes *et al.* (2008) found a few honey bee cell nuclei positive for *N. ceranae* that were also positive for CBPV and DWV, but found no phenotypic signs of diseases. The levels of CBPV were thought to be too low to be able cause symptoms. *Nosema ceranae* found in honey bees can infect bumble bees, although with lower pollen load but higher virulence. Forty-eight percent of bumble bees died within a week of *N. ceranae* exposure (Graystock *et al.* 2013a). Bees that die from *N. ceranae* often have many spores in their bodies (Graystock *et al.* 2013a). A study involving two apiaries in Spain, located 750 km apart, found both apiaries experienced widespread loss of colonies (80% of colonies lost in apiary 1, 34% in apiary 2) with *N. ceranae* being the only pathogen found in all honey bee samples (Higes *et al.* 2009). Cox-Foster *et al.* (2007) found *N. ceranae* was detected in all 30 collapsed colonies, yet was also found in some colonies that did not collapse.

Fumagillin, an antibiotic, can eliminate *N. ceranae* infection in honeybees, but if treatment is stopped after 6 months post winter, colonies again became infected (Higes *et al.* 2008). Another study also found fumagillin lowered the presence of *N. ceranae* in colonies (Giacobino *et al.* 2016). Fumagillin is a potentially useful antibiotic to treat *N. ceranae*, but the bees must be treated continuously to keep them free from infection. Irradiation of pollen, which can kill many pollen transmitted pathogens, is a potentially useful method of limiting pathogens (Graystock *et al.* 2016) but may decrease pollen quality as food. High temperature has also been found to decrease the pathogen load of *N. ceranae* in honey bees (Chen *et al.* 2012).

Flagellates

Trypanosomatids

Trypanosomatids are single-celled, flagellated parasites, some of which are known to infect humans, such as the causative agent of Chagas disease, *Trypanosoma cruzi*, . Trypanosomatids that are known to affect bees are all in the Leishmaniinae subfamily (Kostygov and Yurchenko. 2017). *Crithidia mellificae* and *Lotmaria passim* infect honey bees (Schwartz *et al.* 2015) and *C. bombi* and *C. expoeki* infect bumble bees (Schmid-Hempel and Tognazzo 2010). *Crithidia mellificae* has been found to also infect mason bees (Ravoet *et al.* 2015).

Crithidia mellificae was identified decades ago as the first trypanosomatid widely found in honey bees (Langridge and McGhee 1967). Since *C. mellificae* was the only trypanosomatid thought to infect honey bees, previous literature assumed all trypanosomatids that were isolated from honey bees were *C. mellificae* (Schwarz *et al.* 2015). However, in 2015 Schwarz *et al.* (2015) proposed that many previous reports of trypanosomatid infection in honey bees were may be erroneously attributed as *C. mellificae*. They proposed *L. passim* as a new genus and argued that *L. passim* is currently more prevalent than *C. mellificae*. In South America, *L. passim* has been found in samples dating back from 2007 (Castelli *et al.* 2019).

Crithidia bombi and *C. expoeki* are common parasites of bumble bees (Tripodi *et al.* 2018), although *C. expoeki* was only discovered recently (Schmid-Hempel and Tognazzo 2010). The two are similar but *C. expoeki* strains differ sufficiently to be considered different species (Gerasimov *et al.* 2019). *Crithidia bombi* is a gut parasite of

bumble bees, which is transmitted horizontally among bees without a vector (Durrer and Schmid-Hempel 1994, Schmid-Hempel and Tognazzo 2010). *Crithidia bombi* is not thought have lethal effects on bumble bees, except in stressful conditions (Gegear *et al.* 2006). It has only been found in bumble bees (Graystock *et al.* 2016), where it primarily affects queens. In a study testing 378 bumble bee queens from six different species, *C. bombi* was the only parasite found to infect all six species. Other parasite species tested, but not found, were *Sphaerularia bombi, Apicystic bombi,* and *N. bombi* (Jones and Brown 2014).

Impact

Honey bee colony losses in Belgium were attributed to *C. mellificae* and *N. ceranae*, along with *Varroa* mites (Ravoet *et al.* 2013). *Crithidia mellificae* has been found to have peak levels in January but was detected at every time point between April and January (Runckel *et al.* 2011). Bumble bees infected with *C. bombi* have been shown to exhibit impaired function in foraging decisions; although, with time, impaired bees eventually achieve the same success as unimpaired bumble bees (Gegear *et al.* 2006). With a sample size of 1728 bees, Tripodi *et al.* (2018) found 20.7% of bumble bees tested positive for at least one trypanosomatid. Of these positive samples, 74.6% were positive for *C. bombi* only, 7.6% positive for *C. expoeki* only, and 13.8% were positive for both. Positive samples of both parasites were found throughout the United States. Tripodi *et al.* (2018) did not test if honey bees could have these parasites. In Massachusetts, rates of *Crithidia* per site varied immensely, with some sites having infection rates of up to 80%.

In the two-year study, overall *Crithidia* infection rates jumped from 24% in the first year to 51% in the second year (Gillespie 2010).

In a field study testing the effects of *C. bombi* on bumble bee colonies, *N. bombi* was found to co-infect the bumble bees, with 53.2% of bees co-infected with *N. bombi* (Imhoof and Schmid-Hempel 1999). The *Nosema* infection took longer than the *Crithidia* infection to occur in the bees. *Crithidia bombi* was shown to spread via horizontal transmission and the rate of infection spread as the season grew later (Imhoof and Schmid-Hempel 1999). This study showed the lack of strong virulence in *C. bombi* and the lack of negative effects in a large sized and well-fed colony.

In laboratory studies, *C. bombi* had no effect on the survival of bees. This is potentially due in part to the fact that *C. bombi* seems to only negatively affect bees in stressful conditions (Gillespie 2010). In stressed conditions, *C. bombi* increased mortality increased 1.5 times compared to non-stressed bees (Brown *et al.* 2000). *Crithidia bombi* was found to completely halt colony founding of infected bumble bee queens in a laboratory setting but had no effect on the mortality of the queen during hibernation. (Brown *et al.* 2003).

Hypotheses

I hypothesized that eukaryotic pathogens known to infect honey bees, such as *N. ceranae, C. mellificae,* and *L. passim,* would only be found in honey bees and mason bees. I also hypothesized the eukaryotic pathogens known to infect bumble bees, such as *C. bombi* and *C. expoeki,* would not occur in other pollinators. I hypothesized that locations with high levels of infection of any one of the pathogens would have high levels of

prevalence of other eukaryotic pathogens, as these pathogens have been found to occur together. I expected both the pathogens to be widespread and found in most locations based on results from other studies.

Method and Materials

Sample Collection

Samples were collected from ten different sites across Northern Virginia in the summer of 2018 and 2019. In total 166 bees were caught and used for DNA research. This total includes 52 honey bees, 87 bumble bees and 27 mason bees. Table 2 below summarizes the number of individuals caught at each location. Numbers in parentheses represent individuals caught in 2019. Mason bee species were identified by the curator at Blandy Experimental Farm, while honey bees and bumble bees were identified by the student collector.

Table 2 | Summary of individuals collected for DNA analysis

Site Number	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10	Total
Apis mellifera	-	-	9	-	-	- (3)	11 (1)	- (10)	18	-	38 (14)
Bombus impatiens	-	-	-	-	-	- (11)	8 (7)	- (2)	12	5	25 (20)
Bombus griseocollis	-	-	3	-	-	- (7)	9 (4)	- (1)	4	3	19 (15)
Bombus perplexus	-	-	-	-	-	-	3	-	-	-	3
Bombus auricomous	-	-	-	-	-	-	1	-	1	-	2
Bombus bimaculatus	-	-	-	-	-	-	1	-	1	-	2
Bombus fervidus	-	-	-	-	-	-	-	-	-	3	3

Bombus pensylvanicus	-	-	-	-	-	-	-	-	-	1	1
Osmia taurus	-	-	3	-	-	-	-	-	-	-	3
Osmia cornifrons	-	3	-	1	-	18	1	-	-	-	23
Osmia lignaria	1	-	-	-	-	-	-	-	-	-	1
Total	1	3	15	1	-	18 (21)	34 (12)	- (13)	36	12	120 (46)

Bees in 2018 were caught in the later weeks of July and early weeks of August. Forty-six bees were caught in 2019, in mid-June. Honey bees and bumble bees were caught in the field or off honey bee hives. Bees caught for DNA extraction were placed into 50 ml falcon tubes and then into ice until they were brought back to the laboratory and stored in a -20°C freezer. Mason bee homes established at each location and consisted of simple wood structures, placed in the early months of spring. In the fall of 2018, the mason bee homes were collected, and any bees found in the homes were collected for analysis. The mason bee homes were all designed to hold 12 slots for mason bees to lay their eggs. However, not all homes were populated equally. This led to varied numbers of captured mason bees for each location. Half of all collected mason bee samples were used for RNA analysis.

DNA Extraction and Amplification

DNA extraction for all samples was performed with a PureLink™ Genomic DNA Mini Kit (Thermo Fischer Scientific), according to the manufacturer's protocol. A dedicated, sterile space was used for DNA extraction and polymerase chain reaction (PCR).

A separate hood was used for PCR preparation. Primer sequences used for *Nosema* were from Fries *et al.* (2015) (Table 3). For each *Nosema* PCR, a 10µl reaction volume was used, consisting of 0.5µl of each primer at a concentration of 10µM, 0.04µl of Platinum II *Taq* Hot-Start DNA polymerase (Thermo Fisher Scientific), 2.0µl of Platinum II 5x buffer, 0.3µl of dNTP's, 5.66µl of distilled water, and 1.0µl of DNA template. Thermal cycling began with an initial denaturation period at 94°C for 2 minutes, followed by 34 cycles of the following: a denaturation period at 94C for 15 seconds , an annealing period at 63°C for 30 seconds and then an extension period at 72°C for 30 seconds. Following the final cycle, there was a 5-minute extension cycle at 72°C.

Primer sequences used for trypanosomatids were used from Tripodi *et al.* (2018). Primers can be found in Table 3 below. For trypanosomatids, 10µl reactions were made, consisting of 0.5µl of each primer at a concentration of 10µM, 0.04µl of Platinum II *Taq* Hot-Start DNA polymerase (Thermo Fisher Scientific), 2.0µl of Platinum II 5X buffer, 0.3µl of dNTP's, 5.66µl of distilled water, and 1.0µl of DNA template. Thermal cycling began with an initial denaturation period at 95°C for 5 minutes, followed by 34 times of the following: a denaturation period at 95°C for 30 seconds, an annealing period at 57°C for 30 seconds and then an extension period at 72°C for 1 minute. Following the final cycle, there was a 10-minute extension cycle at 72°C.

Negative controls were used in all PCR reactions. For the negative controls, the DNA template was replaced with purified water. Each batch of PCR reactions, consisting of anywhere between 8 and 62 samples, included one negative control. Samples were run

by gel electrophoresis to detect for the presence of PCR products of the correct size, which indicated positive samples. Gels were run at 90 volts and 400mA for 40 minutes with a 1.2% agarose gel. With each set of primers, PCR pathogen detection was performed on each sample twice. Samples which were visible on the agarose gel (at the correct size) for one of the two PCR reactions were subject to a third PCR reaction. A sample was considered positive if the third reaction showed a correctly sized band (i.e., 2 out 3 PCRs are positive). Positive reactions from individuals were combined (total amount = 16 ul) before DNA purification. This was done to double the amount of PCR product.

Table 3 | Primers used for DNA amplification

Target pathogen	Primer	Sequence (5' to 3')
Trypanosomatids	Trypan-CB-SSUrRNA-F2	CTTTTGACGAACAACTGCCCTATC
	Trypan-CB18SR2	TGCTGGTTTGTTATCCCATGCT
Nosema	SSU-res_NosF	GCCTGACGTAGACGCTATTC
	SSU-res_NosR	GTATTACCGCGGCTGCTGG

Amplicon Purification

After PCR was completed, amplicon purification was performed using 24µl of 1% solids Sera-Mag™ SpeedBeads (MilliporeSigma) according to manufacturer's protocol. Because each PCR reaction used 10µl of DNA product, and 2µl was used for gel electrophoresis, 16µl of PCR product was leftover for amplicon purification. A 1:1.5 ratio of PCR product to SpeedBeads was used for purification. After cleanup, the PCR products

were used to conduct cycle sequencing, using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). For each cycle sequencing reaction, a master mix was made using 1.0 µl of one forward or reverse primer, 1.5µl of BigDye™ Terminator 5X Sequencing Buffer, 0.5µl of BigDye™, 1.5µl of distilled water, along with 2.5µl of cleaned PCR product. In the thermal cycler, samples were denatured at 96°C for 1 minute, followed by 38 cycles at the following conditions: 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. After thermal sequencing was finished, clean products were further purified using Sephadex™ G-50 Superfine Beads (GE Healthcare). These samples were then sequenced on an Applied Biosystems™ PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientific).

Sequence Assembly

Forward and reverse reads for all sequences were assembled in the software *Geneious*, version 9.0.5. Assembled contigs were manually checked and errors fixed. Sequences were entered into NCBI Blast to determine the pathogens' species. Standard nucleotide BLAST was used with default parameters. Non-redundant Blast database (nr/nt) was used for sequence searching and the program was optimized for highly similar sequences (Megablast). *Geneious* aligner was used to align all sequences. All trypanosomatid generated sequences were aligned together and a *C. bombi* isolate (GenBank accession number: MG182417.1) was used as the reference sequence. All *Nosema* sequences were aligned together and a *N. bombi* sequence (GenBank accession number: AY008373.1) was used as the reference sequence. All aligned sequences were manually checked one last time for potential insertions or deletions missed by the software.

Final alignments of the sequences were exported from *Geneious* in a Newick file format and used for further analyses. Phylogenetic trees were constructed using the MrBayes 3.2.6 software (Huelsenbeck and Ronquist 2001) found in *Geneious*. Phylogenetic trees were edited and visualized with Dendroscope 3 version windows 3.7.2 (Huson and Scornavacca 2012).

MrBayes analysis is a Markov Chain Monte Carlo-based Bayesian algorithm which produces the most likely phylogenetic tree with an alignment of nucleotide sequences. MrBayes analysis was performed using its pre-pared default parameters in *Geneious*. These conditions included: gamma-distributed rate variation across sites, chain length of 1,100,000, a subsampling frequency of 200, 4 heated chains, a burn-in length of 100,000, and a heated chain temperature of 0.2. MrBayes gave the Bayesian posterior probabilities of phylogenetic trees and nodes based on the data. Phylogenetic trees were constructed for both *Nosema* and trypanosomatid aligned sequences.

Results

Infection Prevalence

Overall, 166 bees from 10 different sites were captured and tested for the presence of two eukaryotic pathogens, *Nosema* and trypanosomatids. In total, 68 bumble bees, 22 honey bees, and 14 mason bees were found to be infected with at least one pathogen. Of the collected bees, 63.9% (n = 104) tested positive for at least one eukaryotic pathogen and 9.0% (n = 15) were found to be infected for two eukaryotic pathogens.

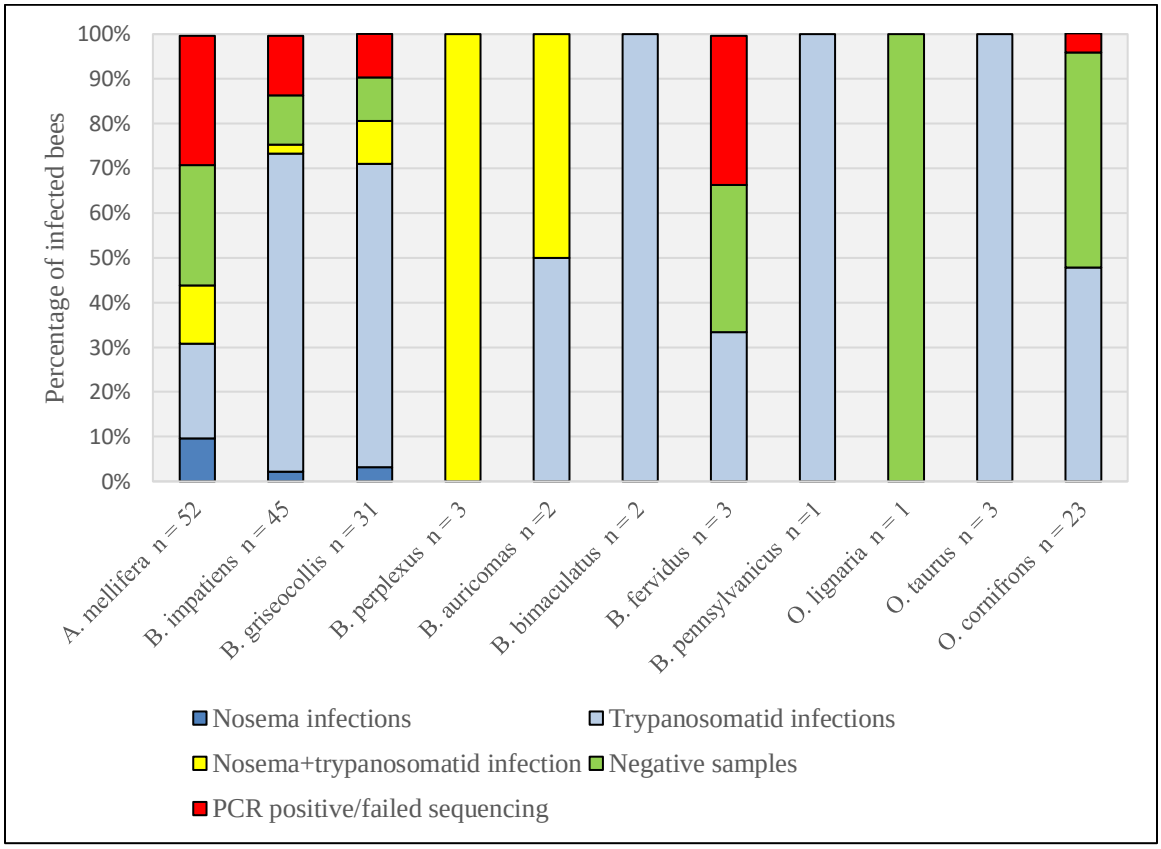

Figure 2 | Percent of samples of bees infected with pathogens, distributed by bee species.

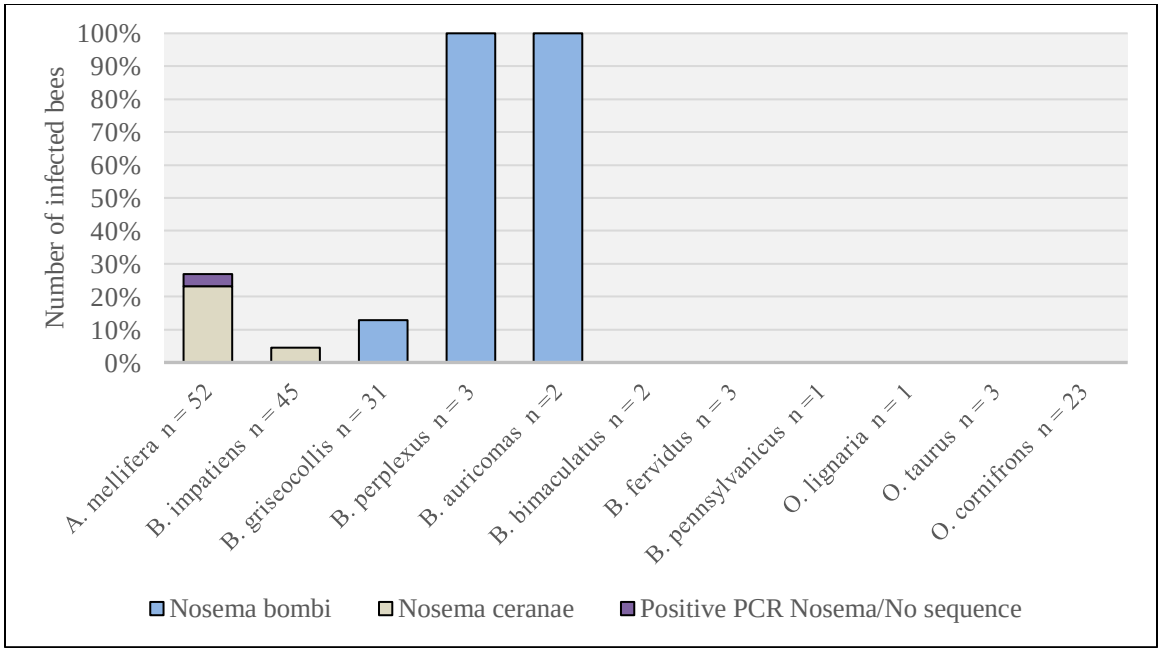

Figure 3 | Distribution of *Nosema* species in different bee species collected.

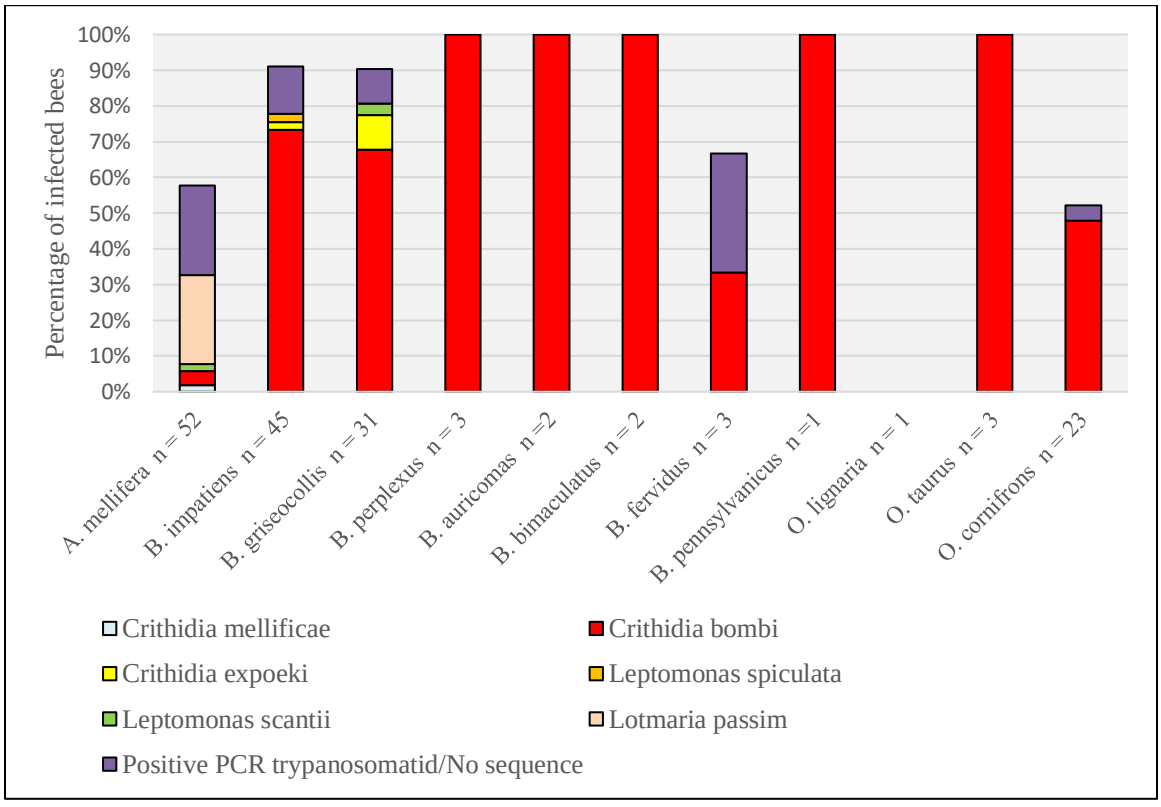

Figure 4 | Distribution of trypanosomatids in different bee species collected.

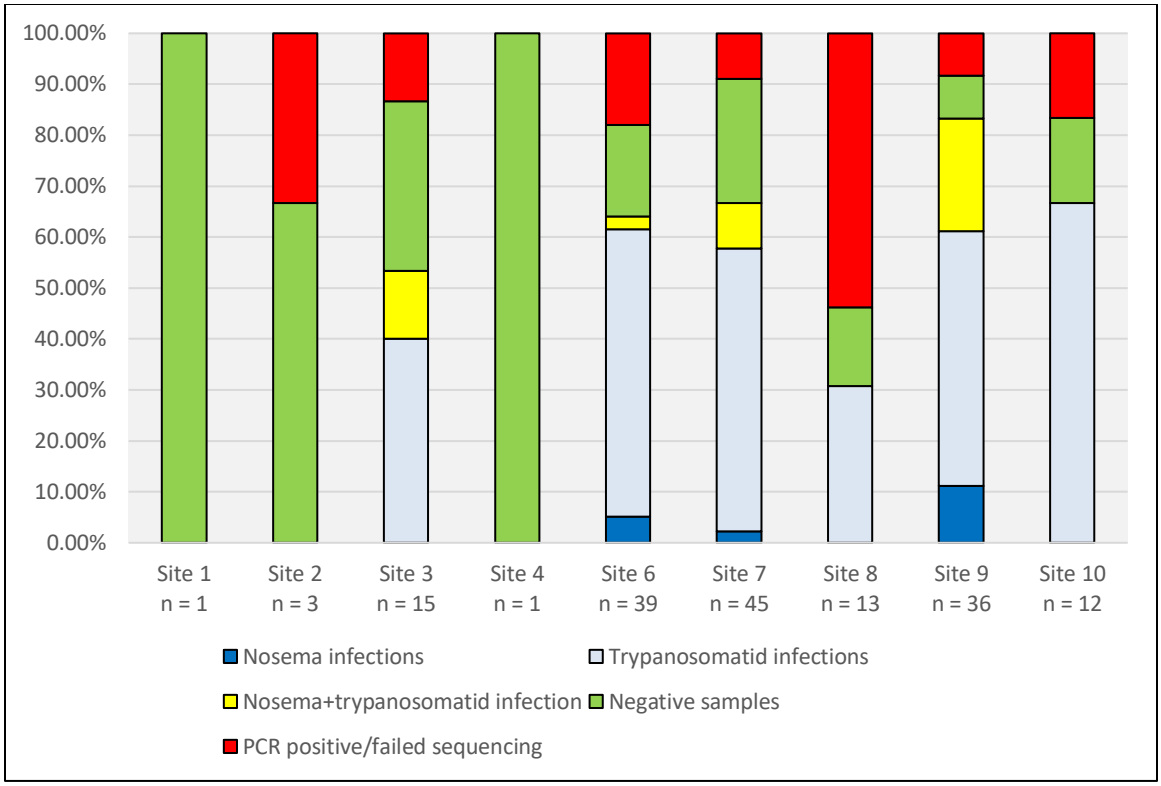

Figure 5 | Percent of samples of bees infected with pathogens, distributed by site. Data from all bee species collected within a site are combined. Site 5 is not represented as zero samples were collected for eukaryotic analysis at this site.

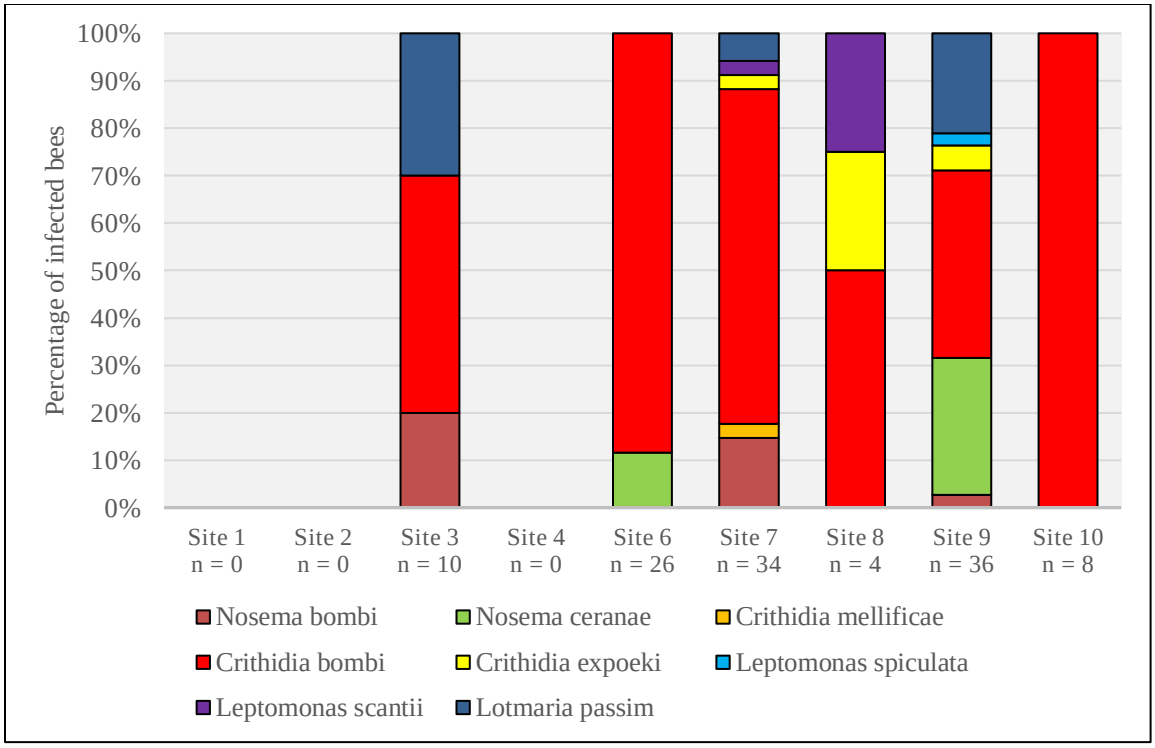

Figure 6 | Comparison of pathogen infection across sites. Data from all bee species collected within a site are combined. Site 5 is not represented as zero samples were collected for eukaryotic analysis at this site.

Figure 6 shows site 9 had the highest raw total number of infected bees, although this site only had the third most collected bees. The most bees were collected at sites 6 and 7, and these two sites had the 2nd and 3rd highest prevalence of eukaryotic infection. The infections at site 6 and site 7 were primarily *C. bombi* infections, although other pathogen species were also present at these sites. Of the six sites where eukaryotic pathogens were found, *C. bombi* was the most prevalent pathogen at all six sites. Figure 4 again shows *C. bombi* was the most prevalent pathogen found. *Crithidia bombi* was most common in *B. impatiens* and *B. griseocollis*, although it was found in every species examined except one, *O. lignaria*. Sites 1, 2, and 4 did not have any honey bees or bumble bees collected at their

sites for eukaryotic research. However, a total of five mason bees were collected from these sites; three from site 2, and one from each of the other two sites. None of these five mason bees tested positive for pathogen. No bees were collected for eukaryotic analysis at site 5.

The location with the highest amount of collected bees was site 7. Thirty-four bees were collected in 2018 and 12 collected in 2019. Only 12 bees, out of the 46 collected, were not infected with any eukaryotic pathogens surveyed. In 2018, 71.4% (n = 20) of collected bumble bees were infected with *C. bombi*. Five of these bumble bees were also infected with *Nosema*, and only one of those bees was not co-infected with both. The lone collected mason bee was unaffected. In 2019, eleven bumble bees and one honey bee were collected and all were infected with trypanosomatids. Two bumble bees were positive for *C. expoeki* and *L. scantii*, respectively. The rest of the bees (including the lone honey bee) were positive for *C. bombi*.

Site 9 had equal numbers of honey bees and bumble bees collected (18 of each). 94.4% (n = 17) of bumble bees collected at this site were positive for a trypanosomatid. Fourteen of the bumble bees were infected with *C. bombi*, two were infected with *C. expoeki*, and one was infected with *L. spiculata*. One bumble bee was co-infected with *N. bombi* and *C. bombi*. 72.2% (n = 13) of honey bees collected here were positive for either *Nosema* or a trypanosomatid and seven of the honey bees were co-infected with both. Eleven honey bees were infected with *N. ceranae*, eight infected with *L. passim*, and one with *C. bombi*.

Figures 2 and 5 show the prevalence of infection across sites and species in broader categories. These figures show either just *Nosema* or trypanosomatid infection

across sites or species. Samples collected that tested negative for any eukaryotic pathogen was also recorded. For most sites and species, the total number of bees infected with at least one pathogen species was higher than the number of unaffected bees. Co-infected bees were found at four of the visited sites with fifteen total co-infected samples. All eight bumble bees positive for multiple pathogens were positive for both *N. bombi* and *C. bombi*. Six out of the seven honey bees positive for multiple pathogens were positive for *N. ceranae* and *L. passim*. One honey bee was positive for both *N. ceranae* and *C. bombi*. All seven honey bees with Nosema – trypanosomatid coinfections were collected at site 9.

***Nosema* Infection**

Table 4 | Prevalence of *Nosema* infection

	N. bombi	*N. ceranae*	Number uninfected	Number infected	Percentage of individuals infected	Percentage of all samples collected	PCR positive/ failed sequencing
A. mellifera	-	12	40	12	30%	7.2%	3
B. impatiens	-	2	43	2	4.7%	1.2%	-
B. griseocollis	4	-	27	4	14.8%	2.4%	-
B. perplexus	3	-	-	3	100%	1.8%	-
B. auricomous	1	-	1	1	50%	0.6%	-
B. bimaculatus	-	-	2	-	0%	0%	-
B. fervidus	-	-	3	-	0%	0%	-

B. pensylvanicus	-	-	1	-	0%	0%	-
O. taurus	-	-	3	-	0%	0%	-
O. cornifrons	-	-	23	-	0%	0%	-
O. lignaria	-	-	1	-	0%	0%	-
Total	-	-	144	22	-	13.3%	3

As table 4 shows, 13.3% (n = 22) of all collected bees were positive for *Nosema* infection. This included ten bumble bees and twelve honey bees; no mason bees were positive for *Nosema*. Eight of the ten bumble bees were found to have *N. bombi* and two bumble bees had *N. ceranae*. All twelve honey bees were found to have *N. ceranae*. Overall, a total of 22 bees were found to be infected with *Nosema*, and 15 bees were co-infected with either *C. bombi* or *L. passim*. Almost a third of all honey bees captured were infected with *Nosema*, while *Nosema* infection widely varied between each bumble bee species. Only three *B. perplexus* were captured, but all three were infected with *N. bombi*. Three honey bees tested positive for *Nosema* spp., but sequencing was not successful on these samples.

Four of the sites visited had confirmed cases of bees infected with *Nosema*. These were sites 3, 6, 7, and 9. The percentage of bees infected with *Nosema* from these locations were as follows: 54.5% (n = 12) from site 9, 22.7% (n = 5) from site 7, 13.6% (n = 3) from site 6, and 9.1% (n = 2) from site 3. Eleven of the twelve instances of *Nosema* infection in honey bees occurred at site 9. Eighteen honey bees were captured at this location with only

five bees not infected with any pathogen. A total of 41 bees were collected at site 6, and only three were honey bees. The rest were *Bombus* or *Osmia*. At this location, there were three instances of positive *Nosema* infection, and all three were found to be *N. ceranae*. However, two of the three infected samples were *Bombus*. Just one honey bee was infected with *N. ceranae*.

Trypanosomatid Infection

Table 5 | Prevalence of trypanosomatid infection

	C. bombi	*C. expoeki*	*C. mellificae*	*L. passim*	*L. spiculata*	*L. scantii*	Number uninfec-ted/ infected	% of infected individuals /all samples	PCR positive /failed sequenc -ing
A. mellifera	2	-	1	13	-	1	35, 17	32.7% 10.2%	15
B. impatiens	33	1	-	-	1	-	10, 35	77.8% 21.1%	5
B. griseocollis	21	3	-	-	-	1	6, 25	80.1% 15.1%	3
B. perplexus	3	-	-	-	-	-	0, 3	100% 1.8%	-
B. auricomous	2	-	-	-	-	-	0, 2	100% 1.2%	-
B. bimaculatus	2	-	-	-	-	-	0, 2	100% 1.2%	-
B. fervidus	1	-	-	-	-	-	2, 1	33.3% 0.6%	1
B. pensylvanicus	1	-	-	-	-	-	0, 1	100% 0.6%	-
O. taurus	3	-	-	-	-	-	0, 3	100% 1.8%	-
O. cornifrons	11	-	-	-	-	-	12, 11	47.8% 6.6%	1
O. lignaria	-	-	-	-	-	-	1, 0	0% 0%	-

Total	79	4	1	13	1	2	66, 100	-, 60.2%	25

As table 5 shows, 60.2% (n = 100) of samples were infected with a trypanosomatid. This includes 69 bumble bees, 17 honey bees, and 14 mason bees. *Crithidia bombi* was the most prevalent trypanosomatid found in a total of 79 bees. Other trypanosomatids found include 13 cases of *L. passim* infection, four cases of *C. expoeki*, two cases of *Leptomonas scantii*, and one case of each *C. mellificae* and *L. spiculata*. High amounts of *B. impatiens* were infected with *C. bombi*, with 75% of that species infected. Albeit with a smaller sample size, over 75% of collected *B. griseocollis* also were infected with *C. bombi*. Twenty-five samples tested positive for a trypanosomatid after PCR, but did sequencing was not successful.

As recorded above, 14 instances of *C. bombi* infection in mason bees were found. All positive samples came from just two locations. One location was site 6, where 18 mason bees were collected and 11 were infected. The other location was site 3, where only three mason bees were collected, but all were infected with *C. bombi*. At site 6, among the honey bees and bumble bees collected, 60% (n = 12) were infected with *C. bombi*. At site 3, nine honey bees and three bumble bees were captured. Of the three bumble bees, two of the bees were co-infected with *N.* bombi and *C. bombi*. Out of the nine collected honey bees, 33% (n = 3) were infected with *L. passim*. One of these *L. passim* sequences was the unique sequence discussed below as part of the phylogenetic analysis.

At site 10, twelve bees were collected, all of which were bumble bees. 67% (n = 8) of those bees were infected with *C. bombi*. At site 8, a total of 13 bees were collected. Ten

were honey bees and three were bumble bees. Only one honey bee, 10% (n =1), was infected and it was infected with *L. scantii*. All three bumble bees were infected with trypanosomatids; two with *C. bombi* and one with *C. expoeki*.

DNA sequence analysis

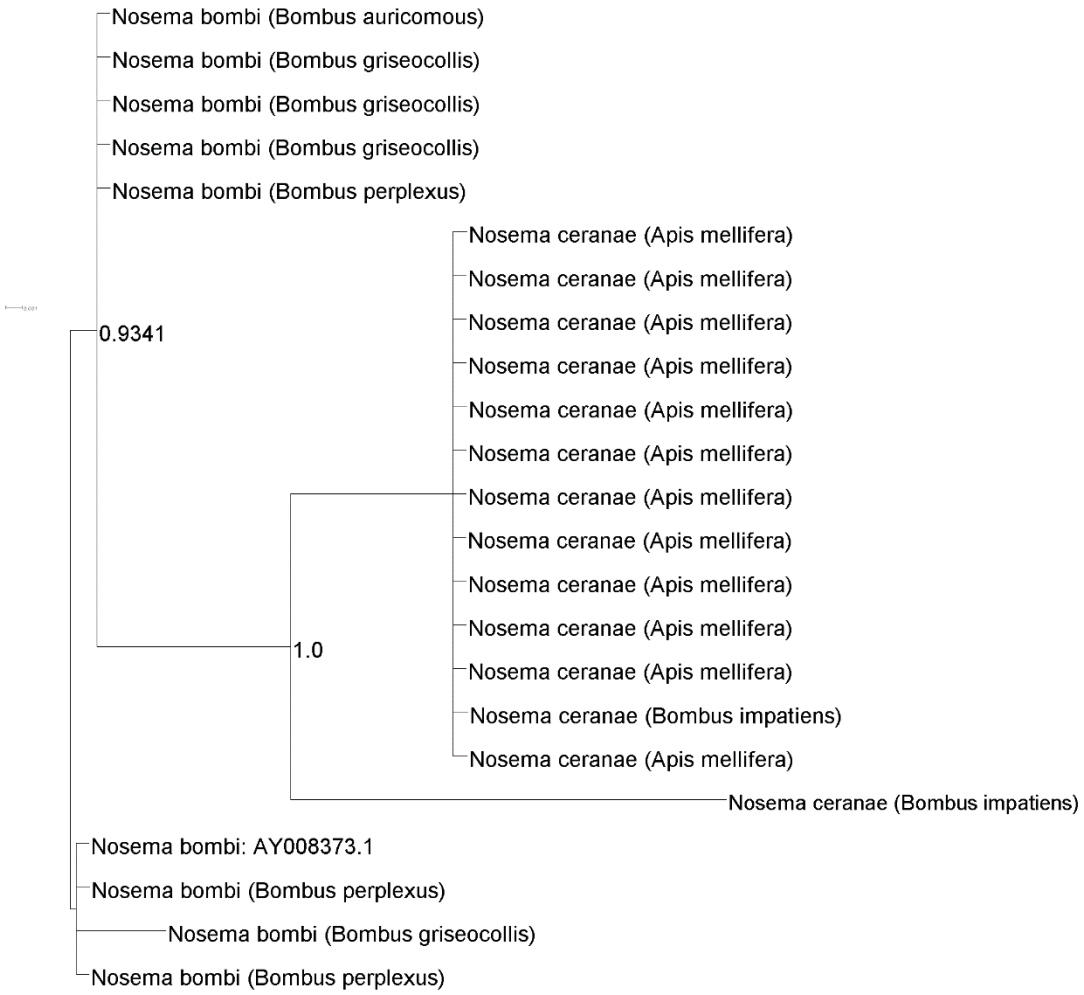

Figure 7 | Phylogenetic tree of *Nosema* sequences of collected bees infected with *Nosema*. Phylogenetic tree of collected bees infected with *Nosema.* Numbers at each node represent Bayesian posterior probability for the node (i.e. node support). Each branch tip includes the pathogen and the species the pathogen was isolated from. Scale of branch length is to the left of 0.9341. The scale reads 0.004.

In the *Nosema* phylogenetic tree, four distinct clades were identified. One *N. ceranae*, although collected at the same site as the other *N. ceranae* sequence found in a bumble bee, was much different than the other *N. ceranae* sequences. This *N. ceranae* sequence was identified as so, because through BLAST, *N. ceranae* showed highest similarity to this sequence. The largest clade included all *N. ceranae* sequences isolated from honey bees and one sequence isolated from a lone *B. impatiens*. This clade had nearly 100% node support of being its own clade. The next largest clade contained five *N. bombi* sequences. These sequences came from three different sites. This clade had 93% node support of being its own clade. The clade containing the reference sequence included three *N. bombi* sequences. These samples were all collected from the same site. Two of the *N. bombi* sequences, in the clade totaling five sequences, were collected from this same site.

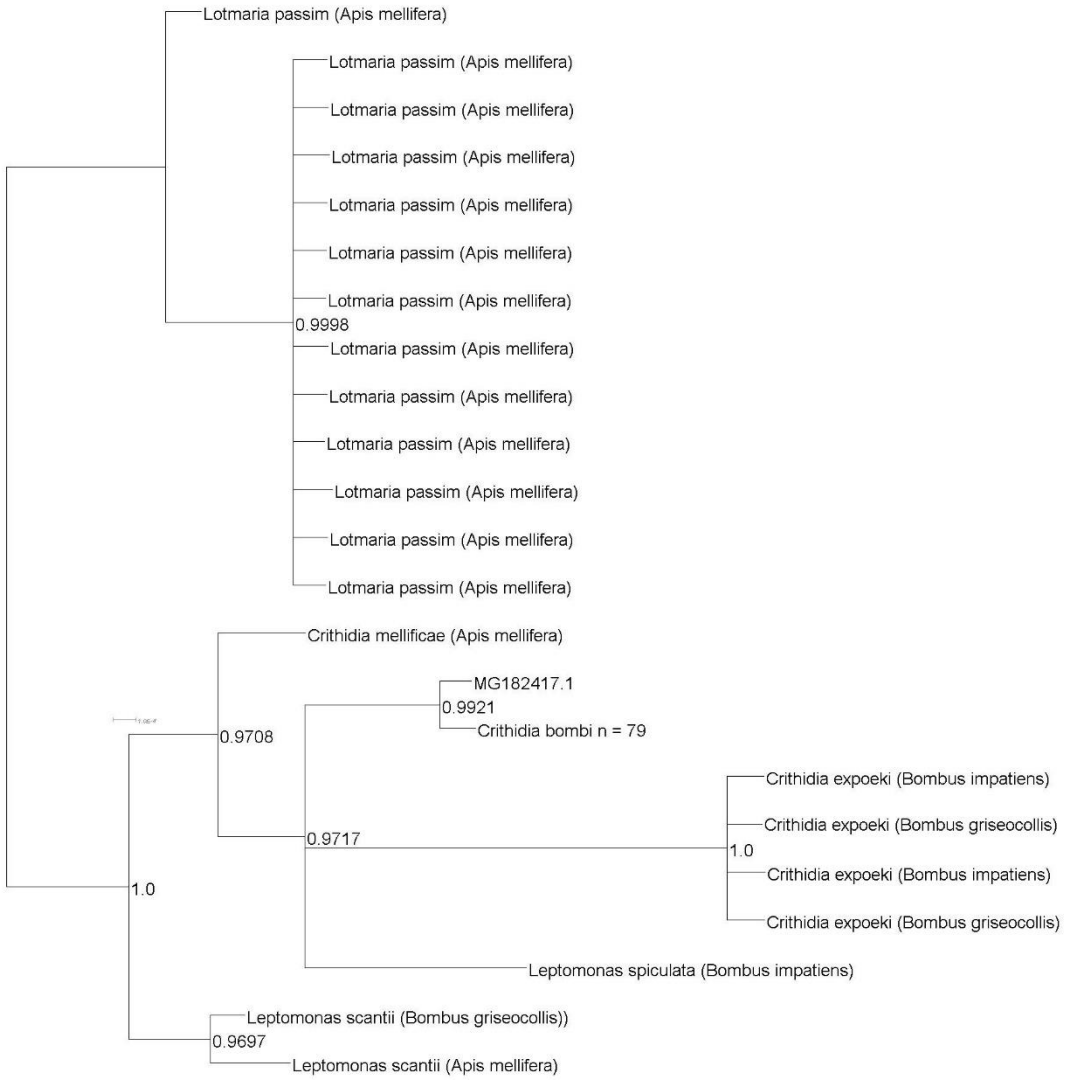

Figure 8 | Phylogenetic tree of trypanosomatid sequences of collected bees infected with trypanosomatids. Numbers at each node represent node support. Each branch tip includes the pathogen and the species the pathogen was isolated from. Scale of branch length is to the left of 0.9708. The scale reads $1.0x10^{-4}$.

In the trypanosomatid phylogenetic tree, seven distinct clades were identified. With the exception of one *L.* passim sequence, all individual species were in their own clades.

The largest clade contained the *C. bombi* reference sequence and included all seventy-nine *C. bombi* sequences. These *C. bombi* sequences were found in all three observed genera of pollinators. The four *C. expoeki* sequences were the most closely related to the reference sequence, with 100% node support that they form their own clade. Sequences found in both *B. impatiens* and *B. griseocollis* were identical. The *L. spiculata, C. mellificae,* and *L. scantii* clades all had around 97% node support. The large clade of *L. passim* sequences had nearly 100% support indicating they were their own clade. There was a one base substitution difference between the lone *L. passim* sequence compared to the other *L. passim* sequences.

Discussion

My first hypothesis stated common eukaryotic pathogens of honey bees such as *N. ceranae, C. mellificae* and *L. passim* would not be found in bumble bees or mason bees. The evidence mostly supported this hypothesis, although there was one instance of *N. ceranae* infection in a bumble bee. Recent studies have found *N. ceranae* can infect bumble bees (Graystock *et al.* 2013a, Sinpoo *et al.* 2019), but infections have not been common. Sinpoo *et al.* (2019) sampled 280 bumble bees and found *N. ceranae* infection ranging from 4.76% to 14.28%, depending on the bumble bee species. In comparison, they found *N. bombi* rates in those same species to be anywhere from 6.7% to 13.98% higher. My study found only one bumble bee infected with *N. ceranae* compared to eight bumble bees infected with *N. bombi*. In honey bees, there were 12 instances of *N. ceranae* infection. I did not encounter widespread *Nosema* infection in my study, but *N. ceranae* was far more common in honey bees. 54.5% of all *Nosema* infections were *N. ceranae* found in honey

bees, while only 9.1% of all *Nosema* infections were *N. ceranae* found in bumble bees. There is almost nothing known about *N. ceranae* infection in mason bees. Bramke *et al.* (2019) found *N. ceranae* infection could be dangerous to *O. bicornis* larvae, they did not find any evidence of *N. ceranae* infection in mason bees. My study found no presence of any *Nosema* infection in mason bee species.

The presence of *Nosema apis* was also examined as part of this research, but no samples were infected with *N. apis*. While *N. apis* was first isolated over 100 years ago by a German researcher (Zander 1909) and is global in distribution, *N. ceranae* has become more the widespread pathogen (Klee *et al.* 2007, Fries *et al.* 2010). *Nosema ceranae* is thought to be more virulent of the two (Betti *et al.* 2014) but co-infection of these pathogens is common. Although *N. ceranae* is the more virulent species and is seemingly becoming the more common species, research has shown *N. ceranae* does not have a competitive advantage against *N. apis* in honey bees (Forsgren and Fries 2010, Milbrath *et al.* 2014). There are several possible explanations why my data did not show any *N. apis* infection. My sample size was relatively small with only 52 honey bees sampled for the presence of eukaryotic pathogens. It is possible the sample size was not large enough, and bees infected with *N. apis* were missed. It is also possible *N. ceranae* has simply overtaken *N. apis* as the dominant *Nosema* species in honey bees, even though research has shown they are able to co-exist and they do not have competitive advantages over each other. It is also possible that co-infection of both *Nosema* species occurred, but if there were low levels of *N. apis* present, then *N. apis* would not be detected through PCR and Sanger sequencing.

While there was one instance of *N. ceranae* infection in bumble bees, there were no instances of *C. mellificae* or *L. passim* in bumble bees or mason bees. *Crithidia mellificae* was the first trypanosmatid widely found in honey bees (Langridge and McGhee 1967), but recently it had been proposed that the old reported cases of *C. mellificae* infection were actually infections of *L. passim* (Schwarz *et al.* 2015). My research found only one instance of *C. mellificae* infection but thirteen instances of *L. passim* infection. If my same research had been performed ten years ago, I may have erroneously stated there were fourteen instances of *C. mellificae* infection. Although *L. passim* was not proposed as a species until 2015, a study in South America found *L. passim* in samples dating back from 2007 (Castelli *et al.* 2019). A laboratory study tested if *C. mellificae* could infect bumble bees, and no recorded infections were found (Ruiz-Gonzalez *et al.* 2006).

My second hypothesis stated eukaryotic pathogens commonly found in bumble bees, such as *C. bombi* and *C. expoeki*, would not be found in honey bees and mason bees. My data mostly supported this hypothesis, but not entirely. *Nosema bombi* was only found in bumble bees, and not in any other species of bee. No previous research that I have found, has data indicating a *N. bombi* presence in honey bees or mason bees. Mason bees are not well studied and so my study is a small piece of evidence indicating *N. bombi* does not affect mason bees. But honey bees are far more well documented, and my research further verifies the lack of *N. bombi* in honey bees.

Crithidia bombi and *C. expoeki* are morphologically similar trypanosomatids, although individual samples are considerably different when comparing nucleotide sequences (Gerasimov *et al.* 2019). Previous research demonstrated that honey bees can be

infected by these pathogens in a laboratory environment (Ruiz-Gonzalez *et al.* 2006), although the presence of these pathogens could not be determined purely through the examination of feces samples under a microscope. Microsatellite analysis was needed to determine *C. bombi* presence. They also concluded honey bees could act as vectors for *C. bombi* transmission (Ruiz-Gonzalez *et al.* 2006). In my research, *C. bombi* was overwhelming found in bumble bees and it was the only eukaryotic pathogen (that was screened for) found in mason bees. Both instances of *C. bombi* infection in honey bees were sites where there was widespread trypanosomatid infection. These honey bees were collected at Blandy Experimental Farm and St. Benedictines Monastery. Although only two honey bees were infected, this research could be the first findings of *C. bombi* infection of honey bees in the wild. This research also could be the first finding of *C. bombi* infection of *O. cornifrons* and *O. taurus* in the wild. There is very little research regarding *L. spiculata* and *L. scantii*. They are similar to the other trypanosomatids, but their effects on pollinators are unknown.

My third hypothesis stated that the pathogens would be widespread throughout all locations and all sites would experience similar infections. My data did support this hypothesis in several ways. For *Nosema*, all but one *N. ceranae* sequence was in the same clade. This sequence was a potentially a highly mutated sequence of *N. ceranae*. The one *N. ceranae* that was distantly related to the rest was one of the two *N. ceranae* sequences found in bumble bees, but the other *N. ceranae* found in a bumble bee was in the same clade as the rest of the *N. ceranae*. Both of those two sequences derived from bees that came from the same site. A key difference between the *N. ceranae* and *N. bombi* sequences

was a twelve base pair stretch in which nine nucleotides were different. All *N. ceranae* sequences had the same nucleotide substitutions from *N. bombi* reference, except for the unique *N. ceranae* sequence. This sequence had two polymorphisms, in this small string of nucleotides, that matched the *N. bombi* sequences. It is also had three polymorphisms differing from either species in the ten nucleotides preceding this base pair stretch. There were also several other polymorphisms scattered around the unique *N. ceranae* sequence that indicated either a similarity to *N. bombi*, or a dissimilarity to both. The three *N. bombi* sequences closest to the reference were all found at the same location. Overall, although *Nosema* was not as widespread as the trypanosomatids, most sites with *Nosema* infection shared similar sequences, indicating *Nosema* species are present throughout the area.

Trypanosomatids were extremely prevalent throughout all sites, especially *C. bombi*. All 79 sequences of *C. bombi* matched the reference sequence. With the exception of one *L. passim* sequence, which represented its own clade, all other species fell into their own distinct clades. These distinct clades were made up of all other members of their species. The unique *L. passim* sequence was found in a location with two other cases of *L. passim*. *Lotmaria passim* was found at sites 3, 7 and 9. These sites span almost the collection range and yet all the sequences were the same, except for one. Evidence showed that both *L. passim* and *C. bombi* are widespread throughout the area. Other areas in the study region that were not sampled are likely to be infected.

Though it is widely known many of these pathogens are prevalent, it is still important to obtain evidence that supports previous studies. I found not only many pathogens, but also more virulent species. Both *N. bombi* and *N. ceranae* are highly virulent

species (Otti and Schmid-Hempel 2007, Betti *et al.* 2014) that have been linked to species decline (Cameron *et al.* 2011, Graystock *et al.* 2013a). More research is needed on solitary bees. There are very few published papers about pathogen presence in solitary bees. Two *Chrysididae* wasp eggs, collected incidentally in this study in a mason bee nest, were tested for eukaryotic pathogens and both were positive for *C. bombi.* This suggests this species may be a possible vector of *C. bombi* transmission or these wasps became positive after parasitizing the mason bee larvae. While my study did not find any *Nosema* infection in mason bees, it did find high rates of *C. bombi* infection. More studies should be performed to determine if *Nosema* is simply unable to infect mason bees, or if it has just not been documented yet. It is important to determine the effects and possible modes of transmission of these pathogens on solitary bees.

CHAPTER 3 | RNA VIRUSES

Introduction

There are at least 24 viruses that infect honey bees, most of which are positive-sense RNA viruses (Remnant *et al.* 2017); although, the first negative-sense RNA virus infecting bees was discovered recently (Levin *et al.* 2017). While the exact mode of the transmission of these viruses is not known, there have been ample sources of evidence suggesting both horizontal transmission (Smith 2012) and vertical transmission (Shen *et al.* 2005, Chen *et al.* 2007). Deformed wing virus (DWV) and black queen cell virus (BQCV) were also found in fecal samples (Chen *et al.* 2006). Queens have been shown to transmit many of the viruses as well. Chen *et al.* (2006) examined ten honey bee queens and found six of the queens were positive for DWV, BQCV, and sacbrood virus (SBV). One hundred percent of the eggs laid by those six queens, tested positive for all three viruses. Of the larvae, 92% tested positive for DWV, 27% for BQCV, and 25% for SBV. DWV and BQCV was also found in the queen's feces. In central Pennsylvania, there was correlation between the prevalence of virus in a honey bee forager and the prevalence of virus in that foragers pollen (Singh *et al.* 2010).

DWV was strongly correlated between foragers and the pollens they carry, while such correlations did not exist for BQCV and SBV (Singh *et al.* 2010). A reason that DWV may have such high infection rates is because DWV was found to be infectious in stored pollen and honey, even after storage for six months (Singh *et al.* 2010). SBV was also

tested in this study, but after five weeks, it was no longer detected. DWV and SBV infected pollen pellets were found with unaffected foragers, suggesting this is a way those two viruses can be transmitted (Singh *et al.* 2010). The viruses found on these pellets were found to still be infective, even after being stored for months. Even though BQCV was the most prevalent of the three viruses, there were few pollen pellets that contained the virus. In contrast to the matching prevalence rates of forager and pollen in DWV, it appears the viruses do not all infect bees the same way. Overall, almost 17% of honey bee foragers tested positive for all three viruses, whereas only 1.5% of pollen pellets tested positive for all three (Singh *et al.* 2010). However, it should be noted that one pollen pellet only represents one foraging trip by a pollinator. Pollinators can make several trips per day, and could become infected on three separate trips, even if each pollen pellet contains only one virus.

Co-infections of multiple viruses in the same bee have been widely reported and published. Bees are not always infected with a single pathogen. McMahon *et al.* (2015) tested several viruses and found 51% of honey bees and 23% of bumble bees were infected with at least one RNA virus. Seven percent of the honey bees were infected with two viruses and 1% were infected with three viruses. For bumble bees, 3% and 0.2% were infected with two and three viruses, respectively. In France, apiaries were tested for six RNA viruses, and 31% of the apiaries contained three different viruses, 36% contained four different viruses, and 25% contained five different viruses (Tentcheva *et al.* 2004). In Austria, 50% of honey bees had two co-infections, 27% had three co-infections, 13% had four co-infections, and one sample was infected with five viruses (Berényi 2006). Samples

also from Poland, Germany, Hungary, and Slovenia all tested positive for DWV and SBV. Infected samples of BQCV, acute brood paralysis virus (ABPV), and chronic bee paralysis virus (CBPV) was also observed in several of these countries. Kashmir bee virus (KBV) was not found in any country, including Austria (Berényi 2006). Berényi (2006) also investigated the presence of viruses and parasites in the same samples. Eighteen percent of samples had both *Varroa* mites and viruses. *Nosema apis* was present in samples positive for ABPV, BQCV, DWV, and SBV.

However, not all the research done on these viruses has calculated the viral loads of these pathogens. Viral loads of these pathogens are not widely known (McMahon *et al.* 2015). Levels of the viral load in bees determines if the bees have low levels of virus and are simply asymptomatic or if there are serious infections. Thus, it is important to perform to quantify the amount of virus infecting bees by performing quantitative reverse transcription polymerase chain reaction (RT-qPCR).

ABPV

Acute bee paralysis virus (ABPV) was first discovered in a lab when researchers noticed it produced similar symptoms to chronic bee paralysis virus (CBPV), but the symptoms appeared quicker, leading to the virus to be named acute bee paralysis virus (Bailey *et al.* 1963). ABPV is similar, in sequence, to Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV), and these are sometimes referred to as the ABPV-IAPV-KBV complex. Francis *et al.* (2013) tested for all three of the viruses using one single assay and one pair of primers. ABPV was not commonly found in bees until the emergence of *Varroa*

mites. Higher amounts of viral loads of the ABPV-IAPV-KBV complex and colony mortality positively correlated with the presence of *Varroa* mites (Francis *et al.* 2013).

McMahon *et al.* (2015) found ABPV to be the most prevalent virus, infecting eleven percent of bumble bees. In his study, he found a wide range of viral loads in bumble bees, ranging from $1.0x10^{4}$-$1.0x10^{11}$ viral copy numbers. These copy numbers represent the initial amount of viral copies inside a sample. Honey bees likely become infected with ABPV when removing feces from previously infected honey bees, but they do not encounter an amount of virus high enough to kill them (Bailey and Gibbs 1964). Highfield *et al.* (2009) found no honey bees affected with ABPV had viral loads higher than $1.0x10^{6}$ copies. Honey bees with viral loads this high were previously found to show no visible symptoms. When bees are feeding, ABPV may be transmitted from bee to bee, but a high amount of virus is needed for infection, around $1.0x10^{11}$ copies per bee. When ABPV was injected into the bees, on the other hand, far less virus was needed to cause paralysis, only about $1.0x10^{2}$ particles (Bailey and Gibbs 1964). This shows the impact *Varroa* mites have on the bees and helps explain why they are such efficient vectors. *Varroa* mites bite the bees and open wounds which allows the virus entry. Tentcheva *et al.* (2004) found ABPV infected colonies with ABPV infected mites, but also found other ABPV positive colonies with no ABPV positive mites. Prevalence of ABPV was highest in late summer and early fall which correlates to the peak of varroa mites (Tentcheva *et al.* 2004, Runckel *et al.* 2011).

ABPV has also been found in Hungary, where several of the colonies also tested positive for *Nosema* (Bakonyi *et al.* 2002) and in Austria (Berényi 2006). Because of the

virality of ABPV, it is unlikely to remain in a colony over winter, unlike other less virulent diseases. Cox-Foster *et al.* (2007) found ABPV found in both Colony Collapse Disorder (CCD) and non-CCD colonies.

BQCV

BQCV was first found in 1975 in honey bees (Bailey and Woods 1977). BQCV affects pupae, causing them to turn black and die; although, the queen is not visibly affected. While it has been suggested BQCV primarily infects queen larvae, others have found little BQCV detections in larvae (2%) but high frequency in adults (58%) (Tentcheva *et al.* 2004). BQCV tends to be more prevalent in the early months of spring and summer, rather than in the fall (Tentcheva *et al.* 2004, Highfield *et al.* 2009, Runckel *et al.* 2011). This is most likely related to high prevalence of *N. apis* in the summer as the two have been shown to be associated. In France, 86% of apiaries were found to have infections of BQCV. BQCV was not found in any *V*arroa mites, but there was a prevalence of *N. apis* with the BQCV samples (Tentcheva *et al.* 2004).

McMahon *et al.* (2015) found viral loads of BQCV did not significantly differ between honey bees and bumble bees. These infections were around $1.0x10^4$-$1.0x10^6$ viral copies, but they did find that BQCV (along with DWV) was more prevalent in honey bees than in bumble bees. In Massachusetts, over 300 honey bees were tested and only five bees were not infected with one of the three viruses (Welch *et al.* 2009). Both BQCV and DWV were prevalent. DWV was found in 98% of the bees in local hives and 72% in migratory hives while BQCV was found in 60% of bees in local hives and 92% in migratory hives.

In France, 86% of apiaries were found to have infections of BQCV (Tentcheva *et al.* 2004). In Austria, BQCV was found in 30% of honey bee samples and had a wide distribution across the country (Berényi 2006). Cox-Foster *et al.* (2007) found BQCV in both CCD and non-CCD colonies. In Iowa, BQCV was found to be common in honey bees but almost non-existent in non-honey bees with only 3% of non-honey bees showing infective levels of BQCV (Dolezal *et al.* 2016). In New Zealand, BQCV was found in over 91% of colonies, the highest proportion for any virus measured. They also found BQCV and SBV exhibited positive correlation with each other in bees, and in mites as well (Mondet *et al.* 2014). In Brazil, bumble bee samples were negative for *Varroa* mites and *N. ceranae,* but all bumble bees tested positive for viruses were all co infected with DWV, BQCV, and SBV. However, four other common pollinator RNA viruses were not detected (Reynaldi *et al.* 2013). Overall, BQCV has a relatively high prevalence in many different areas around the world.

DWV

Deformed wing virus (DWV) is arguably the most prevalent honey bee virus. Symptoms of DWV include body discoloration and deformed wings (Chen and Siede 2007). DWV is not highly pathogenic by itself, but it becomes deadly when the hosts encounter stress factors (Tantillo *et al.* 2015). Without *Varroa* mites, DWV infection rarely leads to visible symptoms (Grau 2017). A wide-ranging study to assess pathogen effects on a colony's strength found that DWV was the only pathogen to have a direct relationship between the presence of that pathogen and a weak colony (Budge *et al.* 2015).

A three-year study investigating the impact of DWV and acute bee virus (ABV) found that if only one to seven DWV infested mites were introduced to a honey bee colony, the colony would collapse in two years. If 15 or more mites were introduced, the colony would only survive one winter before collapsing in the next winter or spring (Martin 2002). On the other hand, ABPV is more virulent, and adult bees with ABPV die before raising offspring. About 10,000 or more ABPV-infected mites are required to kill a colony. In Switzerland, DWV and *Varroa* mites were shown to be positively correlated (Berthoud *et al.* 2010). Because adult bees that carry the virus are relatively unaffected, the mites can overwinter on the bees and the virus will remain present (Francis *et al.* 2013). In the McMahon *et al.* (2015) study, which covered numerous sites across Great Britain, DWV was the most prevalent virus, infecting 36% of honey bees. Honey bees had a higher viral load ($1.0x10^{10}$-$1.0x10^{11}$ per bee) when compared to bumble bees ($1.0x10^{4}$-$1.0x10^{6}$). DWV viral loads in asymptomatic honey bees have also been found to be in range from $1.0x10^{3}$-$1.0x10^{9}$ copies, with levels in symptomatic bees much higher (Highfield *et al*. 2009). Colonies that have both survived or collapsed were found to have levels of DWV in the higher range ($1.0x10^{9}$ per bee) during the summer, showing that high levels of DWV during the summer were not indicative of a colony's eventual survival (Highfield *et al.* 2009). However, colonies with high levels of DWV during the winter months did experience greater losses (Highfield *et al.* 2009). Studies that have detected DWV in bees found most bees were asymptomatic but positive for the disease (Lanzi *et al.* 2006) However, bees that do show symptoms have virus levels 4.4-fold higher than asymptomatic bees (Chen and Siede 2007). This further shows that simply the presence of DWV will not kill a bee and

that bees captured while foraging, are likely to have lower levels of the virus. Bees that show symptoms will be severely affected and will not fly well. Prevalence of DWV has been found to be consistent in honey bees, wild bees, and hive bees (Dolezal *et al.* 2016).

Fürst *et al.* (2014) found a strong spatial autocorrelation between DWV-infected *A. mellifera* and DWV-infected *Bombus*. Their data implied local transmission was the cause as DWV strain variants from specific sites were the same between honey bees and bumble bees. However, they estimated their prevalence was underestimated; bees with high levels of DWV would be unable to fly and would not be sampled. In France, 97% of apiaries were found to have infections of DWV (Tentcheva *et al.* 2004). In Austria, 91% of honey bee samples had DWV. Asymptomatic honey bees had viral loads 126 times lower than honey bee colonies showing symptoms of DWV (Berényi 2006). Cox-Foster *et al.* (2007) found DWV in both CCD and non-CCD colonies.

However, not all studies have found high rates of DWV in sampled bees. A study in Bulgaria testing honey bee viruses found DWV to be the virus with the highest prevalence at slightly over ten percent (Shumkova *et al.* 2018). This proportion is much lower than other reported data on DWV prevalence from other studies. DWV was found in only one pool sample of bees from their apiary 1, with no other viruses detected in Spain (Higes *et al.* 2009).

Hypotheses

I hypothesized that these primarily honey bee viruses will be found in both bumble bees and mason bees, with DWV being the most common of the viruses. I also expected to find locations with high levels of prevalence of both BQCV and *Nosema*. I expected to find

more samples with co-infections of viruses than samples with just single infections of a virus.

Methods and Materials

Sample Collection

Samples were collected from ten different sites across Northern Virginia in the summer of 2018. In total, 136 honey bees, bumble bees, and mason bees were collected and used for RNA research. This total includes 60 honey bees, 46 bumble bees, and 30 mason bees. Table 6 below summarizes the number of individuals caught at each location.

Table 6 | Summary of individuals collected for RNA analysis

Site Number	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10
Apis mellifera	-	10	10	10	-	10	10	-	10	-
Bombus impatiens	11	-	1	-	1	3	9	8	-	-
Bombus griseocollis	-	-	1	-	5	6	1	-	-	-
Bombus perplexus	-	-	-	-	-	-	-	-	-	-
Bombus auricomous	-	-	-	-	-	-	-	-	-	-
Bombus bimaculatus	-	-	-	-	-	-	-	-	-	-
Bombus fervidus	-	-	-	-	-	-	-	-	-	-
Bombus pensylvanicus	-	-	-	-	-	-	-	-	-	-
Osmia taurus	-	-	5	-	-	2	-	-	-	-
Osmia cornifrons	-	3	-	1	-	12	1	-	-	-
Osmia lignaria	1	-	-	-	-	5	-	-	-	-
Total collected	12	13	17	11	6	38	21	8	10	0

Honey bees and bumble bees were caught in the field or off honey bee hives. Bees were caught in 50 ml falcon tubes and then were immediately transferred to 2 ml centrifuge tubes which were then placed in a portable tank of liquid nitrogen, where samples were instantly frozen to preserve the RNA. Mason bees were collected from their homes in late 2018 and live bees were transported on ice until they were transferred into a -80°C freezer.

RNA Standard

A universal RNA standard for the RNA viruses, in the form of a plasmid, was obtained from Carrillo-Tripp *et al.* (2016). These authors created a plasmid which contained the primer sequences of the viruses they tested. This plasmid contained segments of DNA of six RNA viruses (ABPV, BQCV, DWV, IAPV, KBV, and SBV). These sequences were concatenated, giving the plasmid a total length of 912 bases. The lengths of the viral sequences were between 105 and 200 bases long. We used the primers as found in their paper, as those primers would also anneal to the RNA standard. After the plasmid was received from shipping, the plasmid was chemically transformed into OneShot® (Thermo Fisher) competent *E. coli* cells. The plasmid was then linearized with an Xbal restriction enzyme. In a vial, 50 µl competent cells and the plasmid were incubated on ice for 30 minutes, followed by incubation at 42°C for 30 seconds in a water bath. After S.O.C. medium was added to the vial, the solution was placed into a shaking incubator at 37°C for 1 hour. Following this, the solution was plated on standard agar plates and incubated overnight at 37°C. The cells were then plated on kanamycin plates. Cells that successfully

survived ensured the plasmid was successfully taken into the cells. This was done to create more stock of the plasmid. A MEGAscript™ T7 Transcription Kit was used to produce the RNA standard. 100µl of the standard was produced, separated into two 50µl aliquots. Only one aliquot was used for this project. The standard was quantified using a Qubit™ RNA HS Assay Kit (Thermo Fisher) on a Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific). The standard was measured three times and the average concentration of RNA molecules in the solution was calculated as 6.48 ng/µl.

RNA Extraction and Amplification

A dedicated hood was used for RNA extraction and amplification. RNA extraction from bees was performed using a PureLink™ RNA Mini Kit (Thermo Fisher Scientific) according to manufacturer's protocol. To remove DNA, the RNA extract was treated with DNase by adding 80µl of DNase solution. This solution consisted of 62µl of RNase free water, 10µl of resuspended DNase, and 8µl of 10X DNase I Reaction Buffer. This was performed using a PureLink™ DNase Set (Thermo Fisher Scientific). Extracted RNA was stored at -80°C and RNA was aliquoted into two 25µl aliquots. To quantify the number of viral copies per sample, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed. RT-qPCR was performed using a StepOnePlus™ Real-Time PCR System instrument and the SensiFAST™ SYBR® Hi-ROX Kit (Bioline). Each RT-qPCR reaction consisted of 10µl of 2x SensiFAST™ SYBR® Hi-ROX One-Step Mix, 0.8µl of each forward and reverse primers in a 10µM concentration, 0.2µl of reverse transcriptase, 0.4µl of RiboSafe RNase Inhibitor, 3.8µl of H_2O, and 4.0µl of RNA template. Endpoint PCR (classic PCR) is commonly used in research but is only able to give final amplification

products. Quantitative PCR (qPCR) was used in this study as amplicons can be detected in real time, which allows different levels of viral loads to be recorded, with the help of standards. This research used quantitative reverse transcription PCR (RT-qPCR) as RNA needs to be reverse transcribed into DNA before amplification

RNA amplification was performed with an initial heating period to 45°C for 10 minutes. This was followed by a denaturation period of 2 minutes at 95°C. Then, a 5 second period at 95°C followed by an annealing period of 20 seconds at 60°C was repeated 40 times. After each run, a melt curve was produced. This procedure was performed by a period of 15 seconds at 95°C then followed by 1 minute at 60°C. Lastly, the temperature was increased by 0.3°C and was held for 15 seconds until a temperature of 95°C was reached. Reactions were performed with anywhere from eight samples per reaction to 39 samples per reaction. Primers for RNA analysis followed those in the paper, *In vivo and in vitro infection dynamics of honey bee viruses* (Carrillo-Tripp *et al.* 2016) (Table 7).

Table 7 | Primers used for RNA amplification

Primer	**Sequence**
ABPVqRTPCR-F	ACCGACAAAGGGTATGATGC
ABPVqRTPCR-R	CTTGAGTTTGCGGTGTTCCT
BQCVqRTPCR-F	TTTAGAGCGAATTCGGAAACA
BQCVqRTPCR-R	GGCGTACCGATAAAGATGGA
DWVqRTPCR-F	GAGATTGAAGCGCATGAACA
DWVqRTPCR-R	TGAATTCAGTGTCGCCCATA

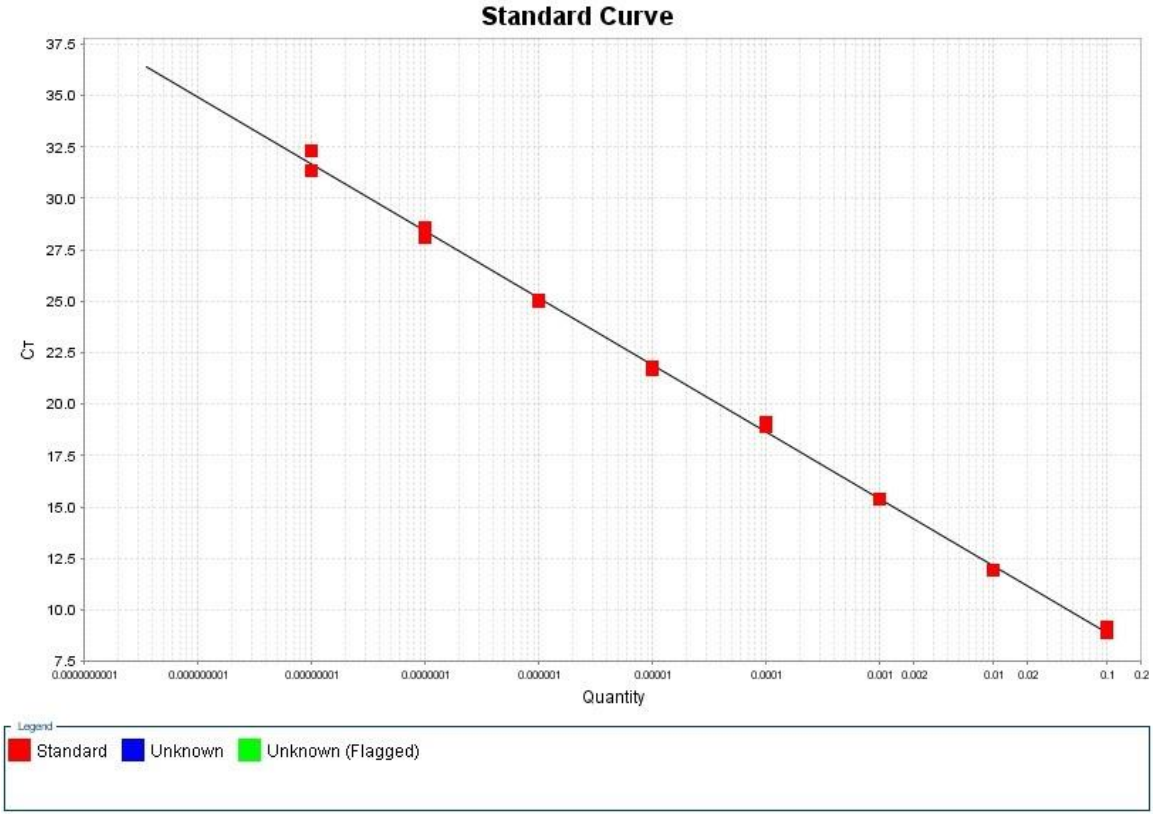

Figure 9 | Standard curve. This is an example standard curve when performing RT-qPCR. This was used with ABPV primers on the RNA standard. Standards were diluted in a 1:10 serial dilution and performed in duplicate.

Serial dilutions of the RNA standard were used to construct a standard curve for each batch of reactions. For each series of serial dilutions, 1:10 serial dilutions were performed. The standard curve for the first reaction consisted of eight serial dilutions. These dilutions ranged from $1.0x10^{-1}$ to $1.0x10^{-8}$. All following reactions consisted of just five serial dilutions, ranging from $1.0x10^{-4}$ to $1.0x10^{-8}$. This was performed because the original concentration of the standard was so high, it was not necessary for dilutions to be above $1.0x10^{-4}$. All samples, including the standards and negative controls, were ran in

duplicate. With each further dilution of the standard, the corresponding Ct values increase. When the standard is serial diluted, this creates the standard curve.

The following process was used to determine the copy number of viruses per ul. First, the length of the sequence covered by each pair of primers in the standard was found. In the standard, the ABPV sequence was 124 base pairs long, the BQCV was 140 base pairs long, and DWV was 130 base pairs long. Next, the concentration in the stock was converted from ng/µl to g/µl. Then, the sequence length was multiplied by 340, which is the average mass of one nucleotide in Daltons. For example, for ABPV, 340 would be multiplied by 124. Lastly, this value was multiplied by Avogadro's number ($6.022414x10^{23}$), to give the final amount of copy numbers inside the template. This was performed according to protocol from a Qiagen qPCR sample assay tech guide for an RNA standard (Qiagen). This was performed for each virus. Final copy numbers for each virus, found in the standard, are listed: $9.26x10^{10}$ copies/ul for ABPV, $8.20x10^{10}$ for BQCV, and $8.83x10^{10}$ for DWV.

Chi-Square Tests

Chi-square tests were performed to measure the potential dependence or independence of the viruses with each other. The Chi-square test is a widely used statistic to test correlations between categorical variables. Chi-square tests can be useful in determining the possible dependence or independence of multiple pathogens affecting organisms. Numerous studies previously cited in this research, showed more than one pathogen can affect the same organism. The null hypothesis for a Chi-square test is that the response variables are independent of each other. If the null hypothesis cannot be rejected,

then one can assume the variables are independent. If the null hypothesis is rejected, the variables are found to be dependent on each other. Three Chi-Square tests were performed; examining the possible relationships between ABPV and BQCV, ABPV and DWV, and BQCV and DWV. These tests examined the presence or absence of virus in individual bees, and whether the presence of these viruses were independent of each other. Any range of viral loads present in a bee count as a "present" for Chi-Square analysis.

Results

Overall Infection Rates

Overall, 136 bees were collected for RNA analysis. Sixty honey bees, 46 bumble bees, and 30 mason bees made up the total sample collection. In total, 84% (n = 115) of bees were found to be infected with at least one virus, 39% (n = 54) of bees were infected with two viruses and 19.9% (n = 27) of bees were infected with all three viruses. Of the 27 bees infected with all three viruses, 25 of them were honey bees. Every single honey bee collected was infected with at least one virus. Only six bumble bees were not infected with any virus. While a majority of honey bees and bumble bees were co-infected with multiple viruses, only three mason bees were co-infected with two viruses. No mason bees were infected with all three viruses. Table 8 below, shows the prevalence of each virus in each of species collected, along with the total prevalence of each virus.

Table 8 | Prevalence of viral infection in collected samples

	Number and percentage of species infected with ABPV	Number and percentage of species infected with BQCV	Number and percentage of species infected with DWV	Number and percentage of species co-infected	Number and percentage of species uninfected	Total number and percentage of species infected

Apis mellifera	26 43.0%	58 96.7%	57 95.0%	56 93.3%	- 0%	60 100%
Bombus impatiens	6 18.2%	29 87.9%	8 24.2%	13 39.4%	3 9.0%	30 91.0%
Bombus griseocollis	5 38.5%	9 69.2%	4 30.8%	6 46.2%	3 23.1%	10 76.9%
Osmia taurus	2 28.6%	2 28.6%	1 14.2%	1 14.2%	3 42.9%	4 57.1%
Osmia lignaria	3 50.0%	2 33.3%	1 16.7%	2 33.3%	2 33.3%	4 66.7%
Osmia cornifrons	5 29.4%	2 11.8%	3 17.6%	3 17.6%	10 58.8%	7 41.2%
Total number and percentage of all collected samples	47 34.6%	102 75.0%	74 54.4%	81 59.6%	21 15.4%	115 84.6%

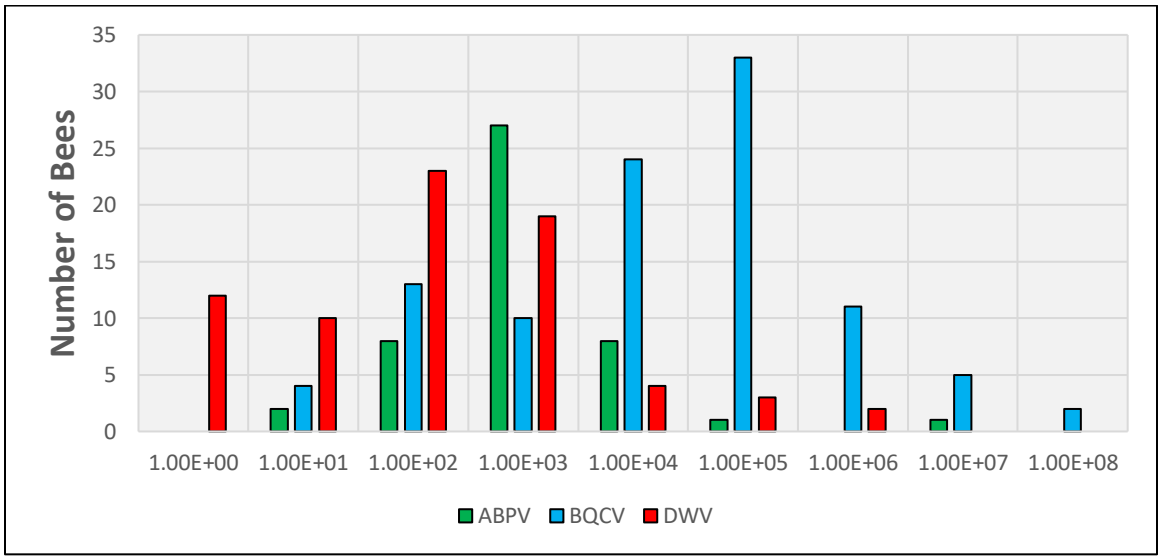

Figure 10 | Range of viral loads per RNA virus. This figure shows the total number of bees infected with one of the three viruses, separated into ranges of viral counts. The x-axis value represents the low-end range of that column. The high-end range ends at the next column.

Table 9 | Viral load comparison per genus

	Total number infected with ABPV	Number and percentage of viral loads of ABPV over $1.0x10^4$	Total number infected with BQCV	Number and percentage of viral loads of BQCV over $1.0 x10^4$	Total number infected with DWV	Number and percentage of viral loads of DWV over $1.0x10^4$
Apis mellifera	26	4 40.0%	57	54 72.0%	59	8 90.0%
Bombus spp.	11	2 20.0%	39	20 26.7%	12	1 10.0%
Osmia spp.	10	4 40.0%	6	1 1.3%	5	- -
Number of infected samples	47	10	102	75	74	9
Percentage infected across all sampled bees	34.6%	7.4%	75%	55.1%	54.4%	6.6%

As shown above in Figure 10 and Table 9, honey bees tended to have higher ranges of viral loads, compared to other genera. 72% and 90% of BQCV and DWV infections, over $1.0x10^4$ viral loads, were found in honey bees. ABPV viral loads were more relatively more consistent across genera. A total of only five mason bees were infected with RNA viral loads above $1.0x10^4$, but four of these viral loads were of ABPV.

ABPV Infection

Thirty four percent (n = 47) of bees were infected with ABPV. Only 7.4% (n = 10) of all bees, had a viral load of $1.0x10^4$ ABPV particles or more (Table 9). The three bees with those viral loads were collected from four different sites, with seven of the bees collected from site 6. Three were honey bees, three were bumble bees, and four were mason bees. Of the nine sites visited for RNA analysis, only sites 5 and 8 had no presence of

ABPV. One of these had very little infections overall. Fourteen bees were collected from these two sites. Although no ABPV was found in samples from these two sites, there was high prevalence of BQCV infection (78.6%, n = 14) and medium rates of DWV infection (35.7%, n = 14).

Site 6 had the highest prevalence of ABPV infections. Thirty-nine bees were collected from this site, split into nineteen mason bees, ten honey bees, and nine bumble bees. All three species were moderately affected by ABPV, with infection rates of 52.6%, 60%, and 55.6% respectively. With the exception of three bumble bees, all other honey bees and bumble bees collected from this site were also infected with both BQCV and DWV. Two mason bees were co-infected with BQCV. According to the data from the other six sites, with the exception of one honey bee, every single other ABPV infected honey bee and bumble bee was co-infected with BQCV. Only six of those bees were also not infected with DWV. Site 6 and site 2 had the highest rates of ABPV infection had rates of 53.8% (n = 21) and 40% (n = 4), respectively. The rates of *Nosema* and trypanosomatids at these same sites were; 7.3% and 68.3%, and 72.2% and 94.4%.

BQCV Infection

Seventy five percent (n = 102) of bees were infected with BQCV. Unlike ABPV, 55.1% (n = 75) of all bees, had a viral load of $1.0x10^4$ BQCV particles or more (Figure 10). The highest viral copy number was $2.58x10^8$ with seven total samples in the range of $1.0x10^7$ or higher. Although bees infected with BQCV had the highest levels of infection, no symptoms were seen in any collected bee. Across all sites, viral copy numbers were higher on average in honey bees compared to bumble bees and mason bees. All sites had

at least one bee with viral loads above $1.0x10^4$. Only six mason bees were infected with BQCV and only one of those had a viral copy number higher than $1.0x10^4$. Three of the mason bees were from site 6, and one mason bee was also found at sites 1, 3, and 4.

Including only honey bees and bumble bees, all bees at Sites 2, 3, 4, 6, and tested positive for BQCV. At four of these sites, all bees infected with BQCV were also co-infected with DWV, although the viral counts of DWV was lower on average. Site 6 only had 52.6% (n = 10) of bees also infected with DWV. As previously mentioned in this paper, site 9 accounted for 54.5% (n = 12) of all recorded *Nosema* infections. This same site had positive BQCV infection in every collected RNA sample, which were all honeybees. With the exception of site 7, where all but three collected bees were positive for BQCV, all sites with recorded infections of *Nosema* occurred at one of the five sites where BQCV was also present in every single honey bee or bumble bee collected.

DWV Infection

Fifty four percent of bees were infected with DWV. Similar for the case for ABPV, a low number of infected bees had relatively high copy numbers (Table 9). Only 6.6% (n = 9) of all sampled bees had a viral load of $1.0x10^4$ DWV particles or higher. The nine bees with those viral loads were collected from five different sites. Eight of the bees were honey bees and one was a bumble bee. Site 3 had all ten of its collected honey bees infected with DWV, and four of those bees had viral loads higher than $1.0x10^4$. Two bumble bees and five mason bees were also collected from this same site, but none were infected except for one mason bee. Site 8 was the location with the highest amount of DWV infected bumble

bees. Half of the captured bees were infected. However, no honey bees or mason bees were caught at this location so the DWV prevalence in those species at this site is unknown.

Along with site 3, sites 2, 4, and 7 were the other sites where all collected honey bees had DWV. Only honey bees and mason bees were collected from sites 2 and 4, and no mason bees were infected with DWV. At site 7, ten bumble bees and one mason bee were also captured, but only one of the bumble bees was infected with DWV. When comparing the *Nosema* and trypanosomatid prevalence at these same sites, none of these sites had any honey bees or mason bees infected with *Nosema*, and only seven bumble bees were infected with *Nosema*. However, a vast majority of the bees collected were infected with trypanosomatids.

Chi-Square Results

Table 10 | Chi-Square table

	Variables	Degrees of Freedom	χ^2 value	P value
Chi-Square Test 1	ABPV and BQCV	1	0.866	0.352817
Chi-Square Test 2	BQCV and DWV	1	14.267	0.000159
Chi-Square Test 3	ABPV and DWV	1	4.982	0.027013

The Chi-Square tests examined potential associations between the presence of two viruses in individual bees. These Chi-Square tests used bee species collected across all sites and included all genera and species. Any range of viral load in a bee was counted as an infection for this analysis. If the p-value was found to be lower than a significance level of

0.05, the null hypothesis of no correlation between infection by the two viruses was rejected. My analysis indicated no association between infection by ABPV and BQCV (Table 10). However, my analyses supported an association between BQCV and DWV and between ABPV and DWV (Table 10).

Discussion

My first hypothesis stated DWV would be the most prevalent virus. My data did not support this as BQCV was the most common virus found. In multiple studies, DWV has been found to be the most common virus (McMahon *et al.* 2015, Tentcheva *et al.* 2004, Berényi 2006). Some of these studies found DWV prevalence as high as 97% (Tentcheva *et al.* 2004) and 91% (Berényi 2006). Other studies have found DWV prevalence only as high as 36% (McMahon *et al.* 2015) or slightly over 10% (Shumkova *et al.* 2018). My study found over 50% of bees were infected with DWV, but viral loads in many of the bees were low. When only accounting for viral loads above $1.0x10^4$, DWV was found in 6% of all bees. Not all previous researchers who measured virus prevalence used RT-qPCR, so it is both unknown what the detection limits of their PCR were and if their viral counts were low or high. For comparison, of these four studies, only McMahon *et al.* (2015) performed RT-qPCR, the other three studies used RT-PCR. McMahon *et al.* (2015), stated 36% of honeybees (n = 237) and 3% of bumble bees (n = 555) were infected with DWV. This would indicate 85 honey bees and 16 bumble bees were infected with DWV, equaling a total sample size of 101 infected bees. But for DWV, the authors make a graph of infection rates with a range of just $1.0x10^4$-$1.0x10^{11}$, with a sample size of just 53. It is unclear if only 53 of the samples had viral particles of $1.0x10^4$ or higher. They used 500ng in their

reactions while I used 4µl in my reactions, although viral copy numbers represent the initial numbers of copies in a reaction. Regardless, it indicates that even the highest levels of DWV found in my samples, are on the low-medium end of other studies.

The presence of DWV is not a death sentence for the bee. Bees may be asymptomatic if they have low levels of viral loads (Chen and Siede 2007). Highfield *et al.* (2009) found bees in the range of $1.0x10^3$-$1.0x10^9$ with symptomatic bees showing higher levels of infection. The lack of bees with high viral loads in this study can be explained several different ways. The first reason may simply be there are no bees with viral loads that high. This is entirely possible as only 136 bees were sampled for RNA analysis. Bees in the area could also be relatively good health. Another possible explanation could be bees showing severe symptoms of DWV would not be able to fly and survive, and thus would not be caught. As its name implies, DWV (deformed wing virus) affects the wings of the bee, among other symptoms. DWV causes the wings to shrink and shrivel up. So highly infected bees would not be easily found and sampled compared to asymptomatic bees.

ABPV was the least prevalent of the three viruses tested with only 34.5% of bees infected. The percentage of bees infected with ABPV with viral counts higher than $1.0x10^4$ was even lower than DWV; 7.4% of bees had viral counts that high. McMahon *et al.* (2015) had ranges of copy numbers of ABPV from $1.0x10^4$-$1.0x10^{11}$ with slightly fewer samples than DWV (n = 42). Highfield *et al.* (2009) found no honey bees affected with ABPV had viral loads higher than $1.0x10^6$. My samples with the two highest loads were honey bees, but neither were higher than the range of $1.0x10^6$. The low prevalence of ABPV collected

should not be related to the season. ABPV is highly correlated with the presence of *Varroa* mites, and the peak of those mites is typically late summer and early fall. Even though a majority of the samples was collected in July and early August, which would probably be classified as mid-summer, ABPV was still the least common virus found.

My second hypothesis stated that locations with high levels of BQCV would have levels of *Nosema* infection. My data did not clearly support this hypothesis. Site 9 had slightly over 54% of all reported *Nosema* infections. Ten honey bees were caught from this same site and all ten were infected, with nine of them over the $1.0x10^4$ threshold. However, BQCV was prevalent throughout all visited sites. The threshold of $1.0x10^4$ viral copies per bee, indicating low or high infection, was chosen based on the study of McMahon *et al.* (2015). In their study, $1.0x10^4$ viral copies per bee was their base value to indicate low levels of infection. From site 9, the highest viral count from this site was in the range of $1.0x10^7$. The highest viral count of BQCV came from site 2, where one honey bee had a viral count in the range of $1.0x10^8$. Ten honey bees were also caught at this site, and all of them had viral counts $1.0x10^4$ or higher. Unfortunately, no honey bees or bumble bees were sampled for this site for DNA analysis, so I could not test for a possible correlation. Three mason bees were observed from that site, but no mason bees from any site was infected with *Nosema*. Tentcheva *et al.* (2004) observed BQCV infection rates were highest in summer, which my data supported. Both BQCV and *N. ceranae* both peak in late summer and early fall (Runckel *et al.* 2011). Bailey *et al.* (1983) found BQCV and *N. apis* were associated with each other. While my study found no instances of *N. apis*, *N. apis* is very closely related to *N. ceranae*.

Overall, 75% of bees were infected with BQCV and slightly over 55% of all bees with viral counts of BQCV of $1.0x10^4$ or higher. These numbers are in stark contrast to DWV and ABPV, as the viral counts for BQCV are far higher. Bumble bees were far more likely to become infected with BQCV, than ABPV or DWV. Twenty-four percent and 26% of bumble bees were infected with ABPV and DWV, respectively, while over 82% of bumble bees were infected with BQCV. Some studies found very little BQCV infection in bumble bees (Dolezal *et al.* 2016), while others found high numbers of infected bees (Alger *et al.* 2019). BQCV was overall far more infective than the other two viruses.

Lastly, my third hypothesis stated there would be more cases of viral co-infection than cases of single infection. My data supported this hypothesis. Slightly 15% of bees were infected with a single virus while just under 60% were infected with two or more viruses. Site 5 was the only site with no instances of viral co-infection, albeit with a sample size of only six bumble bees. Numerous previously cited studies have found high rates of co-infection between several viruses. For example, Tentcheva *et al.* (2004) found over 92% of apiaries were infected by three or more pathogens and 25% of apiaries were infected with five viruses. Chi-square tests indicated that DWV was associated with both BQCV and ABPV. Secondary DWV infections have typically been labeled as covert infections and relatively asymptomatic (de Miranda and Genersch 2010), but a recent study found secondary DWV infections to be more virulent and have longer lasting effects than previously thought (Benaets *et al.* 2017).

ABPV and DWV both peak around the same months *Varroa* mites peak, which is late summer and early fall. When a colony is infested with *Varroa* and a virus, the viral

load typically increases throughout the year, peaking at late summer. The number of mites correlate with the number of viruses. BQCV is not associated with *Varroa* mites and typically peaks in the summer. It is unknown if any of the sites visited, experience *Varroa* infestations. *Varroa* infestations, when combined with ABPV or DWV can be deadly to a colony. If the colonies sampled for this study where not affected with mites, then that could explain the lower amounts found in this research, compared to BQCV.

Overall this research helps provide evidence of pathogen presence in the Northern Virginia area. Three viruses were tested, and they were found in varying amounts. Most of the viruses alone will not kill a colony, but when combined with other pathogen infections or *Varroa* mite infestations, a colony can become overwhelmed. It is important for beekeepers in this area to know what pathogens have been recorded in the area. Although my study utilized RT-qPCR and it could detect viral loads of differing, sequencing was not performed on the RNA samples. Therefore, it is unknown whether different locations or host groups have the same strain of the three viruses. More research should be performed in the Northern Virginia area regarding levels of pathogen viral load in different times of the season. More research also needs to be done regarding solitary bees. Just as in the case for DNA pathogens, there are few papers observing pathogen infection in solitary bees. My research found all three viruses were able to infect solitary bees. More research is needed on the effects of these viruses on solitary bees as well as the possible effect of *Chrysididae* wasps, or other parasites, or inquilines. One *Chrysididae* wasp egg was incidentally found in the mason bees and used for RNA analysis, and it was found to be infected with ABPV. This perhaps suggests that this species may act as a vector for ABPV or this species

acquired the virus from a mason bee, after parasitizing the bee. This paper provides novel baseline data on viral infections in Virginia and expands knowledge of eukaryotic pathogens in the region. More research should be performed to answer questions that this research was unable to answer. Solitary bees are not widely studied, and more information is needed. Also, the RNA pathogens were not sequenced, and thus it is unknown if the species share the same strains or not. Future studies could further explore these unanswered questions. The data discovered during this project will hopefully become a stepping stone to more research in the Northern Virginia region.

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