

Bumble Bee Pathogen Prevalence Determined by Host Species

Thesis

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## Abstract

Bumble bees (Order Hymenoptera) are important pollinators of economically significant crops. In the past 20 years, there has been a decline in several North American species, including the federally endangered rusty patched bumble bee (*Bombus affinis* Cresson, 1863). Once common throughout the upper Midwest and Eastern U.S., *B. affinis* is now absent from 87% of its historical range. Several causal factors contributing to bumble bee decline have been identified, including habitat loss/degradation, the increased use of pesticides, and an increase in pathogens. Pathogens, particularly those originating from commercial colonies, have been implicated in this decline and are an immediate concern for conservationists. However, the combined effects of pathogen distribution and land cover on pathogen prevalence in the U.S.A is largely unknown. Madison, WI is a large metropolitan area, within the extant range of the rusty patched bumble bee and is bordered by suburbs, exurbs, agriculture, and forested areas. During the summers of 2019 and 2020, 2,094 bees were collected from 20 urban and agricultural sites. Each bee was screened for the microparasites *Vairimorpha* spp. (Fantham & Porter, 1914), *Crithidia bombi* (Léger, 1902), *Crithidia expoeki* (Schmid-Hempel & Tognazzo, 2010), and *Apicystis bombi* (Liu et al., 1974) using a multiplex PCR panel. Conopid flies (Order Diptera) and mermithid nematodes (Order Mermithida), both large endoparasitoids, were found across sites. *Bombus impatiens*, the most abundant species, had the highest

pathogen prevalence and the most prevalent pathogen in this species was *Apicystis bombi*.

## **Dedication**

Hey ma, this is for you.

## Acknowledgments

This thesis took a village. First, I would like to thank my advisor, Dr. Jamie Strange, for giving me this opportunity, for being patient while I was learning, and for teaching me the way of the bee. I will be forever grateful. Dr. Mo Christman who was always available at the last minute to help me with r, editing, data interpretation, and advise. Dr. Pinar Barkan who was always willing to edit my terrible writing, distract me with endless conversations, and for providing me with a kind shoulder to lean on. Dr. Ellen Klinger for always being willing to discuss statistics, how best to work with my data, and for being on my committee. Dr. Kayla Perry who included and supported me from the very beginning and for the hours spent practicing presentations, writing code, and helping me interpret my data. Dr. Carol Anelli for the interesting conversations, providing support, and for being on my committee. Iliana Moore for being a supportive lab mate and for allowing me to tag along during field work and dumpster diving. Taylor Tai and Dr. Claudio Gratton for sharing their bees and hard-earned data. Many folks helped me with lab work: Claire Nagy, Gwen Stamper, Mary Kozlowski, and especially Elizabeth Blackford. Without their assistance, I would still be neck deep in bees. And lastly, ma'colleague, Al, for always being willing to jump in the truck to head off on our next adventure, I love you.

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## **Fields of Study**

Major Field: Entomology

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## Introduction

Bumble bees (*Bombus*) are social insects in the order Hymenoptera, which includes bees, wasps, ants, and sawflies. There are approximately 265 bumble bee species widely distributed throughout the Holarctic, Palearctic, and Neotropical regions of the world (Williams et al. 2014). Their unique thermoregulatory adaptations (i.e. facultative endothermy) enable them to inhabit diverse habitats including temperate, subarctic, and boreal regions (Heinrich 2004, Hines 2008). Within North America, 46 species occur at varying distributions across a variety of habitat types such as wetlands, deserts, prairies, montane environments, savannas, agricultural landscapes, and even urban centers (Williams et al. 2014, Cameron and Sadd 2020, Williams and Jepsen 2021). Species such as *Bombus impatiens* Cresson, 1863 and *Bombus griseocollis* Cresson, 1863 have large geographic ranges, whereas *Bombus affinis* Cresson, 1863 and *Bombus vosnesenskii* Radoszkowski, 1862 are more limited and persist in much smaller ranges (Williams et al. 2014). The local abundance and diversity of bumble bee species is reliant on a continuous supply of pollen and nectar throughout the growing season (Williams et al. 2014).

North American bumble bees have an annual lifecycle (Figure 1.1) that begins in the early spring as mated gynes emerge from their winter hibernacula. They spend their first weeks collecting pollen from spring ephemerals and searching for a nest cavity. Bumble bees rely on abandoned rodent dens, hollow logs, and even suitable man-made

structures for nesting which they modify to fit their needs (Goulson 2010). Nesting types, locations, and conditions are species specific (Goulson 2010). A gyne bumble bee will create honeypots within the newly founded nest for nectar storage, and form pollen masses where she will lay fertilized eggs, which will develop into female workers (Goulson 2010). The gyne becomes a queen when she lays eggs and incubates the larvae as they feed directly on the pollen until eclosion from the pupal cells. Once the first female workers emerge as adults, they take over the tasks of nectar and pollen gathering, while the queen stays in the nest to continue producing brood. By late summer, as floral resources begin to diminish, the queen will start producing unfertilized eggs that develop into male bees (drones), and fertilized eggs destined to become the next year's queens (gynes). After they emerge from the nest, gynes will mate and seek out a suitable place to overwinter until the following spring (Goulson 2010) as the cycle continues.

Bumble bees feed on carbohydrates, lipids, and proteins found in plant nectars and pollen (Vaudo et al. 2018). Female workers forage on flowering plants throughout the season and gather pollen in large pellets on their corbiculae, which are specially modified areas of the hind legs evolved to store and transport collected pollen. The foraging range of bumble bees varies between species and resource availability. Previous studies have found that they frequently travel as far as 5km from their nest but distances as far as 20 km have been recorded for *Bombus terrestris* (Linnaeus, 1758) (Osborne et al. 1999, 2008, Williams et al. 2014). As such, bumble bees are capable of covering immense distances over their lifetimes as they search for sparsely scattered floral resources across the landscape (Heinrich 2004). During a single foraging trip, a worker

bee might visit hundreds of individual flowers (Heinrich et al. 1977). Any pollen that has incidentally accumulated on the setae covering her body is transferred from the anthers of one plant to the stigma of another, which completes the act of pollination. The act of foraging is essential for bumble bee health as sufficient resource provisioning and nutrition is linked to successful colony maintenance and reproductive output (Vaudo et al. 2018).

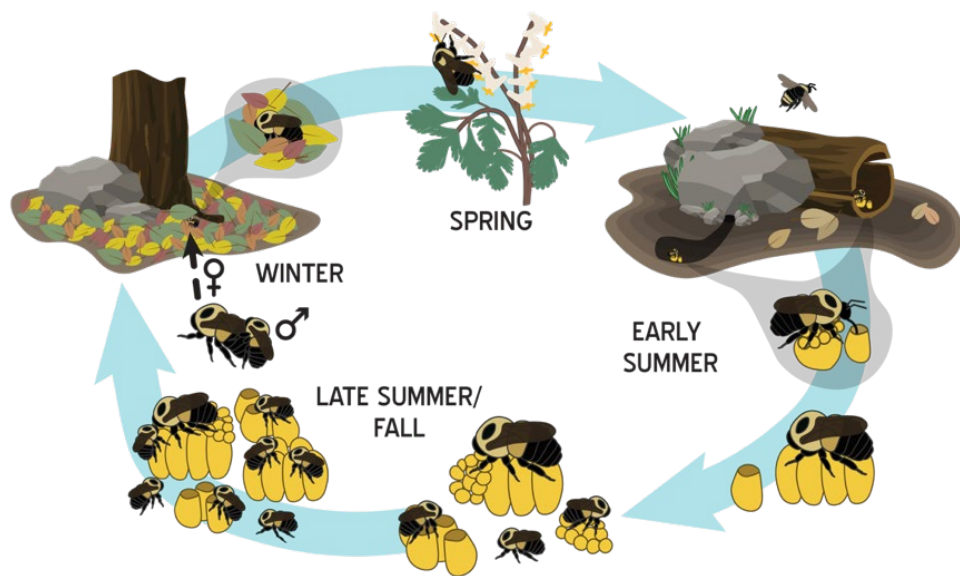


Figure 1.1: Bumble bee annual lifecycle. Gynes emerge in early spring to feed on spring ephemerals before founding a colony in early summer. As the season progresses, the queen produces workers and eventually reproductive offspring. Figure from Jeremy Hemberger 2020.

For life to persist on Earth, essential ecosystem services including nutrient and water cycling, climate regulation, and pollination are critical. The majority of flowering crops and wild plants (75%) require pollination for sexual reproduction (Kearns et al. 1998, Klein et al. 2007). A large number of flowering plants are partially or entirely dependent on bumble bee pollination, sometimes with a specific *Bombus* species favored for seed set (Goulson et al. 2005, Biesmeijer et al. 2006, Ollerton 2021). The pollination of flowering plants by bumble bees represents a critical ecosystem service that benefits humanity ecologically, culturally, and monetarily (Kearns et al. 1998). In the United States, bumble bees contribute \$65-70 billion USD (Kevan and Viana 2003) in pollination services each year. While it is generally thought that managed honey bee colonies provide sufficient pollination services to crops (Allen-Wardell et al. 1998, Klein et al. 2007, Angelella et al. 2021, Senapathi et al. 2021), they neither maximize pollination nor fully substitute the contributions of diverse wild bee assemblages (Garibaldi et al. 2013). At sufficient densities, bumble bees pollinate select high-value crops more efficiently than honey bees (Stubbs and Drummond 2001, Goulson et al. 2008) and wild pollinators, like bumble bees, can double fruit set independent of the presence of honey bees (Garibaldi et al. 2013, Senapathi et al. 2021). However, the sparsity of diverse, wild bee assemblages in agricultural landscapes has historically pushed growers to supplement with honey bees or commercial bumble bees (Goulson et al. 2008).

The efficiency of bumble bees as pollinators has led to a commercial industry in which several *Bombus* species are produced for greenhouse and open-field crop

pollination. *Bombus impatiens* is currently the most economically significant bumble bee species managed in North America. It is shipped nationwide, including outside its native range for greenhouse use. Additionally, *B. vosnesenskii* was recently approved for commercial use in 2022 and is available throughout its native range in California, Oregon, and Washington (Strange 2015, Strange et al. 2023). *Bombus huntii* Greene, 1860 can also be purchased for use in western Canada.

### **Threats to Bumble Bees**

Broadly speaking, insects are in decline across the planet (Goulson 2019) with flying insects potentially experiencing some of the greatest losses (Hallmann et al. 2017). Bees, in particular, are declining at alarming rates (Goulson et al. 2008, Cameron et al. 2011, Cameron and Sadd 2020). Since the 1990s, there has been a precipitous decline in several bumble bee species world-wide (Cameron et al. 2011). In North America, multiple species, including *B. affinis* (87% estimated reduction), *Bombus occidentalis* Greene, 1858 (28% estimated reduction), and *Bombus pensylvanicus* De Geer, 1773 (23% estimated reduction) have experienced a steep decline in both population and distribution (Cameron et al. 2011). Of the 46 North American species, 26% are listed as threatened, vulnerable, or critically endangered by the International Union for Conservation of Nature (Cameron and Sadd 2020)

Several anthropogenic factors have been implicated in the decline of bumble bees in North America. Habitat loss and fragmentation are considered leading factors in the decline of overall diversity (Foley et al. 2005). Agricultural intensification and urban

development in prairie landscapes have led to a loss of floral resources and nesting sites, resulting in a decline of suitable bumble bee habitats (Goulson et al. 2008, Liczner and Colla 2019, Pereira et al. 2021). Further, remaining prairie habitats are severely fragmented leading to genetic isolation and inbreeding in remaining populations (Zayed 2009). Another known threat to wild bumble bees is the increasing prevalence of pathogens (Cameron et al. 2016, Janousek et al. 2023), especially those that “spill over” from commercial bumble bee rearing facilities and managed colonies (Colla et al. 2006, Otterstatter and Thomson 2008, Whitehorn et al. 2013). In declining bumble bee species pathogens are more prevalent than in co-occurring stable species found in the same regions (Cameron et al. 2011, Colla et al. 2012, Arbetman et al. 2013, 2017, Cameron and Sadd 2020). In addition, there is a high chance for transmission of pathogens between bees. Prior studies have found that as many as 540 unique colonies visit and forage on the same floral resources (McGrady et al. 2021) illustrating the potential for a “pathogen hub” when interacting with commercially managed honey and bumble bees.

### **Pathogens and Spillover**

Pathogens are an inherent part of ecological processes; they make up a large portion of the biomass on the planet (Fischhoff et al. 2020) and they have direct impacts on the ecosystem services provided by plants and animals (Paseka et al. 2020) but disturbances such as changes in land cover (Bradley and Altizer 2007, Faust et al. 2018), the introduction of non-native species (Brown and Paxton 2009, Cameron et al. 2016, Arbetman et al. 2017, Meeus et al. 2018), and pathogen spillover from commercially



managed animals (Power and Mitchell 2004, Colla et al. 2006, Whitehorn et al. 2013, McMahon et al. 2015) have been linked to higher levels of pathogen prevalence and reduced stressor tolerance in wildlife (Bradley and Altizer 2007, Janousek et al. 2023). Spillover occurs when pathogen densities proliferate within a domesticated or semi-domesticated host population and transmitted to an alternative host (i.e., wild animals) (Daszak et al. 2000, Power and Mitchell 2004, Colla et al. 2006). For example, urban racoons have significantly higher loads of *Baylisascaris procyonis*, an endoparasitic roundworm, compared to rural populations (Prange et al. 2003). Runoff from developed urban areas into coastal waters transmits *Toxoplasma gondii* to vulnerable wild otter populations (Miller et al. 2002). White-nose syndrome, *Pseudogemnoascus destructans*, is a fungal pathogen introduced from Europe and first documented in North American in 2006. Capable of infecting seven species of bats, it is responsible for large bat die-offs across 32 states (Welch and Leppanen 2017). Chestnut blight fungus, *Cryphonectria parastica*, was introduced to North America from Japan in the early 1900s and has devastated American Chestnut populations across the United States (Dutech et al. 2012) fundamentally altering eastern hardwood forests. Pathogens have also been linked to the decline of several bumble bee species, such as *Bombus occidentalis* whose populations began to decline after the outbreak of the microsporidian parasite, *Vairimorpha bombi* (Fantham & Porter, 1914), in commercial bumble bee rearing facilities in the United States (Janousek et al. 2023) and the decline of *Bombus dahlbomii* Guérin-Méneville, 1835 in Argentina began when commercial colonies of *B. terrestris* were introduced for their pollination services and were likely infected with the neogregarine parasite,

*Apicystis bombi* (Liu et al, 1974), at the time of introduction (Colla et al. 2006, Arbetman et al. 2013, 2017).

As mentioned above, bees are important pollinators to high-value crops; this has led to the commercialization of bumble bees for crop pollination. Since the 1990's commercially managed bumble bee colonies have been available for pollination in open-field crops or in greenhouse systems. The colonies are reared in closed facilities with lab-raised queens (Huang et al. 2015). Once the queens produce 50-100 workers, the colonies are shipped out to growers who deploy them (Velthuis and van Doorn 2006, Strange et al. 2023). Currently, there are no formal guidelines or regulations regarding the management of pathogens in commercial bumble bee rearing facilities (Strange et al. 2023). Bumble bee producers make efforts to maintain disease-free populations (Huang et al. 2015, Strange et al. 2023) however, deployed colonies have the ability to amplify existing pathogens in wild populations and to sustain high pathogen prevalence over long periods (Power 1991, Faust et al. 2018) thus increasing the likelihood of spillover. These disturbances alter basic pathogen-host variables such as host population structure and density (Daszak et al. 2000, Power and Mitchell 2004) and often favor pathogen populations (Power 1991, Foster et al. 2003). Commercial bumble bees used in greenhouse systems regularly escape and forage outside on wild plants (Morandin et al. 2001, Colla et al. 2006, Otterstatter and Thomson 2008) thus increasing their interactions with wild bee populations (Colla et al. 2006, Fürst et al. 2014). Commercially managed bees have high population densities and pathogen loads compared to wild bees (Whittington and Winston 2003, Otterstatter and Thomson 2008, Mallinger et al. 2017).

As a result, wild bumble bees foraging around greenhouses that employ commercial pollination services have been found to harbor higher pathogens loads than bees foraging further away (Colla et al. 2006, Otterstatter and Thomson 2008). This is particularly concerning for imperiled bumble bee species located in areas with high greenhouse density (Szabo et al. 2012), where there is an increased risk of infection. Pathogen spillover has occurred and declining bumble bee species located in areas with high greenhouse density are increasingly exposed to infection sources (Colla et al. 2006, Colla and Packer 2008, Williams and Osborne 2009, Szabo et al. 2012). Because of this, pathogens have been recognized as significant threats to bumble bee diversity around the world and are responsible for the extinction or decline of multiple bumble bee species (Cameron and Sadd 2020).

### **Pathogens of Concern**

*Crithidia* spp. (Kinetoplastea: Trypanosomatidae) (Figure 1.2) are common extracellular parasites that occur in the midgut and rectum of bumble bees (Cordes et al. 2012, Cameron and Sadd 2020). Infected queens are reproductively delayed (Shykoff and Schmid-Hempel 1991a) and can experience an overall 40-50% reduction in fitness compared to uninfected queens (Koch and Schmid-Hempel 2011). Infected workers experience impaired foraging abilities and cognitive function (Gegear et al. 2006), and are less likely to carry pollen back to the colony (Shykoff and Schmid-Hempel 1991a, 1991b, Gegear et al. 2006). Additionally, the ovaries of infected workers are more likely to be developed and egg-producing which leads to non-specific egg cannibalism,

increases in aggressive behavior among nest mates, and reduced colony efficiency (Shykoff and Schmid-Hempel 1991b). In general, *Crithidia* spp. exhibit low levels of virulence to bumble bees if environmental conditions are favorable to the host (Brown et al. 2000). However, in suboptimal conditions (i.e. overcrowding, adverse weather, or sparse resources), presence of *Crithidia* spp. are associated with high host mortality (Schaub 1994). The queen is usually the initial source of infection for the colony (vertical transmitting it to their offspring), but *Crithidia* spp. can also be transmitted horizontally between queen and worker bees via shared floral resources (Durrer and Schmid-Hempel 1994). Large-scale studies have found *Crithidia* spp. infections in nearly 20% of examined bee specimens of various species (Malfi and Roulston 2014, Tripodi et al. 2018), but *Crithidia* presence across the landscape can be variable (Shykoff and Schmid-Hempel 1991a, Gillespie 2010, Cordes et al. 2012, Tripodi et al. 2018). *Crithidia bombi* Léger, 1902 is more commonly detected in bees than *Crithidia expoeki* Schmid-Hempel & Tognazzo, 2010 (Tripodi et al. 2018), however, precise numbers can be a result of a conflation between the two species prior to the description of *C. expoeki*. Generally, *C. bombi* is found in smaller bodied (Malfi and Roulston 2014), more common bumble bee species and infrequently detected in declining species (Cordes et al. 2012). Commercially reared bumble bees employed in greenhouses have been shown to escape and transmit *C. bombi* to wild bees within a 2 km radius of the greenhouse (Durrer and Schmid-Hempel 1994, Goulson et al. 2015). *Crithidia expoeki* is newly described (Palmier et al. 2020) with little known regarding its effects on and prevalence in wild bumble bees. *Crithidia mellificae*, a honey bee parasite, has also been documented in bumble bees (Michalczyk

and Sokół 2022). However, little is known about its effects on non-*Apis* species or if bumble bees can function as vectors to honey bees.

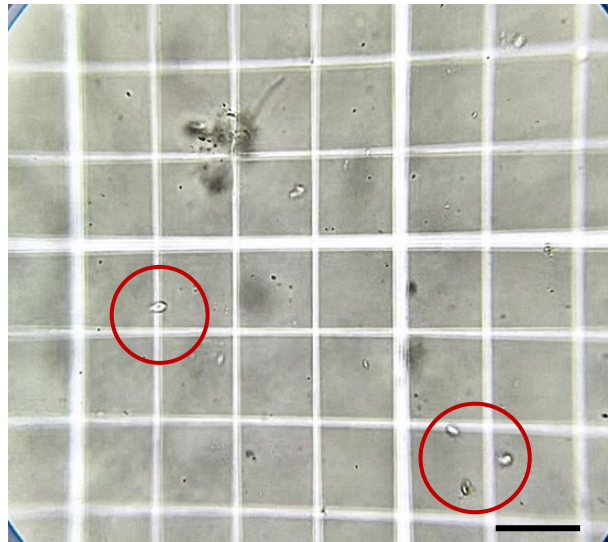


Figure 1.2: *Crithidia bombi* at 400x indicated by red circles. Scale bar represents 50  $\mu\text{m}$ . Photo by Emily Runnion, 2022.

*Vairimorpha bombi* (Fantham & Porter, 1914) and *Vairimorpha ceranae* (Fries et al., 1996) (Microsporidia: Nosematidae, formerly classified as *Nosema*) (Figure 1.3) are obligate intracellular parasites and close relatives of *Vairimorpha apis* (Zander, 1909), a major pathogen in honey bee colonies. *Vairimorpha* spp. are highly virulent to bees (Otti and Schmid-Hempel 2007), have broad host ranges (Kissinger et al. 2011), can be transmitted both horizontally and vertically (Rutrecht and Brown 2008a), and are

widespread in the UK and North America (Cordes et al. 2012). Infective spores are released into the environment via feces and onto shared floral resources (Otti and Schmid-Hempel 2007). *Vairimorpha spp.* primarily colonize the Malpighian tubules of the host and secondarily the midgut, connective tissues, and the fat body; infected bees may have distended abdomens and often times become paralyzed (Macfarlane et al. 1995). The queen is assumed to be the primary source of initial colony infections (Otti and Schmid-Hempel 2007, 2008). Infected queens have a smaller oocyte yield (Shykoff and Schmid-Hempel 1991a) and impaired behavior (Otti and Schmid-Hempel 2007). While this does not significantly impact her ability to initiate a colony (Fisher and Pomeroy 1989), it does impact overall colony size and the number and fitness of the reproductive individuals produced (Müller and Schmid-Hempel 1992, Otti and Schmid-Hempel 2008). Offspring are clumsy and less hygienic than uninfected bees which consequently inhibits foraging and colony maintenance. Males tend to have a higher prevalence of *V. bombi* than gynes; infected males produce almost no sperm and have a significantly lower survival rate than uninfected individuals (Gillespie 2010). Infected gynes do not readily mate (Otti and Schmid-Hempel 2007), likely due to their swollen abdomens, thus reducing future generations. An analysis of museum specimens revealed low prevalence of *V. bombi* in declining bumble bee species prior to the commercialization of pollinators in the 1990's (Cameron et al. 2016). Additionally, increased prevalence of pathogens starting in the 1990s co-occurred with the decline of bumble bees (Cameron et al. 2016, Brown 2017). Detectable amounts of *V. bombi* are significantly higher in declining bumble bee populations such as *B. occidentalis* and *B.*

*pensylvanicus* (Cameron et al. 2011, Cordes et al. 2012, Malfi and Roulston 2014) and *Bombus occidentalis*, once the second most common bumble bee species collected in berry fields and natural vegetation in western North America (Colla and Ratti 2010), has declined dramatically (Cameron et al. 2011), partly due to an outbreak of *Vairimorpha bombi* (Fantham & Porter, 1914) in rearing facilities (Szabo et al. 2012, Strange et al. 2023).

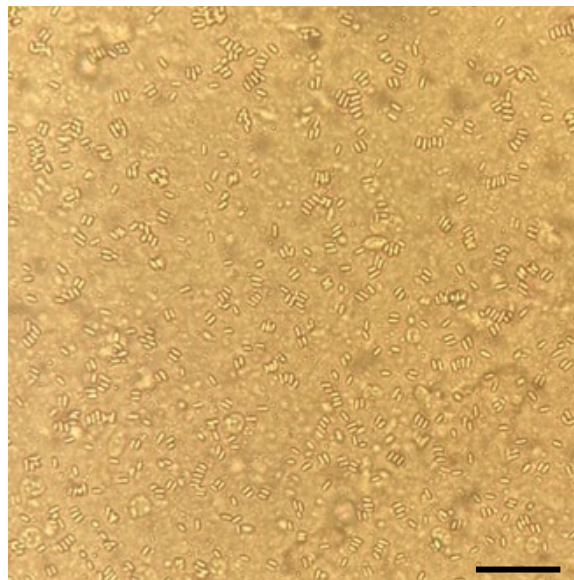


Figure 1.3: *Vairimorpha bombi* under 400x. Scale bar represents 25  $\mu\text{m}$ . Photo by James Strange, 2021.

*Apicystis bombi* (Liu et al, 1974) (Neogregarinida) (Figure 1.4) is a widely distributed and highly virulent protozoan that infects the fat body of its host. It has been

confirmed in more than 20 bumble bee species in Europe and North America (Lipa and Triggiani 1996, Schmid-Hempel 2001, Rutrecht and Brown 2008b, Plischuk et al. 2011, Arbetman et al. 2013, Ravoet et al. 2014) but many species have not been analyzed for *A. bombi* so this number might increase as more research is conducted. *Apicystis bombi* is likely transmitted as bees forage on floral resources (Graystock et al. 2016) and ingest oocytes in fecal material (Lipa and Triggiani 1996). As a result, *A. bombi* moves readily between commercially managed honey and bumble bee colonies and their wild counterparts (Plischuk et al. 2009, 2011, Graystock et al. 2016, Cameron and Sadd 2020). For example, the introduction of non-native *B. terrestris*, infected with *A. bombi* for commercial pollination led to the rapid decline of native *Bombus dahlbomii* in South America (Meeus et al. 2011, Arbetman et al. 2013). This is due to the severe physical and behavioral effects *A. bombi* has on bumble bees. Infected bees have a significant depletion of fat bodies, which are essential energy reserves for worker bees foraging in habitats with scattered floral resources and for over-wintering bumble bee gynes (Plischuk et al. 2011). Reduced energy reserves negatively impact the infected gynes ability to establish a successful colony the following spring. Further, both workers and queens experience increased mortality rates of up to 18% (Rutrecht and Brown 2008b, Plischuk et al. 2011, Graystock et al. 2016).





Figure 1.4: *Apicystis bombi* under 400x indicated by red circle. Scale bar represents 30  $\mu\text{m}$ . Photo by Amber Tripodi, 2015.

#### *Knowledge Gaps and Research Objectives*

There is a large deficit in knowledge concerning pathogens and their role in bumble bee declines, including how commercially managed bee colonies contribute to pathogen prevalence in wild bee populations (Cameron et al. 2011, Woodard et al. 2015, Aguirre and Adler 2022, Janousek et al. 2023, Strange et al. 2023). The majority of studies concerning pathogens and their effects on bumble bees have been conducted in western Europe and have focused on *B. terrestris* and *B. lucorum*. Little is known about the pathogen complex of North American *Bombus* species (Shykoff and Schmid-Hempel 1991b, Korner and Schmid-Hempel 2005, Rutrecht and Brown 2008b, Cordes et al. 2012) and prior to the use of molecular tools to detect the presence of pathogens, infections were determined with microscopy leading to a likely underestimation of the

number of bumble bee pathogens (Cameron et al. 2016, Brown 2017). Therefore, bumble bee susceptibility to pathogens needs to be evaluated for a number of species (Goulson et al. 2005, 2008, Cameron et al. 2011, Cameron and Sadd 2020). The threat of pathogens on vulnerable bumble bee species does not happen in a vacuum; other stressors, such as habitat loss (Szabo et al. 2012), climate change (Janousek et al. 2023), pesticides (McArt et al. 2017, Iverson et al. 2019), etc, can function as synergists, amplifying the impact that pathogens have. For example, the interaction between neonicotinoid application rates and drought (brought about by climate change) has been shown to decrease the presence of *B. occidentalis* and further increase the prevalence of *V. bombi* in the western U.S. (Janousek et al. 2023). There is also a need for basic ecological data outside of the well-researched agricultural systems that can be used to predict the future distribution and abundance of bumble bees in North America (Brown and Paxton 2009). For bumble bee conservation to be effective, we must understand the pathogen communities that impact them and the dynamics that drive infection (Daszak et al. 2000, Colla and Packer 2008, Graves et al. 2020). To address some of these knowledge gaps, my research in Madison, WI attempts to elucidate potential landcover associations with bumble bee pathogen distribution, prevalence, and diversity.

# Chapter One: Bumble Bee Pathogen Prevalence Determined by Host Species and Land Cover

## Abstract

Bumble bees (Hymenoptera: Apidae: *Bombus*) are social insects and important pollinators in both rural and urban landscapes. There has been a precipitous decline in bumble bee species across North America over the past 20 years with more than 25% of the 46 species becoming threatened, vulnerable, or critically endangered. Human-modified landscapes and the spread of pathogens have been documented as causal factors in the decline of bumble bees. Despite the growing threat of pathogens to bees, little is known about the influence of landscape factors on pathogen distribution and prevalence. I evaluated the effect of land cover on pathogen prevalence and diversity in agricultural and urban sites in Madison, WI, USA. To collect baseline bumble bee pathogen data, I screened 2,094 bumble bees for *Vairimorpha* spp., *Crithidia bombi* Léger, 1902, *Crithidia expoeki* Schmid-Hempel & Tognazzo, 2010, and *Apicystis bombi* from 20 sites across Madison, WI. I then characterized the dominant land cover (agricultural or urban) of each collection site at a 1.5 km buffer. Using PCR and species-specific primers, I detected *A. bombi* in 32% of the screened bumble bees, while *Crithidia* spp. and *V. bombi* were present in very few individuals. Land cover was not a consistent factor regarding pathogen prevalence as each year resulted in different significance, however *A. bombi* was detected in *B. impatiens* significantly more than either *B. griseocollis* or *B. bimaculatus*. Annual variation in pathogen prevalence was significant indicating that

temporal factors may be important in determining pathogen loads. Evaluating landscape and temporal drivers of pathogen distribution and prevalence will increase the understanding of bumble bee declines and help us make more informed choices concerning pollinator conservation.

## **Introduction**

Bumble bees (Hymenoptera: Apidae: *Bombus*) are beneficial social insects. There are approximately 265 species worldwide and 46 species in North America, with most species occurring in temperate northern latitudes (Williams et al. 2014). Bumble bees are important pollinators of both wild plants (Ollerton 2021) and high-value crops (Klein et al. 2007). Their efficiency as pollinators has led to the commercialization of bumble bee colonies for greenhouse and crop pollination (Velthuis and van Doorn 2006). Despite our ability to recognize the importance of bumble bees, they are in decline worldwide (Goulson et al. 2008) with multiple species imperiled in North America (Colla and Packer 2008, Grixti et al. 2009, Cameron et al. 2011, Koch 2011, Colla et al. 2012). These declines are anthropogenically driven (Kearns et al. 1998, Winfree et al. 2009) and are often attributed to habitat loss and fragmentation (Foley et al. 2005), the introduction of novel pathogens (Arbetman et al. 2013), the increased use of pesticides and herbicides (Goulson et al. 2015, Janousek et al. 2023), and spillover from commercially managed colonies (Power and Mitchell 2004, Colla et al. 2006, Szabo et al. 2012).

Habitat loss, by way of urban development, agricultural intensification, and fragmentation, is a primary driver of bumble bee declines that has broad implications for

species distribution, disease transmission between bees, and loss of stress tolerance (Potts et al. 2010, Janousek et al. 2023). Critical bumble bee habitats like the tallgrass prairies that once dominated the interior of North America, have largely been converted to farmland with roughly 1% of the original area remaining. Since 2016, nearly 10 million acres of flower-rich grasslands have been converted to large-scale agriculture or suburban developments (World Wildlife Fund 2022), which represents the single largest reduction of a major North American ecosystem (Samson and Knopf 1994). This type of habitat loss and fragmentation restricts gene flow between bee populations (Zayed 2009, Jha and Kremen 2013), reduces nesting site availability (Winfree et al. 2009) and reduces resource abundance and diversity (Wilson et al. 2016), which are essential to wild bumble bee populations (Winfree et al. 2009, Williams et al. 2010, Goulson et al. 2015). With limited habitat, the density of bumble bees visiting shared floral resources is higher, increasing the risk of bee to bee pathogen transmission (Schmeller et al. 2020). These effects of habitat loss greatly hinder bees' ability to handle additional stressors like climate change and pesticide exposure (Goulson et al. 2015). Generally, bumble bee diversity is negatively correlated with increased urbanization and agricultural intensification (Ahrné et al. 2009) as developed and managed areas filter out floral specialists bee species that rely on specific flowering plants which are commonly missing from these habitats. Further, functional trait diversity, such as diet and nesting preference (Goulson and Darvill 2004, Goulson et al. 2015), body and colony size (Westphal et al. 2006), and emergence time (Fitzpatrick et al. 2007) are often negatively impacted with increasing habitat loss. The impact of habitat loss and fragmentation is not uniform across

all bumble bee species. For example, the common eastern bumble bee (*Bombus impatiens* Cresson, 1863) is not experiencing population declines and is putatively a stable species (Lozier and Cameron 2009). In contrast, species such as *Bombus affinis* and *Bombus pensylvanicus* have faced steep declines in both population and distribution since declines were first recorded in the 1990's.

Pathogens are naturally occurring components of any ecosystem and play an important role in regulating the population of their hosts (Power and Mitchell 2004). The virulence of a particular pathogen is a measure of its disease-producing power and its impact on the reproductive fitness of its host (Vega and Kaya 2012). Virulence is dependent on several factors including host population density (Ebert and Bull 2008), pathogen density (Vega and Kaya 2012), and environmental stressors (Brown et al. 2000). The context-dependent virulence of pathogens allows for “strategic” tradeoffs between virulence and transmission ensuring the pathogen persists within a host population (Brown et al. 2003). Pathogens that replicate and kill their host quickly are considered highly virulent, especially if they disrupt the host's ability to reproduce (Vega and Kaya 2012). Under normal conditions, the host-pathogen relationship, while dynamic, is balanced by the availability of resources needed to maintain a healthy host population, such that host species are able to persist regardless of the presence of pathogens. However, if environmental conditions deteriorate, the shifting dynamic often reduces the host's ability to tolerate infection and the pathogen population increases. Generally, declining bee species are observed to have higher pathogen levels (Cameron et al. 2011) which has been largely attributed to the commercialization and mass production

of colonies for crop pollination (Colla et al. 2006). Commercial bumble bee colonies are produced in high density rearing facilities, which can increase the risk of exposure to pathogens (Power and Mitchell 2004). Further, these commercially produced colonies are shipped across the country to pollinate greenhouses and open-field crops which poses a risk for rapid disease spread (Strange et al. 2023). Once deployed, commercial bumble bees often escape management and interact with wild bees in the area, increasing the likelihood of a spillover event (Colla et al. 2006, Strange et al. 2023). Spillover of pathogens from commercial bee colonies to wild bees has been documented in Canada (Colla et al. 2006), England (Murray et al. 2013), Japan (Meeus et al. 2011), and Argentina (Arbetman et al. 2013). Pathogen outbreaks in the U.S. have also been tied to the abandonment of *Bombus occidentalis* Greene, 1858 as a commercial pollinator (Szabo et al. 2012).

The impacts of pathogens on bumble bee individuals varies between host and pathogen species. *Crithidia* spp. (Kinetoplastea: Trypanosomatidae) are frequently found in commercial bee colonies and are known to spread horizontally on shared floral resources (Durrer and Schmid-Hempel 1994), causing reproductive delays and reduced fecundity in queens (Shykoff and Schmid-Hempel 1991b, Koch and Schmid-Hempel 2011). *Vairimorpha bombi* (Fantham & Porter, 1914) (Microsporidia: Nosematidae, formerly named *Nosema bombi*) outbreaks in commercial rearing facilities led to the subsequent decline of *B. occidentalis* populations in the western U.S. (Velthuis and van Doorn 2006). *Vairimorpha bombi* is highly virulent to bumble bees (Otti and Schmid-Hempel 2007), can be transmitted both horizontally and vertically (Rutrecht and Brown

2008a), and impacts colony fitness and reproductive output. *Apicystis bombi* (Neogregarinida) is also highly virulent (Lipa and Triggiani 1996) and cause a significant depletion of fat bodies (Plischuk et al. 2011), which are an essential energy reserves for overwintering queens. The effects of *A. bombi* are currently unresolved but previous studies have found that it can infect several *Bombus* and non-*Bombus* pollinators in Europe (Lipa and Triggiani 1996), South America (Plischuk et al. 2011), and Japan (Morimoto et al. 2013). Ultimately, these three pathogens reduce colony reproductive outputs, decrease bee tolerance to stressors and cause higher mortality than observed in populations of uninfected bees.

The combined effects of habitat loss/fragmentation and increased pathogen prevalence on bumble bees is not well understood. Previous work has found that significant changes in land cover caused by urbanization and agricultural development are strongly associated with increased pathogen prevalence (Foley et al. 2005). Therefore, in this study I evaluated the influence of host species and land cover on pathogen prevalence, distribution, and diversity in urban and rural habitats in Madison, WI.

## **Methods**

### *Bumble Bee Collection*

Bumble bees workers were collected via aerial netting from early June to late August in 2019 (16 sites, Table 3) and 2020 (19 sites, Table 4) in Madison, WI (Figure 7). In 2019, 7 agricultural and 9 urban sites were sampled for four bumble bee species and retained for pathogen analysis. In 2020, only *B. impatiens* was collected from 9



agricultural and 10 urban sites due to labor complications caused by the SARS-CoV-2 pandemic. Approximately 70 bumble bees were collected from each site in 2019 and 50 from each site in 2020. The collected bumble bees were identified to species using taxonomic keys (Williams et al. 2014) and preserved in 3.5mL vials with 70% EtOH.

#### *Bumble Bee Dissection and DNA Extraction*

Bumble bee dissections were performed under 10x–50x magnification (Meiji Techno, EMT-2). The bee was positioned dorsal side down exposing the ventral portion of the abdomen. Incisions were made along the lateral sides to remove the abdominal exoskeleton and expose the body cavity. Gut tissues (Malfi and Roulston 2014, Tripodi et al. 2018) and fat bodies (Meeus et al. 2010, Figueroa et al. 2020) were collected in an empty 3.5mL vial and homogenized using a sterile pestle using methods similar to Tripodi et al. (2018). DNA was extracted from the homogenate using a “desalting” procedure based on Sambrook and Russel (2006) in which 300µL of cell lysis solution was added to each homogenized gut/fat body sample and then frozen overnight to facilitate cellular disruption. Once the samples thawed, 100µL of protein precipitation solution (7.5 M ammonium acetate) was added. The samples were then washed with 300µL of isopropanol and ethanol, dried, and stored at -40°C. Each sample was rehydrated as needed using 50µL of 10mM Tris-HCL DNA hydration solution. Samples were stored at -40°C until further analysis.

#### *PCR Reactions and Diagnosis*

PCR analysis was performed by loading either 1.2 $\mu$ L of DNA extract or 1.2 $\mu$ L of distilled water (for a negative control) into a 96-well plate. Samples were amplified using a multiplex panel (Table 2.1) as described in Mullins et al. (2019). The reaction consisted of 1 $\mu$ L of 20  $\mu$ M forward and 1 $\mu$ L of 20  $\mu$ M reverse primer for each pathogen, 0.5 $\mu$ L of 20  $\mu$ M forward and 0.5 $\mu$ L of 20  $\mu$ M reverse Apidae18S-rRNA primer (for positive control), 12.5 $\mu$ L of 2X Taq PCR Master Mix (ApexBio – Houston, Texas, USA), and 4.3 $\mu$ L of sterile double distilled water bringing the volume to 25 $\mu$ L per well. PCR cycles specifications are provided in Table 6. The amplified PCR products were loaded into a 2% agarose gel and ran at 105 V for 1 hr. and 25 min or until reference bands were sufficiently separated. Gels were stained with 2.5x MilliporeSigma™ GelRed™ (Darmstadt, Germany), and bands were visualized under a UV transilluminator. Positive bands were sized using Thermo Scientific™ GeneRuler DNA ladder. Samples with the presence of a band at 584, 357, and 233 base pairs were considered as trypanosomatid-positive, neogregarine-positive, and microsporidia-positive, respectively. Positive samples were further analyzed using species specific primers for *Crithidia expoeki* (163bp product) and *Crithidia bombi* (279bp product). As most *Vairimorpha* infections in bumble bees are *V. bombi*, I did not pursue further analysis.

Table 2.1: Multiplex PCR panel modified from Mullins et. al. (2019).

Multiplex PCR Panel				
Detect	Name	Sequence	Volume	Product (bp)
Trypanosoma	CB-SSUrRNA-F2	CTTTTGACGAACAACACTGCCCTATC	1 $\mu$ L	584
	CB18SR2	TGCTCCTTTGTTATCCCATGCT	1 $\mu$ L	
Neogregarine	ApicysITSF	AGCGATGGATGTCTTGGGTC	1 $\mu$ L	357
	ApicysITSR	CCTAGTTAGTTTCTTTTCCTCCGC	1 $\mu$ L	
Microsporidia	MSporDegR	GGTGTGTRCAAAGAACAGGG	1 $\mu$ L	270
	MSporF2	AGTGGTGCATGGCCGTTTTTC	1 $\mu$ L	
Apidae	Apid18SF	GTCCTATCGGTGGGCTTAGC	0.5 $\mu$ L	233
	Apid18SR	ATGCCCCCATCTGTCCCTAT	0.5 $\mu$ L	

### *Land Cover Classification*

The dominant land cover type at each collection site was calculated using data obtained from the National Land Cover Database (NLCD 2019) provided by the Multi-Resolution Land Characteristics Consortium ([www.mrlc.gov](http://www.mrlc.gov)). Using QGIS (Version 3.22), a 1500m buffer (Osborne et al. 2008) was created around the center of each collection site. FRAGSTATS (Version 4.2) was used to calculate the class area (CA) of the land cover within each 1500m buffer. Urban land cover included urban greenspace, developed-open space, developed-low intensity, developed-medium intensity, and developed-high intensity land. Agricultural land cover included barren land, grassland, shrubland, and agricultural fields. Dominant land cover was determined by the land cover accounting for > 50% within the buffers.

### *Statistical Analysis*

All analyses were performed in R version 4.2.0 (R Core Team 2022). Because the collection effort was different between 2019 and 2020 due to the SARS-CoV-2 pandemic, analyses regarding the pathogen prevalence within bumble bee species was only performed in 2019. A two-sample z-test for proportions (Oksanen et al. 2022) as conducted to compare the proportions of pathogen in bees between land cover types in both 2019 and 2020. A binomial logistic regression was used to analyze the relationship between both land cover and host species and pathogen presence. Another two-sample z-test for proportions was conducted to compare pathogen proportions in *B. impatiens* between 2019 and 2020. P-values less than 0.05 were used to determine significance. Bees unable to be identified to species were included in land cover and by-year analyses but were excluded in *Bombus* species analysis.

### **Results**

In 2019 and 2020, a total of 2,094 bumble bees were collected from 9 agricultural and 11 urban sites in Madison, WI: 1,165 in 2019 and 929 in 2020 (Table 2.2). In 2019, *B. impatiens* was the most abundant species sampled (63%) followed by *Bombus bimaculatus* Cresson, 1863 (20%), *Bombus griseocollis* De Geer, 1773 (13.3%), and *Bombus vagans* Smith, 1854 (0.2%). An additional 39 unidentified bumble bees were collected and retained for pathogen analysis. In 2020, *B. impatiens* accounted for 99% of the bees collected due to the reduction in labor necessitated by the COVID-19 pandemic. A total of 11 unidentified bumble bees were collected and again retained for pathogen

analyses. In 2019, 395 bumble bees were positive for at least one pathogen and 12 had co-infections (more than one pathogen species). In 2020, 316 bumble bees were infected by one pathogen and 4 were co-infected.

In 2019, we found *Vairimorpha* spp. in 2.56% of *B. bimaculatus*, 1.94% of *B. griseocollis*, and 2.32% of *B. impatiens*. *Crithidia bombi* was found in 1.71% of *B. bimaculatus*, 1.29% of *B. griseocollis*, and 0.82% of *B. impatiens*. *Crithidia expoeki* was found in one *B. impatiens*. *Apicystis bombi* was found in 16.24% of *B. bimaculatus*, 12.90% of *B. griseocollis*, 40.19% of *B. impatiens*, and 66.67% of *B. vagans*. In 2020, 1.09% of *B. impatiens* were infected with *Vairimorpha* spp. and 33.13% were infected with *A. bombi*. No *C. bombi* or *C. expoeki* infections were detected in 2020. Due to the low detections of the pathogens *C. bombi*, *C. expoeki*, and *Vairimorpha* spp. and of *Bombus vagans*, they were not included in further statistical analyses.

Table 2.2: A total of 2,094 bumble bees collected from Madison, WI in 2019 and 2020 by land cover.

Year	Land Cover	<i>Bombus</i> Species	No. Collected	No. w/ <i>Crithidia bombi</i> (%)	No. w/ <i>Crithidia expoeki</i> (%)	No. w/ <i>Vairimorpha</i> spp. (%)	No. w/ <i>Apicystis bombi</i> (%)
2019	Agriculture	<i>B. bimaculatus</i>	39	0 (0.00)	0 (0.00)	0 (0.00)	3 (7.69)
		<i>B. griseocollis</i>	45	0 (0.00)	0 (0.00)	1 (2.22)	3 (6.67)
		<i>B. impatiens</i>	286	3 (1.05)	1 (0.35)	10 (3.50)	131 (45.8)
		<i>B. vagans</i>	2	0 (0.00)	0 (0.00)	0 (0.00)	2 (100)
		Unidentified	7	0 (0.00)	0 (0.00)	0 (0.00)	1 (14.3)
2019	Urban	<i>B. bimaculatus</i>	195	4 (2.05)	0 (0.00)	6 (3.08)	35 (17.9)
		<i>B. griseocollis</i>	110	2 (1.69)	0 (0.00)	2 (1.82)	17 (15.5)
		<i>B. impatiens</i>	448	3 (0.67)	0 (0.00)	7 (1.56)	164 (36.6)
		<i>B. vagans</i>	1	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		Unidentified	32	0 (0.00)	0 (0.00)	0 (0.00)	12 (37.5)
2020	Agriculture	<i>B. impatiens</i>	383	0 (0.00)	0 (0.00)	4 (1.04)	104 (27.1)
		Unidentified	2	0 (0.00)	0 (0.00)	0 (0.00)	2 (100)
2020	Urban	<i>B. impatiens</i>	535	0 (0.00)	0 (0.00)	6 (1.12)	200 (37.4)
		Unidentified	9	0 (0.00)	0 (0.00)	0 (0.00)	4 (44.4)
Grand Total			2,094	12 (0.57)	1 (0.05)	36 (2.72)	678 (32.4)

In 2019 (Table A.2), 36.94% of bumble bees collected in agricultural sites were infected by *A. bombi* and 29.01% were infected in urban sites. Overall percent of bees that were positive for *A. bombi* in bumble bees was significantly greater in agricultural sites than urban sites ( $z = 2.66$ ,  $df = 1$ ,  $p = 0.004$ ). Further, the odds that *A. bombi* infections would be present was 18% higher in agricultural sites than urban (odds ratio = 0.82,  $z$ -value = -0.14,  $p$ -value <0.001).

In 2020 (Table A.3), 27.53% of *B. impatiens* collected were infected from agricultural sites that showed presence of *A. bombi* and 37.50% were infected in urban sites. Unlike 2019, overall percentage of bees positive for *A. bombi* in 2020 was

significantly greater in urban sites than agricultural sites ( $z = 3.10$ ,  $df = 1$ ,  $p < 0.001$ ). Further, the odds that *A. bombi* infections would be present was 140% higher in urban sites than agricultural sites (odds ratio = 1.60,  $z$ -value = 3.24,  $p$ -value = 0.001).

#### *Pathogen prevalence as a response to host species in 2019*

In 2019, 40.19% of *B. impatiens*, 16.24% of *B. bimaculatus*, 12.90% of *B. griseocollis* were infected with *A. bombi*. *Bombus impatiens* had significantly higher odds (232%) of *A. bombi* infections than *B. bimaculatus* (odds ratio = 3.32,  $z$ -value = 6.16,  $p < 0.001$ ). Meanwhile, the odds that *A. bombi* was present was 25% less in *B. griseocollis* than *B. bimaculatus* (odds ratio = 0.74,  $z$ -value = -0.99,  $p = 0.32$ ). In 2020, 33.1% of *B. impatiens* were infected with *A. bombi*. *Apicystis bombi* infections in *B. impatiens* were significantly higher in 2019 than 2020 ( $z = 2.92$ ,  $df = 1$ ,  $p = 0.001$ ).

## **Discussion**

This study analyzed four species of bumble bees collected from two land cover types in one year and bumble bees from a single species across two years and two land cover types to evaluate the prevalence of pathogens within the bumble bee community. I found few occurrences of *Crithidia* spp. and *Vairimorpha* spp. and high occurrence of *A. bombi*. While land cover was not a consistent factor in the prevalence of *A. bombi*, host species was. Additionally, *A. bombi* prevalence among years was significant in the analysis.

Pathogen populations can be temporally variable (Runckel et al. 2011) experiencing periods of high and low abundance, both seasonally and interannually, that are influenced by many factors including competition and host community composition. Further, in a multi-host, multi-pathogen system, such as in this study, competition between pathogen species could reduce the overall occurrence of some pathogens (Cordes et al. 2012). These factors might explain the low prevalence of *C. bombi*, *C. expoeki* and *Vairimorpha* spp. across land covers and host species. The results from this study are consistent with the prevalence found in previous studies (Cameron et al. 2011, Cordes et al. 2012). There is a lack of research assessing the temporal changes, or seasonality, of most bumble bee pathogens in North America, but such studies would give some insight into the variability of pathogen prevalence over time and would clarify when pathogen communities are at their highest and potentially, most virulent.

Our study analyzed four co-occurring *Bombus* species with stable population densities (Cameron et al. 2011) in the north central United States. The high overall infection prevalence of *A. bombi* in these common species might seem unexpected given that prior studies have found that declining species are generally associated with a higher prevalence of pathogens (Cameron et al. 2011). However, a previous study in South America identified *A. bombi* in both stable and declining species (Arbetman et al. 2017). Therefore, further research needs to be conducted on a broader range of host species to evaluate *A. bombi* prevalence more comprehensively throughout the United States.

The high *A. bombi* infection counts in this study could possibly be explained by each bumble bee species' ability to tolerate infections without large fitness costs as it's



conceivable that tolerance is variable between bumble bee species (Cordes et al. 2012, Arbetman et al. 2017). Secondly, highly virulent pathogens, like *A. bombi*, may act as ecological filters in which individuals belonging to susceptible bumble bee species are either absent or if they survive infection, are likely to be found free of pathogens (Arbetman et al. 2017). This study suggests that *A. bombi* might be less virulent in *B. impatiens* than other co-occurring species. If this is true, *B. impatiens* may function as a reservoir, essentially ensuring that *A. bombi* is present and transmissible to other *Bombus* species to which *A. bombi* virulence is potentially higher. This may explain the low prevalence in co-occurring species. However, there have not been any studies regarding the virulence of *A. bombi* in any North American bumble bee species so more research is needed to determine if this is the case.

Comparative studies of bumble bee pathogen virulence and host tolerance are needed to reveal how each bumble bee species manages infection. Unlike *Crithidia* spp. and *Vairimorpha* spp., *A. bombi* is grossly understudied. With so little known about *A. bombi* and its impacts on bumble bee species, reports of high infection prevalence are alarming. These results suggest that both temporal variables and bumble bee community composition are likely more influential than land cover in this region. However, given that only two years of data were included in this study, I was unable to elucidate any patterns regarding pathogen prevalence and land cover in this region or to predict whether these patterns would be similar in other environments or across larger timescales. Moving forward, future studies could include (1) comparing current *A. bombi* prevalence with those of museum specimens, if possible; (2) a comparative analysis of *A.*

*bombi* virulence in both stable and declining species, and (3) comparing *A. bombi* prevalence among regions.

## Chapter 2: Conopid Larvae and Rarely Reported Mermithid Nematodes Parasitizing Bumble Bees

### Abstract

Bumble bees (Hymenoptera: Apidae: *Bombus*) are important pollinators in both agricultural and urban landscapes. They host a suite of pathogens and parasites, including endoparasitic conopid flies (Diptera: Conopidae), found in many hymenopterans. In contrast, endoparasitic mermithid nematodes (Nematoda: Mermithidae), while commonly found in many invertebrate species, are not generally associated with bumble bees and have only been found in 17 bumble bees prior to this survey. Little is known about the influence of landscape factors on the prevalence of these two endoparasite groups in their bumble bee hosts. I sought to determine if urban and agricultural land cover types were predictive in the prevalence of conopid larvae near Madison, WI, USA. I screened 2,094 bumble bees for conopid larvae. During dissections, eight mermithid nematodes were found parasitizing bumble bees and the occurrence is reported here. I then characterized the dominant land cover (agricultural or urban) of each collection site. Conopid parasitism occurred in 11% of bees collected across four common species across two years of the study. Land cover type was not a factor in conopid prevalence in the two years analyzed. Mermithid nematode parasitism was found in 8 bumble bees collected

from urban sites. This study adds to the few existing records of both conopid presence and mermithid parasitism in bumble bees in North America.

## **Introduction**

Bumble bees (Hymenoptera: Apidae: *Bombus*) are social insects in the order Hymenoptera. Relying solely on nectar and pollen for nutrition, bumble bee workers spend their adult lives foraging on flowers and are important pollinators of both wild plants and commercial crops across the globe. However, several species are in decline globally with some showing a >60% reduction (Cameron et al. 2011). Population declines are often attributed to habitat loss, increased use of pesticides, and an increase of pathogens and parasites. Bumble bees are hosts to a number of parasites and pathogens (Macfarlane et al. 1995, Schmid-Hempel 2006, Evans et al. 2023) some of which have been implicated in their declines (Cameron et al. 2011, Cameron and Sadd 2020).

Conopid flies (Diptera: Conopidae) (Figure 3.1) are obligate endoparasitoids of adult Hymenoptera, with several genera commonly associated with bumble bees (*Bombus* spp.) (Schmid-Hempel 2001, Abdalla et al. 2014). The incidence of conopid parasitism varies widely by location and host species; reports of conopid parasitism in bumble bees range from 70% in Europe (Schmid-Hempel et al. 1990, Müller and Schmid-Hempel 1992), 80% in the eastern U.S. (Gillespie 2010, Malfi et al. 2014), and 10% in Canada (Otterstatter 2001) during peak growing season. Conopid fly geographic ranges overlap with bumble bees throughout Europe (Schmid-Hempel and Durrer 1991), Asia (Maeta and MacFarlane 1993), South America (Abdalla et al. 2014), and North America where

the flies are commonly found foraging on nectar rich flowers (Freeman 1966). Conopid parasitism has been documented in several *Bombus* spp. in North America including *Bombus impatiens*, *Bombus griseocollis*, *Bombus bimaculatus*, *Bombus perplexus*, *Bombus fervidus*, and *Bombus auricomus*. While the presence of the parasite is easy to confirm upon dissection, conopid larvae species verification is challenging due to the lack of keys available.

While conopid host choice is not well understood, host size and host species has been found to contribute to successful larval development (Otterstatter 2004). Generally, female conopids are more likely to parasitize larger bumble bees (Schmid-Hempel and Schmid-Hempel 1996a) and some observations indicate certain functional traits, like long tongue length, have very low conopid parasitism, suggesting floral associations and diet may influence parasitoid exposure (Schmid-Hempel and Schmid-Hempel 1988, Hawkins et al. 1997, Otterstatter 2004).

Conopid females attack bumble bees foraging on flowers (Goulson 2010) and oviposit a single egg into the bee's abdomen. The egg develops through three instar stages (Malfi et al. 2018) over a 10–13 day time period (Abdalla et al. 2014). First instar larvae move freely through their host's abdomen, feeding on hemolymph. Second and third instar larvae are not free swimming and must attach to the bumble bee host's tracheal system for respiration (Evans et al. 2023). Third instar larvae feed on the bumble bee host's gut tissues until large enough to fill the abdominal cavity, which signals for fly pupation (Schmid-Hempel and Schmid-Hempel 1996b). To reduce exposure to extreme temperatures, desiccation and predators and to increase survival, the larva induces a

“grave digging” behavior in the host bumble bee prior to fly pupation, resulting in a stoppage of foraging behavior (Müller 1994). As a result, through this additional energy expenditure, conopid parasitism shortens the lifespan of the bumble bee by about a third relative to unparasitized bees (Schmid-Hempel and Schmid-Hempel 1988) Parasitism also leads to lower thoracic temperatures and increased torpidity within the bumble bee (Müller 1994). Parasitized workers spend more time out of the colony than unparasitized workers, but are less likely to forage and return to the colony (Schmid-Hempel and Müller 1991). Consequently, the reduction of workers available to gather resources for a colony may diminish if many bees are infected. In turn, this can reduce colony resource procurement and the number and/or quality of reproductive individuals (gynes and males) able to be produced in the colony (Schmid-Hempel and Durrer 1991, Müller and Schmid-Hempel 1992, Otterstatter 2001). In particular, bumble bee colony success can be greatly impacted by conopid parasitism during times of low resource availability (Malfi et al. 2018).

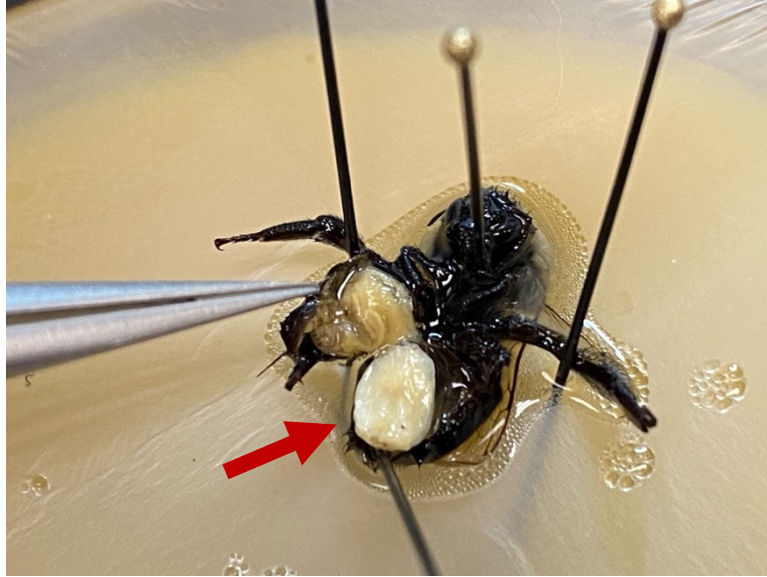


Figure 3.1: Bumble bee dissection reveals a third instar parasitoid thick-headed fly (Order Diptera: Family Conopidae) larva, indicated by the red arrow. Female conopids lay their eggs inside of bumble bee abdomens where they develop until ready to pupate, inducing a grave digging behavior prior to killing the host.

Mermithid nematodes (Nematoda: Mermithidae) (Figure 3.2) are obligate endoparasites of many aquatic and terrestrial invertebrates and commonly found in insects. Most mermithids are thought to parasitize a specific host species, genus, or family; however, some species are capable of infecting several insect orders. This diversity allows them to occur in environments otherwise hostile to nematodes (Poinar 2012). Infections in honey bees (*Apis mellifera*) have been reported in the U.S. by two mermithid genera: *Agamomermis* and *Mermis* (a common grasshopper parasite) (Milum 1938, Morse 1955, Nickle 1972). Additionally, there have been 8 reports of 17 mermithid

occurrences in bumble bees, only two of which included nematode species identification (Table 11) (Rao et al. 2017, Tripodi and Strange 2018). Due to limited molecular data available and a “troublesome taxonomy” particularly for immature stages (Tripodi and Strange 2018), identification of mermithids remains elusive.

Mermithid life cycles are typically monoxenous, requiring a single host to complete their development (Vega and Kaya 2012). However, one genera, *Pheromermis* spp., requires a paratenic or secondary transport host (Poinar 2012, Rao et al. 2017) in order to complete its life cycle. Mermithids have a six-stage life cycle: egg, four juvenile stages, and adult. Infective juveniles can be transmitted to a host via ingestion or a wound (Poinar 1975, Vega and Kaya 2012); however, many are thought to be transmitted to insect hosts ingesting water containing eggs. Once ingested the nematode develops through the larval stages in the haemocoel. Prior to emergence from the host as an adult, infection effects range from inconsequential to sterilization; however, infections almost always end in death as the adult nematode exits the host’s body (Poinar 1975). The effects of mermithid infections on bumble bee populations has not been explored and parasitism rates are assumed to be extremely low, but there are certainly deleterious consequences for individual bee hosts (Tripodi and Strange 2018). With so few records (Table A.4) of mermithid infections in bumble bees coupled with the difficulty of identifying mermithids, there is still a great deal of information unknown about bumble bees-mermithid dynamics including if they are incidental infections or if they can complete development in bumble bee hosts.





Figure 3.2: Mermithid sp. found inside a *Bombus huntii* abdomen during dissection.

Mermithid parasitism is rare in bumble bees having only 17 known records prior to this study. Photo by Jess Mullins, 2018.

Changes in land cover have been linked to higher pathogen prevalence (Faust et al. 2018) but it is not known if conopid parasitism is affected by different land cover types. Urban landscapes often lack the floral resources needed to support both bumble bee hosts and conopid fly populations (Dufлот et al. 2014). Conversely, urban areas have higher bee diversity (Eggenberger et al. 2019) and consequently greater host choice for conopid females.

Our objective for this study was to investigate the prevalence of conopid parasitism of bumble bees in urban and agricultural sites to determine if land cover is a significant factor in their prevalence. This research adds to the growing knowledge of bumble bee parasites and the hosts they chose including substantially adding to the known records of mermithid parasitism of bumble bees.

## **Methods**

Bumble bees were collected using aerial insect nets from early June to late August in 2019 (16 sites) and 2020 (19 sites) in and near Madison, WI (Figure A.1). In 2019, 7 agricultural and 9 urban sites were sampled, and in 2020, 9 agricultural and 10 urban sites were sampled. All collected bumble bees were identified to species in the field using taxonomic keys (Williams et al. 2014) and preserved in 3.5mL vials with 70% ethanol. In 2019, four species of bumble bees were retained for analyses: *Bombus impatiens*, *B. bimaculatus*, *B. griseocollis* and *B. vagans*. In 2020, due to labor issues related to the SARS-CoV-2 pandemic, only *B. impatiens* were retained for analyses. All collected bumble bees were shipped to the Rothenbuhler Bee Laboratory in Columbus, OH, USA.

Each bumble bee specimen was dissected and examined for conopid fly larvae and mermithid nematodes by microscopic inspection of the abdominal cavity, where they are known to occur. Dissections were performed under 10x–50x magnification (Meiji Techno, EMT-2) by making incisions along the lateral sides of the bumble bee abdomen, which was then opened ventrally to expose the contents of the abdominal cavity. If present, endoparasites were extracted, identified to family using taxonomic keys

(Schmid-Hempel and Schmid-Hempel 1996), placed in dry 3.5 mL vials, and stored at -40°C.

### *Land Cover Classification*

The dominant land cover type at each collection site was calculated using data obtained from the National Land Cover Database (NLCD 2019) provided by the Multi-Resolution Land Characteristics Consortium ([www.mrlc.gov](http://www.mrlc.gov)). Using QGIS (Version 3.22), a 1500m buffer (Osborne et al. 2008) was created around the center of each collection site. FRAGSTATS (Version 4.2) was used to calculate the class area (CA) of the land cover within each 1500m buffer. Urban land cover included urban greenspace, developed-open space, developed-low intensity, developed-medium intensity, and developed-high intensity land. Agricultural land cover included barren land, grassland, shrubland, and agricultural fields. Dominant land cover was determined by the land cover accounting for > 50% within the buffers.

### *Statistical Analysis*

Statistical analyses were performed in R version 4.2.0 (R Core Team 2022). Overall parasite prevalence was calculated by dividing the number of parasitized bees by the total number of bees analyzed. A two-sample z-test for proportions was conducted to compare conopid parasitism between land cover types in both 2019 and 2020. A binomial logistic regression was used to analyze the relationship between host species and conopid parasitism. Results were reported in terms of odds ratios, which were obtained by

exponentiating the outputs. P-values less than or equal to 0.05 were used to determine significance. Unidentified bees were included in land cover and by-year analyses. P-values less than 0.05 were used to determine significance. Due to the low number of occurrences, no analyses were performed for mermithid infections, but we report the occurrence data and host species here.

## Results

In total, 2,094 bumble bees were collected from 9 agricultural and 11 urban sites in Madison, WI. Of the 1,165 bumble bees collected in 2019, 63% were *Bombus impatiens* Cresson, 1863, 20% were *Bombus bimaculatus* Cresson, 1863, 13.3% were *Bombus griseocollis* De Geer, 1773, 0.33% were unidentified *Bombus* spp., and 0.26% were *Bombus vagans* Smith, 1854 (Table A.2). In 2020, 929 bees were collected, 98.9% were *B. impatiens* and 1.1% unknown *Bombus* spp. were collected (Table A.3). Due to the labor restrictions imposed during the pandemic no other species were sampled in 2020.

### *Conopid Parasitism*

A total of 229 conopid larvae were found within the 2,094 bumble bees (Table 3.1) across 2019 and 2020. Of the 1,165 bumble bees collected in 2019, 176 were parasitized. In 2020, 53 of the 929 bumble bees were parasitized by conopid larvae. Based on distribution maps from iNaturalist.org and previously documented host

associations (Freeman 1966), it is likely that the conopid larva found in this study belonged to the genera *Physocephala* or *Myopa*.

In 2019, 12.40% of bumble bees were parasitized by conopid larvae in agricultural sites and 16.41% were parasitized in urban sites. In 2020, 5.97% of *B. impatiens* were parasitized by conopid larvae in agricultural sites and 5.51% were parasitized in urban sites. Conopid parasitism was significantly higher in bumble bees collected from urban sites than agricultural sites 2019 ( $z = 1.70$ ,  $df = 1$ ,  $p\text{-value} = 0.0442$ ), but not in 2020 ( $z = 0.1537$ ,  $df = 1$ ,  $p\text{-value} = 0.4389$ ).

In 2019, conopid larvae were found in 10.76% of *B. impatiens*, 23.50% of *B. bimaculatus*, 33.33% of *B. vagans*, 20.0% of *B. griseocollis*, and 26% of unidentified *Bombus* species. Meanwhile, in 2020, conopid larvae were found in 5.77% of *B. impatiens*; no conopids were found in unidentified bees. The odds that conopids larvae were present was 59% less in *B. impatiens* than *B. bimaculatus* (odds ratio = 0.41,  $z\text{-value} = -4.471$ ,  $p < 0.001$ ). Additionally, the odds that conopid larvae were present was 16.5% less in *B. griseocollis* than *B. bimaculatus* (odds ratio = 0.84,  $z\text{-value} = -0.709$ ,  $p = 0.48$ ).

Table 3.1: Conopid parasitism in bumble bees caught in Madison, WI during the summers of 2019 and 2020.

<b>2019 Bumbles Bees Parasitized by Conopid Larvae</b>		
<b>Species</b>	<b>No. Collected</b>	<b>No. of Conopid Larvae (%)</b>
<i>B. impatiens</i>	734	79 (10.7%)
<i>B. bimaculatus</i>	234	55 (23.5%)
<i>B. griseocollis</i>	155	31 (20.0%)
<i>B. vagans</i>	3	1 (33.3%)
<i>Bombus</i> spp.	39	10 (25.6%)
<b>Total</b>	<b>1165</b>	<b>176 (15.1%)</b>

<b>2020 Bumbles Bees Parasitized by Conopid Larvae</b>		
<b>Species</b>	<b>No. Collected</b>	<b>No. of Conopid Larvae (%)</b>
<i>B. impatiens</i>	918	53 (5.7%)
<i>Bombus</i> spp.	11	0 (0.00%)
<b>Total</b>	<b>929</b>	<b>53 (5.7%)</b>

#### *Mermithid Nematode Occurrence*

Overall, eight bumble bees were parasitized by mermithid nematodes. In 2019, two were found in *B. impatiens*, two in *B. griseocollis*, and one *B. bimaculatus*, all collected from urban sites. In 2020, three mermithids were found in *B. impatiens* collected from urban sites. Due to the low number of specimens, no analyses were performed.

#### Discussion

The objective of this study was to determine if land cover or host species were significant factors in conopid larva infections. I found that *B. bimaculatus* has significantly higher incidences of conopid parasitism than both *B. impatiens* and *B.*

*griseocollis*. Land cover was not a consistent factor in parasitism prevalence.

Additionally, conopid parasitism was significantly greater in 2019 than 2020. This study also added 8 occurrences of mermithid parasitism.

Conopid prevalence in this study was within the range of previously reported data in North America (Kissinger et al. 2011: 0 – 30%; Malfi and Roulston 2014: 25%). Bumble bees collected from urban sites had higher rates of parasitism by conopid larva than agricultural sites in 2019, but not in 2020. While the main factors that influence conopid parasitism rates are unknown, the variance across sites bears further investigation. Inconsistencies with the impact of land cover on parasitism rates could be due to host availability, host species composition, or other density dependent factors.

In 2019, *B. bimaculatus* was more frequently found in urban sites than agricultural sites. Further, *B. bimaculatus* had greater odds of being parasitized than either *B. griseocollis* or *B. impatiens*. One explanation for higher conopid parasitism in *B. bimaculatus* is that conopid parasitism is more often found larger bees (Müller et al. 1996, Malfi and Roulston 2014) like *Bombus bimaculatus* whose workers are generally larger than either *B. impatiens* or *B. griseocollis* (Williams et al. 2014). Secondly, the peak colony population for *B. bimaculatus* occurs earlier in the season, around late June/early July, compared to either *B. griseocollis* or *B. impatiens* (Colla et al. 2011) whose peak populations occur around mid-July and early August respectively.

Conopid flies each have their own host preference that can be determined by catching a parasitized bumble bee and allowing the conopid larva to pupate, but this process is difficult and requires providing appropriate temperatures for the developing

fly. Most conopid occurrences in bumble bees are discovered during dissections but identification is problematic as keys are not available for conopid larvae identification. I observed that conopid parasitism varied year to year; however, this two-year study did not reveal any consistent pattern of conopid parasitism related to land cover. Other factors, such as host sex or abundance (Malfi and Roulston 2014), likely have a greater effect than land cover. Further longitudinal studies could identify the longer-term host-parasite population dynamics and landscape factors moderating conopid parasitism in bumble bees.

Mermithid parasitism identified in this study is especially interesting given the lack of historical records, which indicate that this phenomenon is rare. I document the first records of bumble bee parasitism by mermithid nematodes in Wisconsin and the surrounding states. The mermithids in this study were large (10 - 15 cm) and found tightly coiled filling the abdominal cavity. This suggests that they were able to successfully grow and develop within their bumble bee host, a significant finding since it is not known if bumble bees are intentional or accidental hosts (Tripodi and Strange 2018). Mermithid nematodes can be effective regulators of host population densities and are often used as biocontrol agents in crop systems (Vega and Kaya 2012), but bumble bees are not targets for control and appear to rarely serve as hosts (Tripodi and Strange 2018). Keys for immature mermithids do not exist and many species remain undescribed. Based on geography and host species, it is likely that these specimens are *Mermis nigrescens* Dujardin, 1842, the grasshopper nematode, but that remains unresolved.



Bumble bees risk encountering many parasites, such as conopid flies and mermithid nematodes, as they forage for floral resources in their habitats. While neither parasite is considered to be responsible for the current decline of bumble bees, high parasitism rates by conopid flies can have detrimental impacts on colony health and resource procurement (Schmid-Hempel and Durrer 1991, Müller and Schmid-Hempel 1992, Otterstatter 2001) and exacerbate other stressors on population health.

In conclusion, this study adds important data to two bumble bee parasites in a novel study site. I determined that land cover is not a consistently significant factor in conopid larvae in the region and that *B. bimaculatus* were more likely to be parasitized than other co-occurring species. Other factors, such as host availability or host species availability, likely play a larger role. Secondly, I was able to add eight new instances of mermithid parasitism, more than doubling the records in North America and extend the range of this host parasite relationship to a new geographic region.

While not addressed here, the risk of parasitism is variable between bumble bee species (Malfi and Roulston 2014), so additional research is needed to better understand host choice selection especially within the conopid flies. Future studies could include choice assays or host functional trait analyses to elucidate the factors involved in host choice. Additionally, parasitism risk is species dependent based on the bumble bees species phenology (Novotny et al. 2021) and various behavioral traits (Otterstatter 2001).

## Appendix



Figure A.1: Twenty collection sites in Madison, WI representing urban and agricultural land covers.

Table A.1: Parameters for the multiplex PCR panel.

	94°C	2 min
10x	94°C	30s
	60°C	30s
	72°C	45s
30x	94°C	30s
	57°C	30s
	72°C	45s
	72°C	5 min

Table A.2: Total of four bumble bee species collected and infections of *Apicystis bombi*, *Crithidia expoeki*, *Crithidia bombi*, and *Vairimorpha* spp. from 16 locations in Madison, WI, June - August 2019

<b>Four Bumble Bee Species Collected from Madison, WI in 2019</b>						
<b>Location</b>	<b>Dominant Land Cover</b>	<b>No. w/ Bees Collected</b>	<b>No. w/ <i>Apicystis bombi</i></b>	<b>No. w/ <i>Vairimorpha</i> spp.</b>	<b>No. w/ <i>Crithidia bombi</i></b>	<b>No. w/ <i>Crithidia expoeki</i></b>
Badger State Trail	Agriculture	101	34	6	0	0
Cemetery	Urban	72	33	0	0	0
Cherokee Heights	Urban	52	25	0	0	0
Cherokee Marsh	Agriculture	53	26	2	0	0
Cottage Grove	Agriculture	59	8	0	0	0
Lakeview Park	Urban	70	20	3	0	0
McKee Road	Urban	82	28	0	0	0
Middleton Airport	Urban	122	41	1	3	0
North Star	Urban	132	25	6	5	0
Pheasant Branch	Agriculture	50	19	0	1	0
Sauk Creek	Urban	99	17	0	0	0
Sun Prairie	Agriculture	50	20	3	1	0
Sycamore Park	Urban	98	28	2	0	0
Waubesa School	Urban	59	11	3	1	0
Waubesa Wetlands	Agriculture	53	29	0	1	1
Windsor	Agriculture	13	4	0	0	0
	<b>Total</b>	<b>1,165</b>	<b>368</b>	<b>26</b>	<b>12</b>	<b>1</b>

Table A.3: Total *Bombus impatiens* collected and infections of *Apicystis bombi*, *Crithidia expoeki*, *Crithidia bombi*, and *Vairimorpha spp.* detected at 19 locations in Madison, WI, June - August 2020

<b><i>Bombus impatiens</i> collected from Madison, WI in 2020</b>						
<b>Location</b>	<b>Dominant Land Cover</b>	<b>No. w/ <i>Bombus</i> Collected</b>	<b>No. w/ <i>Apicystis bombi</i></b>	<b>No. w/ <i>Vairimorpha</i> spp.</b>	<b>No. w/ <i>Crithidia bombi</i></b>	<b>No. w/ <i>Crithidia expoeki</i></b>
Atwood	Urban	50	28	0	0	0
Badger State Trail	Agriculture	54	15	0	0	0
Capitol	Urban	48	25	2	0	0
Cemetery	Urban	51	27	2	0	0
Cherokee Heights	Urban	52	20	1	0	0
Cherokee Marsh	Agriculture	38	17	0	0	0
Cottage Grove	Agriculture	49	21	0	0	0
Elver Park	Agriculture	46	5	0	0	0
Holy Wisdom	Agriculture	50	7	0	0	0
Lakeview Park	Urban	44	2	0	0	0
McKee Road	Urban	50	3	0	0	0
Middleton Airport	Urban	51	12	1	0	0
North Star	Urban	50	18	0	0	0
Sauk Creek	Urban	49	18	0	0	0
Sun Prairie	Agriculture	50	16	3	0	0
Sycamore Park	Urban	49	25	0	0	0
Waubesa School	Urban	50	26	0	0	0
Waubesa Wetlands	Agriculture	49	13	0	0	0
Windsor	Agriculture	49	12	1	0	0
	<b>Total</b>	<b>929</b>	<b>310</b>	<b>10</b>	<b>0</b>	<b>0</b>

Table A.4: Previously reported occurrences of mermithid worms (Nematoda: Mermithidae) in bumble bees. Mermithids are widespread but very rarely recorded in bumble bees.

Previously Reported Occurrences of Mermithid Parasitism in Bumble Bees					
Year	Location	No. of Mermithids	Identification	<i>Bombus</i> Host Species	Source
1966	Indiana, U.S.	1	unidentified	Not reported	MacLean, 1966
1995	Sweden	unreported	unidentified	Not reported	Durrer and Schmid-Hempel, 1995
2012	Japan	2	unidentified	<i>B. terrestris</i>	Kosaka et al., 2012
2016	Japan	1	unknown; not <i>Pheromermis</i> sp.	<i>B. pseudobaicalensis</i>	Kubo et al., 2016
2017	Uruguay	2	unidentified	<i>B. bellicosus</i>	Plischuk et al., 2017
2017	Massachusetts, U.S.	1	<i>Pheromermis</i> sp.	<i>Bombus impatiens</i>	Rao et al., 2017
2018	Delaware, Pennsylvania, Vermont, and Arkansas, U.S.	6	<i>Pheromermis</i> sp. and <i>Mermis nigrescens</i>	<i>B. bimaculatus</i> , <i>B. impatiens</i> , <i>B. vagans</i> , <i>B. griseocollis</i>	Tripodi and Strange, 2018
2021	Massachusetts, U.S.	3	unidentified	<i>B. impatiens</i> , <i>B. vagans</i> , <i>B. bimaculatus</i>	Averill et al., 2021

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