Developing a Commercial Bumble Bee Clean Stock Certification Program: A white paper of the North American Pollinator Protection Campaign *Bombus* Task Force

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Abstract

The commercial production and subsequent movement of bumble bees for pollination of agricultural field and greenhouse crops is a growing industry in North America and globally. Concerns have been raised about the impacts of pathogen spillover from managed bees to wild pollinators, including from commercial bumble bees. This white paper discusses the need to develop a program to mitigate disease risk in commercial bumble bee production, which will in turn reduce disease stressors on wild pollinators and other insects. We give an overview of what is known about bumble bee pathogens, parasites and other symbionts and methods for their detection, quantification, and control. We also provide information on assessment of risk for select bumble bee symbionts and highlight key knowledge gaps. Finally, we provide recommendations for the components of a clean stock program with specific best management practices for rearing commercial bumble bees including related products such as pollen, wax and other nesting material.

Introduction

Bumble bees (*Bombus* spp.) are important pollinators of commercially grown crops, a variety of garden vegetables, and native flowering plants. Approximately 40 bumble bee species are native to the United States and Canada (Williams et al., 2014) and three of them are commercially available in those countries. By far the most economically important managed bumble bee species in the United States and Canada is *Bombus impatiens*, a native to the eastern United States and Canada (Velthuis & van Doorn, 2006). However, *Bombus huntii* is available for use in western Canada and *Bombus vosnesenskii* was recently approved for use in California and is now being sold commercially, and it is expected that use of these new

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commercial species will grow in the western United States and Canada in the future. Currently, these species are produced in facilities in Michigan (United States) and Ontario (Canada) and shipped throughout North America for crop pollination, most notably, greenhouse grown tomatoes (Strange, 2015; Velthuis & van Doorn, 2006)

While the commercial producers of bumble bees make efforts to maintain clean stock in production facilities (Huang et al., 2015), and provide guidelines to end-users for containment when bees are sold outside of their native range, commercial bumble bee hives are not isolated from wild bumble bee communities because they often forage outside of greenhouses via vents (Whittington et al., 2004). Bumble bees are deployed frequently in open-field situations to augment pollination of field tomatoes, tree fruit, and berry crops. The use of these bees where they can come into contact with wild bees poses a clear risk for the movement of pathogens and parasites within and beyond the bumble bee community (Colla et al., 2006; Fürst et al., 2014; Murray et al., 2013). Managed bumble bees have the potential to amplify existing pathogens and parasites in the wild bumble bee community, through pathogen spillback (Pereira et al., 2021), but the introduction of pathogens and parasites with managed colonies represents a greater concern. High pathogen incidence has been correlated to facilities that deploy commercial bumble bees, leading to concerns of pathogen spillover (Colla et al., 2006; Murray et al., 2013).

Notably, declining bumble bee populations in the United States (Cameron et al., 2011) and Canada (Colla & Packer, 2008) have been linked to higher levels of pathogens (Cordes et al., 2012; Kent et al., 2018). However, a clear causative link between population status and infection remains elusive, due to a lack of baseline data on differential susceptibilities. Declines in some species have raised concerns about extinction risk and over 20% of North American species have been identified through the International Union for the Conservation of Nature Red List as 'at risk' (updated at https://www.iucnredlist.org/). In addition, several species are legally recognized in the United States and Canada as endangered, including *Bombus affinis*, the

Rusty Patched Bumble Bee, which is federally protected in both countries. The impacts of commercial bumble bees on these declining species are poorly understood, but previous disease outbreaks in rearing facilities have been implicated in declines (Flanders et al., 2003).

Commercial bumble bee production begins in captivity when lab-raised queens are provisioned with honey bee-collected pollen and sugar syrup and confined to a nest box where they commence nesting (Huang et al., 2015; Velthuis & van Doorn, 2006). Within a few days of confinement, the queen bumble bee will oviposit on the pollen mass and begin brooding her developing offspring. More pollen is provided as needed as the developing nest remains in isolation in the facility. As worker bees reach adulthood and the nest grows, the nest is moved to a shipping box and is ready for sale about 60 days after nest initiation. Once colonies reach a desired size (*e.g.*, 50-100 workers), the nests are shipped from the production facilities to growers, and do not return (Huang et al., 2015), nor are the nesting materials from sold colonies returned to the facility; however a percentage of the colonies reared in facilities must be retained to supply future reproductive individuals for the operation (Huang et al., 2015; Velthuis & van Doorn, 2006). Growers dispose of the colonies once their crop has completed flowering or the colony starts producing reproductive individuals instead of workers.

Although the bumble bee production environment is closed, the rearing system does have external inputs. Notably, sugar and pollen must be supplied to developing colonies, and nesting material is also essential (Huang et al., 2015). Nesting boxes from major bumble bee producers are currently composed of plastic boxes that are manufactured for the purpose and arrive as clean, sterile plastic into the system. Similarly, sugar syrup is provided, generally in a proprietary nutrient and preservative mixture, and this is sterilized before delivery (Velthuis & van Doorn, 2006). Pollen must be obtained in large quantities for commercial production and this necessitates purchasing bulk pollen that has been collected by beekeepers from honey bee hives (Velthuis & van Doorn, 2006). The collection of pollen from honey bee hives is done using standard pollen traps deployed on the entrance of a honey bee colony. The traps remove pollen

from the corbiculae of returning honey bee foragers and collect this pollen in trays that the beekeeper can empty. Because the pollen is retrieved from a biological system and has had contact with honey bees in a hive, it is frequently contaminated with pathogens (Chen et al., 2006; Gilliam et al., 1988; Graystock, Yates, Darvill, et al., 2013; Higes et al., 2008) and detritus, and may be contaminated with pesticides or other environmental contaminants (Mullin et al., 2010). Pollen sourcing thus represents a significant risk to bumble bee production. It is not known to what extent new queens or males for mating are brought into rearing facilities to increase genetic diversity and avoid inbreeding of captive stock, but that is another possible external input.

A large number of pathogens and parasites are known to attack and infect bumble bees (Goulson, 2010); however, not all of the pathogens pose risks on an economically important scale. Likewise, some parasites that are already abundant in the wild would seem to pose little threat of being spread by captive reared bees, due to their complex life cycles. Furthermore, some pests such as wax moths or Indian meal moths can become a problem in rearing facilities, but probably pose little threat to bees in native communities. Yet, some pathogens such as *Varimorpha bombi*, *Apicystis bombi*, *Crithidia bombi,* a variety of viral diseases, and potentially emergent pathogens can infect commercial colonies and be moved quickly through shipments across the continent. **Bee movement regulation and clean stock guidelines are needed to ensure tolerable levels of pathogens are not exceeded and so that outbreaks are quickly detected and contained.** Implementation of a clean stock program would align with needs identified in the National Strategy on Pollinators in the Pollinator Research Action Plan from the Pollinator Health Task Force (2015) and the National Strategy for Biosurveillance (2012) both of which highlight the need for detection and monitoring of diseases with potential to impact agricultural production. A clean stock certification program would help reduce the threat and impacts that managed bumble bees have on wild bee populations, and help commercial companies avoid economic costs associated with outbreaks.

In this document we adopt the term "potentially deleterious symbiont" to mean organisms (including viruses) that have a known or suspected deleterious association with bumble bees in captivity or the wild. Not all symbionts are thought to have ecologically or economically relevant impacts. We define "clean stock program" as a documented system 1) to detect pathogens of concern in commercial rearing facilities that pose a threat to wild bees, 2) to prevent the spread of infections both within and outside of facilities, and 3) to produce actionable information for federal, state, and provincial regulators and conservation professionals if a suspected disease outbreak occurs. The clean stock program can be applied equally to laboratories rearing bumble bees for research or conservation purposes.

The goals of this report are to:

1) summarize known bumble bee potentially deleterious symbionts

2) produce a summary symbiont list identifying potentially deleterious symbionts of concern for clean stock and commercial bumble bee rearing (Appendix A)

3) provide recommended methodology for detection and quantification of bumble bee symbionts of concern

4) summarize treatment for symbionts of concern, control methods, and management strategies, if they exist

5) point out knowledge gaps and the risks they pose

6) provide recommendations for a clean stock program for commercial bumble bees including related products such as wax and pollen

The development of a clean stock program would enable producers, regulators, conservation groups, and end users of bumble bees to ensure that all reasonable measures are being taken to maintain healthy bumble bee communities in both production and wild systems.

1) Identifying known bumble bee potentially deleterious symbionts

In this section, we address some of the most important, most commonly encountered, and most discussed potentially deleterious symbionts of bumble bees, particularly those that are of interest in captive rearing environments. This is far from a complete list (See Appendix A, Supplementary Symbiont List) but interested readers who wish to read about some of the more obscure organisms associated with bumble bees are encouraged to seek the works on parasitism (Beaurepaire et al., 2020; de Miranda et al., 2013; MacFarlane et al., 1995; Schmid-Hempel, 1998) and bumble bee natural history (Alford, 1975; Goulson, 2010). Additionally, we use the term "parasite" broadly to refer to organisms of all taxa, including viruses that sustain themselves at the expense of their hosts and have the potential to cause harm to their hosts, a definition which, for our purposes, also encompasses the term "pathogen".

Viruses

To date, all of the named viruses detected in bumble bees were previously known only from honey bees. There are approximately 60 honey bee viruses currently known, although next-generation sequencing technologies are allowing for the exploratory discovery of additional viruses of managed honey bees and wild bees (Beaurepaire et al., 2020; de Miranda et al., 2013; Remnant et al., 2017; Schoonvaere et al., 2016). A single virus, perhaps specific to bumble bees, was noted in three North American species in the 1980s (present in *B. pensylvanicus, B. impatiens,* and *B. fervidus;* absent in *B. bimaculatus* and *B. vagans*), although nothing is known about these "entomopoxvirus-like particles", aside from their original description (Clark, 1982). Most honey bee-associated viruses found in bumble bees are single-stranded, positive-strand RNA (ss-RNA) viruses. The structure of these ss-RNA viruses allows for the diagnosis of active replication through detection of the negative (replicating) strand. Although negative-strand detection has indicated that the so-called honey bee viruses do

replicate within bumble bees (Fürst et al., 2014; Li et al., 2011; Radzevičiūtė et al., 2017), the effects of infection on individuals and colonies are largely unknown, and it is not clear whether presence of these viruses is maintained largely through spillover or if substantial transmission occurs within the wild bee community (Manley et al., 2015). Many honey bee viruses persist within honey bee colonies as non-apparent, chronic infections that exhibit symptoms only when the colony is exposed to additional stressors or intracuticular exposure, such as seen with the strains transmitted by *Varroa* mites (McMenamin et al., 2016). Although these viruses are considered honey bee viruses, there is little known of their true host ranges or their ability to cause disease in non-*Apis* hosts (Tehel et al., 2016).

Deformed Wing Virus (DWV) is one of the most commonly detected honey bee viruses in both Europe and North America (Dolezal et al., 2016; McMahon et al., 2015). DWV is known to affect colonies negatively and can be transferred by feeding on infected pollen. Although infected individuals often eclose as adults with crippled wings, cryptic, asymptomatic infections are known, and other factors can deform the wings of bees during pupation, including infections of *Vairimorpha bombi* (Rutrecht & Brown, 2009). The first detection of the virus in bumble bees was based on visual inspection of overt pathology. In a commercial rearing facility in Europe, about 10% of new *B. terrestris* queens exhibited characteristic crumpled wings upon eclosion, and these, as well as asymptomatic honey bees in a co-located apiary, were shown to be harboring DWV (Genersch et al., 2006). The host range of DWV might be quite broad with replicating DWV found in a number of insect orders, including Blattodea and Dermaptera, and even in *Varroa destructor,* a member of the class Arachnida and an ectoparasite of honey bees (Gisder et al., 2009; Manley et al., 2015).

Using molecular means, DWV has been detected across a broad spectrum of wild bee hosts in many families. In the United Kingdom, asymptomatic cases of DWV have been detected in wild, flying individuals of *B. terrestris* and *B. pascuorum,* as well as in the wasp *Vespula vulgaris* (Evison et al., 2012). Prevalence of DWV is often quite high in some of the

insect populations surveyed, (*e.g., Apis mellifera* (100%); *B. terrestris* (29%), and *V. vulgaris* (30%))*,* although other species of bumble bees surveyed at these same sites were free of DWV (Evison et al., 2012). DWV has also been detected in North American bumble bee species, including field-collected *B. ternarius* and *B. vagans*, wild and lab-reared *B. huntii,* and commercially sourced *B. impatiens* (Levitt et al., 2013; Li et al., 2011; Sachman-Ruiz et al., 2015; Singh et al., 2010). The virus has also been observed in bumble bees from commercially sourced colonies in Europe (Graystock, Yates, Evison, et al., 2013). In the US, active replication of DWV has been observed in *B. huntii, B. impatiens,* and *B. vagans* (Levitt et al., 2013; Li et al., 2011)*.* There were no measurable differences between quantified levels of virus in wild bees and wild-caught honey bees in a study in the US, although wild-caught honey bees had much higher levels in a quantification study in the United Kingdom (Dolezal et al., 2016; McMahon et al., 2015). Few experiments have addressed the incidence of disease in DWV-infected bumble bees, but DWV has been shown to increase mortality in experimentally infected individuals both alone and on co-infection with the protozoan *Apicystis bombi* (Fürst et al., 2014; Graystock, Meeus, et al., 2016). However, a laboratory study considering the efficacy of proposed natural transmission routes suggested that transmission in the wild may be limited (Gusachenko et al., 2020). The closely related *Varroa destructor* viruses (VDVs) and kakugo virus (KV) are considered by some to be variants of a DWV species complex (McMahon et al., 2015). Alger et al., (2019) examined spillover of honey bee viruses to wild bumble bees and found DWV and Black Queen Cell Virus (BQCV) to be higher in bumble bees foraging in areas where apiaries were found. Additionally, they confirmed the presence of these viruses on flowers near apiaries, which indicates the potential of bee viruses to be spread due to shared flower use in agricultural landscapes where managed bees are most commonly used.

Acute Bee Paralysis Virus (ABPV), Kashmir Bee Virus (KBV), and Israeli Acute Paralysis Virus (IAPV) are closely related and considered strains of the same virus complex (AKI-complex) (Gisder et al., 2009; McMahon et al., 2015). ABPV was the first honey bee virus to be

detected in bumble bee hosts, and all bumble bee species tested are susceptible to

experimental infection and show classic symptoms, although its occurrence in natural

populations and effect on bumble bee health through natural infection routes are unknown

(Bailey & Gibbs, 1964). In honey bees, ABPV causes trembling, loss of motor control, and

eventual death within a few days of infection (Bailey & Gibbs, 1964). ABPV is systemic but it

can be found in high concentrations within the salivary glands of honey bees and can be

transmitted through pollen, honey, and trophallaxis (Bailey & Gibbs, 1964; Benjeddou et al.,

2001). The virus is shed in large quantities in feces and remains infectious for months (Bailey &

Gibbs, 1964). A recent survey in the United Kingdom found ABPV to be the most common virus

detected in bumble bees, and that ABPV was more common in bumble bees than in honey bees

collected from the same sites (McMahon et al., 2015). Commercial colonies of *B. impatiens* in

Mexico also tested positive for ABPV (Sachman-Ruiz et al., 2015). Although KBV has been

reported from bumble bees in North America and New Zealand, these records are vague and do

not include which species were infected (Singh et al., 2010; Ward et al., 2007). However, one

colony of commercial *B. impatiens* tested positive for KBV in Mexico (Sachman-Ruiz et al.,

2015). KBV is detectable in feces, suggesting this may be a relevant infection route for foraging

bees sharing floral resources (Hung, 2000).

In addition to detection within *Bombus* spp., there is some information on the

transmission and virulence of viruses in the AKI-complex for *Bombus.* IAPV causes shivering,

paralysis, and death in infected honey bees, with increased mortality in the presence of *Varroa*

(Gisder et al., 2009; Palacios et al., 2008). IAPV has been detected in commercially reared *B.*

impatiens, and cross-infectivity studies suggest that transmission between honey bees and

bumble bees can occur through shared food sources (Sachman-Ruiz et al., 2015; Singh et al.,

2010). The route of infection may be very important to the virulence of this virus complex. Orally

administered IAPV and KBV did not induce mortality in infected *B. terrestris* individuals, but KBV

infected microcolonies suffered slower colony establishment and lower offspring production,

with the latter also seen for IAPV (Meeus et al., 2014). A subsequent study has shown that oral administration can result in acute infections with associated virulence, but at much higher doses (Wang et al., 2018). Another study showed that injections of as few as 20 particles of IAPV into *B. terrestris* caused rapid mortality, with all experimental bees dead after only eight days; in contrast, bees injected with as many as 20,000 particles of another, unrelated virus, Slow Bee Paralysis Virus (SBPV), showed no increase in mortality over control bees (Niu et al., 2016). Yet, SBPV virulence can be condition-dependent, with even orally administered SBPV increasing *B. terrestris* mortality under nutritional limitation (Manley et al., 2015). SBPV has also been detected in bumble bees from the UK, at a slightly, but non-significantly, higher prevalence than honey bees, whereas IAPV was not detected in either host (McMahon et al., 2015).

In honey bees, Chronic Bee Paralysis Virus (CBPV) is recognizable by the presence of congregations of trembling bees at the hive entrance, yet infections rarely impact colonies unless other stressors, such as overcrowding or nutritional stress, are also present (Allen & Ball, 1996). Replicating CBPV has been detected in non-*Apis* organisms, including the mite *Varroa destructor*, which is a parasite of honey bees, and the ant *Camponotus vagus,* which opportunistically feeds on dead honey bees, suggesting a wider host range for this virus than is currently documented (Celle et al., 2008). CBPV and ABPV were the most common viruses detected in commercial colonies of *B. impatiens* in Mexico (Sachman-Ruiz et al., 2015), and CBPV has also been detected in native bumble bees in Argentina (Fernandez de Landa et al., 2020). Cloudy Wing Virus (CWV, initially described as CW Particle) is a similar, but likely unrelated virus (Bailey et al., 1980). There are few data about the pathology of this virus, even in honey bees. It appears to exist primarily as an asymptomatic infection in honey bees, although under some circumstances, it may cause rapid mortality (Bailey et al., 1980; Carreck et al., 2010). In Korea, the virus has been detected in captive, field-deployed colonies of *B. terrestris* and *B. ignitus*, and may have been an agent of mortality when present in combination with other viruses, such as KBV and Sacbrood virus (SBV) (Choi et al., 2010).

Black Queen Cell Virus (BQCV) is one of the most common honey bee viruses and has been detected in multiple hymenopteran hosts, including ants, wasps, and bees including mining bees, sweat bees, carpenter bees, leaf-cutting bees, and bumble bees (Levitt et al., 2013; Peng et al., 2011; Ravoet et al., 2014; Singh et al., 2010; Zhang et al., 2012). The distribution of the virus is largely unknown, but, due to its prevalence in honey bees (*e.g.* 98.5% of sampled honey bees in Pennsylvania) (Singh et al., 2010), it is expected to be widespread. Bumble bees from commercial facilities have been recorded harboring the virus in the United States (Singh et al., 2010), Mexico (Sachman-Ruiz et al., 2015), and Argentina (Reynaldi et al., 2013), as have both laboratory-reared and field-caught *B. huntii* in Utah (Peng et al., 2011). Replicating BQCV in bumble bees has also been detected in multiple sites across Europe (Radzevičiūtė et al., 2017). Field surveys show that BQCV is common in both honey and bumble bees in the United Kingdom (McMahon et al., 2015), but a study in Iowa detected very few bumble bees with the virus, in spite of high prevalence in apiaries (Dolezal et al., 2016). BQCV has been detected in pollen loads harvested from honey bee workers (Singh et al, 2010), and in wild bumble bees foraging near apiaries (Alger et al., 2019; McNeil et al., 2020). BQCV replicates in the tissues of the midgut of *B. huntii* and is distributed throughout the body, yet infected individuals show no overt symptoms (Peng et al., 2011). In honey bees, infection by BQCV is more detrimental to larvae, with adults only suffering from infection when coinfected with *Vairimorpha apis* (Ball & Bailey, 1999). If such age-specific effects of BQCV infection are also present in bumble bees, it may be difficult to assess the presence and effects of BQCV infections.

Sacbrood virus (SBV) is a disease that causes mortality in honey bee larvae. Infected individuals cannot molt and eventually die, leaving distinctive carcasses full of virus-laden ecdysial fluid that are usually removed from the colony by vigilant workers (Bailey, 1975). Although the effect of SBV infection on bumble bees is unknown, it has been detected in non-*Apis* hosts on three continents, including in *B. ternarius, B. vagans, B. atratus, Andrena* spp.,

and the paper wasp *Polistes metricus* (Ravoet et al., 2014; Reynaldi et al., 2013; Singh et al., 2010). The virus can also be detected in pollen collected by foraging honey bees (Singh et al., 2010), suggesting a possible transmission route to captive-reared bumble bees. In a sample of 33 wild bumble bee individuals from Iowa, SBV was the most commonly detected virus of five tested for, with 52% testing positive for SBV (Dolezal et al., 2016). However, there have not been any studies that have tested for replicating strands of SBV or examined the impacts of SBV infection on bumble bees, so the impact of this virus is unknown (Gisder & Genersch, 2017).

Bumble bees have been surveyed for only a few honey bee viruses, yet these pathogens appear common among many species and across a wide geographic range. There will likely be more honey bee viruses detected in bumble bees, given that others, such as *Apis mellifera* Filamentous Virus (AmFV), have been detected in more distantly related solitary bees, such as *Andrena vaga, Andrena ventralis, Osmia bicornis* and *O. cornuta* (Ravoet et al., 2014). Unraveling the infection dynamics, routes of transmission, and distinct physiological and colony-level effects of these viruses on bumble bee hosts will be necessary to determine the impacts of honey bee viruses on bumble bee hosts (Tehel et al., 2016).

Bacteria

Little is known about bacterial diseases in bumble bees, but early reports speculated that pathogenic bacteria were responsible for some larval mortality (Frison, 1926). More recently there has been a focus on the beneficial effects of core bacteria associated with the gut of Apid bees (Kwong & Moran, 2016), and how these microbes may aid in resistance against parasite infection (Koch & Schmid-Hempel, 2011a, 2011b; Mockler et al., 2018). While bacterial diseases of honey bees such as American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius*) can be devastating, there are few homologous reports of bacterial infections in bumble bees (Fünfhaus et al., 2018). Many bacteria that have been found

in bumble bees to date appear to be largely either neutral or beneficial, though further work is warranted on this topic. Bacteria that have been identified from bumble bees include *Bacillus cereus, B. pumilus, Brevibacillus laterosporus, Burkholderia cepacia, Enterobacter* (formerly *Aerobacter*) *cloacae, Lysinibacillus* (as *Bacillus*) *fusiformis, Paenibacillus glucanolyticus*, *Spiroplasma apis* and *S. melliferum* (Ahmed et al., 2007; MacFarlane et al., 1995; Marche et al., 2016; Meeus et al., 2012; Přidal, 2001, 2002; Přidal et al., 1997; Schmid-Hempel, 1998). *Spiroplasma melliferum* and *S. apis* are pathogenic bacteria that are associated with May disease in honey bees and both are known to cause mortality (Clark et al., 1985; Meeus et al., 2012). Although both are normally associated with honey bees, they have been detected on the surface of flowers and within the hemolymph and guts of numerous flower-visiting insects, including *B. impatiens, B. pensylvanicus, B. pascuorum, B. pratorum,* and *B. atratus,* and the leaf-cutting bees *O. cornifrons* and *O. bicornis* (Clark et al., 1985; Gamboa et al., 2015; Meeus et al., 2012; Ravoet et al., 2014). The presence of high levels of bacteria, like *Spiroplasma spp.*, in bumble bee guts may indicate their potential as a pathogen in bumble bees (Clark et al., 1985), but this has not been verified. In honey bee queens, *E. cloacae* causes B-melanosis, a disease of the ovaries that sterilizes the queen (Fyg, 1964), but its effect in bumble bees is unrecorded (Schmid-Hempel, 1998). Bumble bees have rarely been screened for the presence of *Wolbachia,* but there are records of this bacterium being detected in European bumble bee species (Evison et al., 2012; Gerth et al., 2015). The effects of *Wolbachia* on hosts are complex (Werren et al., 2008); it is predominantly vertically transmitted and not always pathogenic. To date, we have no knowledge of the kind of association this bacterium has with bumble bees. Research on impacts of bacterial infections and microbiome studies are needed to understand better how bacteria should be managed in a clean stock program.

Protozoans

The trypanosomatid *C. bombi* is an intestinal parasite found in species throughout the genus *Bombus,* with a worldwide distribution (Schmid-Hempel & Tognazzo, 2010). The distribution of this parasite within *Bombus* remains relatively poorly studied and most information on its pathology comes from *B. terrestris*. A close relative, *C. expoeki*, was described from *Bombus* samples collected in both Europe and North America and is assumed to be a similar pathogen (Schmid-Hempel & Tognazzo, 2010). In a survey throughout the United States, *C. bombi* was far more common than *C. expoeki* and co-occured in the same hosts (Tripodi et al., 2018). Similarly, genetic data indicate another undescribed species, nicknamed "*C. mexicana*", that was detected in bumble bee samples from southern Mexico (Gallot-Lavallée et al., 2016), and additional undescribed trypanosomatids in the United States (Tripodi et al., 2018). In the US, *C. bombi* prevalence is highly variable, but can be quite high, for example ranging from 0 - 82% in Massachusetts (Gillespie, 2010). An extensive survey of bumble bees in the US found *Crithidia* to be widespread, yet at low prevalence across species at the sites sampled (Cordes et al., 2012), however another study found regional variation in infection rates (Tripodi et al., 2018). In addition to *Bombus, C. bombi* has been detected in the non-Apidae hosts *Andrena vaga* and *O. bicornis* in Europe (Ravoet et al., 2014), including experimental evidence for active replication in *O. lignaria* and *M. rotundata* (Figueroa et al., 2021; Ngor et al., 2020), though nearly nothing is known about the pathogenicity of *Crithidia* in non-*Bombus* hosts (Figueroa et al., 2021). The honey bee trypanosomatid parasite *Lotmaria passim* has been detected molecularly, but may not be a true parasite of bumble bees (Tripodi et al., 2018).

Crithidia parasites are flagellated single-celled eukaryotes found in the gut lumen of the host bee, anchoring to the ileum epithelium with their flagellum (Koch et al., 2019). Infection in bumble bees can impair the foraging abilities of infected workers (Gegear et al., 2005; Otterstatter et al., 2005), reduce queen hibernation survival (Fauser et al., 2017), and reduce colony founding success (Brown et al., 2003). Although acute mortality is rarely observed (Brown *et al.,* 2003), under conditions of nutritional stress, infected workers are 50% more likely

to succumb to infections than their well-fed counterparts (Brown *et al.,* 2000). In general the outcomes of infection are considered to be context- and condition-dependent (Sadd & Barribeau, 2013).

Crithidia is shed in the feces and can be transmitted through feeding. Experimental evidence shows that bumble bees can contract *C. bombi* infections while feeding on flowers that have been previously visited by infected bees (Adler et al., 2020; Durrer & Schmid-Hempel, 1994). Transmission dynamics on flowers vary by plant species and environmental conditions, with deposition and acquisition for foraging *B. impatiens* varying by flower parts, and exposure to UV radiation significantly reducing pathogen survival on flowers (Figueroa et al., 2019). Moreover, differences among plant species in transmission potential for individual *B. impatiens* workers (Adler et al., 2018), can affect colony-level infection patterns (Adler et al., 2020), highlighting the role of flowers in mediating transmission and prevalence in this bumble bee species. However, there is very limited understanding of *C. bombi* transmission patterns via flowers beyond *B. impatiens* and *B. terrestris* (Ruiz-González et al., 2012). Bees from commercial rearing facilities have tested positive for this pathogen upon delivery (Gegear et al., 2005; Graystock, Yates, Evison, et al., 2013; Murray et al., 2013; Otterstatter et al., 2005). Higher infection levels of this parasite were found in bumble bees near greenhouses that had deployed commercial bumble bees than in wild populations far removed from such sites, lending support to the "pathogen spillover hypothesis" (Colla et al., 2006; Graystock et al., 2014).

The neogregarine, *Apicystis bombi*, is a widely distributed parasite of multiple bumble bee species (Lipa & Triggiani, 1996). In bumble bees, although there are few experimental assessments of virulence, the parasite can have severe effects. *Apicystis bombi* decimates the fat body of infected individuals, and field-collected infected spring queens of European species die before founding colonies (Jones & Brown, 2014; Rutrecht & Brown, 2008). Commercially-sourced colonies of *B. terrestris* were found to harbor this parasite, suggesting a real danger of pathogen spillover of this organism from captive to wild populations (Graystock, Yates, Evison,

et al., 2013). Unlike *Crithidia, Apicystis* was not associated with greenhouse sites in a Canadian study, although a study in the United Kingdom did see higher prevalence of both parasites near greenhouse sites (Colla et al., 2006; Graystock et al., 2014). Population genetics of *A. bombi* from Argentina, Colombia, Mexico, and Europe also suggest that *A. bombi* in Argentina originated from the recent importation of non-native *B. terrestris* from Europe to Chile as commercial pollinators (Aizen et al., 2018; Maharramov et al., 2013). (Please refer to the 2006 NAPPC Bombus Task Force White Paper "Importation of Non-Native Bumble Bees into North America: Potential Consequences of Using *Bombus terrestris* and Other Non-Native Bumble Bees for Greenhouse Crop Pollination in Canada, Mexico, and the United States") However, *B. terrestris* is not present in Colombia, thus the high prevalence of *A. bombi* in South America might be due to more complex factors (Gamboa et al., 2015). Feeding experiments show that *A. mellifera* are susceptible to *A. bombi* infections, and this parasite has been infrequently reported from *A. mellifera* in Europe, Japan, and South America (Graystock, Yates, Darvill, et al., 2013; Lipa & Triggiani, 1996; Morimoto et al., 2013; Plischuk et al., 2011; Ravoet et al., 2014). Additionally, it has been detected in European specimens of *A. vaga, A. ventralis, Heriades truncorum, O. bicornis,* and *O. cornuta* (Ravoet et al., 2014).

Fungi

The microsporidian *Vairimorpha bombi* (formerly *Nosema bombi*) (Tokarev et al., 2020) has a cosmopolitan distribution (Cameron et al., 2016; Koch & Strange, 2012; Li et al., 2011) and is found throughout the genus *Bombus*; however, evidence suggests that some species and/or subgenera are differentially infected (Cameron et al., 2011; Cordes et al., 2012). Furthermore, some declines of bumble species have been linked to presumed epizootic events involving *V. bombi,* including the recent declines of the North American subgenera *Bombus* sensu stricto and *Thoracobombus* (Cameron et al., 2011; Malfi & Roulston, 2014). However, while the incidence of *V. bombi* in North America has increased in recent times, there is no

evidence to support the hypothesis that contemporary strains of the parasite were exotic or introduced from Europe (Cameron et al., 2016). *Vairimorpha bombi* has frequently been detected in commercially-sourced colonies and greenhouse-associated wild populations, but the evidence for spillover remains inconsistent and inconclusive (Colla et al., 2006; Graystock, Yates, Evison, et al., 2013; Murray et al., 2013; Sachman-Ruiz et al., 2015; Whittington & Winston, 2003). Recent molecular screening of *V. bombi* in wild bee communities across old fields and wildflower strips in upstate New York found the pathogen to be virtually absent across two years of sampling (Figueroa et al., 2019; Graystock et al., 2020), highlighting that factors which contribute to differing prevalence rates are not sufficiently understood.

Infections of *V. bombi* occur through the digestive tract, with spores usually concentrated in the Malpighian tubules, the tissues of the midgut and the fat body, although spores can also present in muscles, and the accessory glands, ovaries, accessory testes, and testes of reproductive adults (Larsson, 2007; Otti & Schmid-Hempel, 2007). Bumble bee colonies that are infected with *V. bombi* can suffer from a reduction in reproductive capacity (van Der Steen, 2008). Mortality is higher in infected males, and the survivors produce fewer viable sperm, while infected gynes exhibit swollen abdomens and are more hesitant to mate than their uninfected counterparts (Otti & Schmid-Hempel, 2007). Infections of colonies early in the colony cycle lead to an absence of the production of sexuals (Otti & Schmid-Hempel, 2008). However, other studies have found *V. bombi* to have no effect upon colony growth or reproductive output (Whittington & Winston, 2003). Much of what is known about the pathology of *V. bombi* infections is from a limited number of species (*B. terrestris* and *B. lucorum*), and species may be differentially affected by the disease (Brown, 2017). For example, although infected colonies of *B. lucorum* were less likely to produce gynes, when they were produced, they were fully functional and capable of mating, unlike the gynes produced in *B. terrestris* colonies (Rutrecht & Brown, 2009). Recently, *B. impatiens* males were shown to have a high tolerance to experimentally established *V. bombi* infections (Calhoun et al., 2021).

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Most microsporidian infections in bumble bees have been attributed to *V. bombi,* however bumble bees in Argentina, Colombia, the United Kingdom, the United States, and Uruguay have tested positive in molecular tests for *Vairimorpha ceranae* (formerly *Nosema ceranae*)*,* and those in the United Kingdom also exhibited low prevalence of *V. apis* (formerly *Nosema apis*)*,* both of which are infective agents in honey bees (Arbulo et al., 2015; Figueroa et al., 2019; Fürst et al., 2014; Gamboa et al., 2015; Graystock et al., 2014, 2020; Plischuk et al., 2009). Additionally, *V. ceranae* infections have been confirmed infectious via microscopy in bumble bee hosts from Argentina, Uruguay, and the United Kingdom (Brown, 2017). Experimental feeding experiments with *B. terrestris* have shown that bumble bees are susceptible to *V. ceranae* infection, and that workers suffer increased mortality (Graystock, Yates, Darvill, et al., 2013). Bumble bees in China, Thailand, and Mexico also carried *V. ceranae,* novel strains of *Vairimorpha* that might be undescribed species, and some species of *Vairimorpha* not associated with bee hosts, but the infection status of these novel detections remains unclear (Gallot-Lavallée et al., 2016; Li et al., 2011; Sinpoo et al., 2019). A new genus and species of microsporidian, *Tubulinosema pampeana* was recently described from true tissue infections in *B. atratus* hosts from Argentina, and it has also been detected in the same species in Uruguay (Plischuk et al., 2015, 2017). The only microsporidians that have been shown to cause true infections in wild bumble bees are *V. bombi, V. ceranae,* and *T. pampeana* (Brown, 2017). In addition to *A. mellifera* and *Bombus, V. ceranae* has been detected in wild European specimens of *A. ventralis, H. truncorum, O. bicornis,* and *O. cornuta* (Ravoet et al., 2014)*,* with increasing evidence of active infections in *O. bicornis* (Bramke et al., 2019; Müller et al., 2019). The health impacts of *V. ceranae* on wild bee communities, especially alongside co-occurring stressors, is largely unknown.

There are a few records of ascomycetes fungi infecting bumble bees, but many members of this group are primarily saprophytic and only opportunistically pathogenic, while others are obligate pathogens of bees (Foley et al., 2014; Jensen et al., 2013; MacFarlane,

1976). MacFarlane (1976) cultured a number of fungi from living and dead bumble bees, including a species of *Aspergillus,* but did not show that these fungi were capable of causing infection. In honey bees, *Aspergillus* species are the causative agents of stonebrood, a rarely observed larval malady of honey bees (Foley et al., 2014). On the whole, the *Aspergillus* are considered more saprophytic than pathogenic, but many species are capable of infecting immunocompromised hosts (both vertebrate and invertebrates) and some strains have been shown to be fully pathogenic to seemingly healthy honey bees (Foley et al., 2014; Jensen et al., 2013; Leatherdale, 1970). The species *Aspergillus candidus* and *Aspergillus niger* have been recorded from bumble bee hosts, but their pathogenic roles are unclear (MacFarlane, 1976; Schmid-Hempel, 1998). The 28 species of *Ascosphaera* are known as bee specialists and have been described from the nests and larvae of dozens of wild bee species, with all known cases of pathogenic *Ascosphaera* reported from larvae and causing a suite of characteristic symptoms leading to the common name chalkbrood (Wynns et al., 2013). However, recent research has reported *Ascosphaera apis* infecting adult bumble bees in Oregon (Maxfield-Taylor et al., 2015). In a captive-rearing experiment, the body cavities of wild-caught queens that died prior to producing colonies were filled with vegetative and sporulating *Ascosphaera* species that the authors genetically identified as *A. apis.* Whether or not the fungus was responsible for the death of the queens or whether bumble bee larvae are also susceptible to the disease remains to be seen. *Ascosphaera apis* is the causative agent of chalkbrood, a larval disease of honey bees, and fungal spores are commonly found in the honey bee sourced pollen fed to captive bumble bees (*e.g.,* Graystock, Yates, Evison, et al., 2013; Maxfield-Taylor et al., 2015). However, none of the ascomycetes recorded from bumble bees have been conclusively shown to be pathogenic by satisfying Koch's postulates, so their true status as pathogens in bumble bees is uncertain (MacFarlane, 1976).

Experiments to see whether bumble bees could vector the biological control fungus *Beauveria bassiana* throughout greenhouses have shown that, at high doses, the fungus is

capable of causing mortality to bees (Kapongo et al., 2008). Similar results were seen in efforts to use bumble bees as vectors of *Metarhizium anisopliae* (Smagghe et al., 2013). It is unknown how frequent infections of these fungi are in wild bumble bees, but these two fungi have been isolated from bumble bees in North America (MacFarlane, 1976). Yeasts in the genus *Candida* (many now classified as *Metschnikowia*) have been cultured from bumble bees, nests, and flowers, but these are typically considered to be nectar yeasts, and likely only facultatively pathogenic to bees (Batra et al., 1973; Brysch-Herzberg, 2004; MacFarlane, 1976). There are other sporadic records of entomopathogenic fungi associated with bumble bees, including *Hirsutella* sp., *Acrostalagmus* sp., *Lecanicillium* (formerly *Cephalosporium* or *Verticilium*) *lecanii*, *Geomyces* (formerly *Chryososporium*) *pannorum*; *Parascedosporium* (formerly *Doratomyces*) *putredinis*, *Penicillium* sp., *Isaria* (formerly *Paecilomyces*) *farinosus* (Batra et al., 1973; Goulson, 2010; MacFarlane, 1976; Schmid-Hempel, 1998; Zimmermann, 2008). An unidentified mass of hyphal growth was also described infecting the gut tissue of living adult bumble bees collected in Illinois and Oregon, but the identity of this fungus remains unknown (Kissinger et al., 2011).

Nematodes

The nematode *Sphaerularia bombi* has a worldwide distribution with infection records in dozens of bumble bees species from North America, South America, Europe, and New Zealand (Goldblatt & Fell, 1984; Lubbock, 1861; Lundberg & Svensson, 1975; Macfarlane & Griffin, 1990; McCorquodale et al., 1998; Plischuk & Lange, 2012; Poinar & Van Der Laan, 1972). This parasite exclusively infects bumble bee queens, and upon infection, the queen is effectively sterilized. Although infected queens may live as long as uninfected queens (MacFarlane et al., 1995), they do not initiate nests upon emergence, but rather resume hibernaculum-seeking behavior (Alford, 1969). Because infection with this parasite prevents queens from initiating colonies, it has the potential to impact populations severely.

Mated *S. bombi* females infect bumble bee queens as they overwinter in soil cells. They develop within the hemocoel of the host throughout the winter, maturing upon bumble bee emergence in spring. Mature, gravid females control the corpora allata of host queens, suppressing chemical signals that allow uninfected queens to mature and seek nesting sites upon emergence (Macfarlane & Griffin, 1990). Each female can produce over 100,000 eggs, which are released and hatch in the hemocoel of the host queen (Macfarlane & Griffin, 1990). At the third stage, juvenile nematodes burrow into the midgut of the host. These juveniles are subsequently excreted into shallow pits in the soil excavated by the infected host queen, where they will mature and wait for the next generation of overwintering queens (Poinar & Van Der Laan, 1972). Because the nematodes drop into the soil to await transmission to the next generation of queens, *S. bombi* is not expected to be a pest of captive-reared bumble bees.

There are few records of mermithid parasites in bumble bee hosts, but they are geographically widespread, with records from North America, South America, Europe, and Asia (Durrer & Schmid-Hempel, 1995; Kosaka et al., 2012; Kubo et al., 2016; MacLean, 1966; Mullins et al., 2020; Plischuk et al., 2017; Rao et al., 2017; Tripodi & Strange, 2018). Because the parasitic stage of mermithids are devoid of morphological characters that would allow their identification, the identity of these parasites is largely unknown. One record of a mermithid infecting a *B. impatiens* worker collected in Wakefield, Massachusetts was identified to the genus *Pheromermis,* but nothing is known of its life history or whether bumble bees are its primary host (Rao et al., 2017). Like *S. bombi,* these parasites require a free-living stage in the soil, so they are unlikely to present an issue in rearing facilities. Mermithids kill their hosts upon exiting the host's body, but with so few occurrences, they are unlikely to have an impact on the population level (Tripodi & Strange, 2018).

Acarines

There are many mites associated with bumble bees, yet most that are found on the host's exterior are considered to be harmless nest commensals. *Scutacarus acarorum*, an inquiline of bumble bee nests known to feed primarily on fungus (Jagersbacher-Baumann & Ebermann, 2013) has incorrectly been described as an occasional parasite of bumble bee larvae (Jagersbacher-Baumann, 2015). Other bumble bee-associated mites that are thought to have non-parasitic life histories include *Kunzia americana, K. affinis, Parasitellus* spp., *Parasitus* spp., *Proctolaelaps longisetosus,* and *P. bombophilus* (Delfinado & Baker, 1976; Eickwort, 1990; Goldblatt & Fell, 1984; L. Richards & Richards, 1976). Most of these mites are thought to be scavengers or fungivores within nests, although some are predatory and may benefit the bumble bees by consuming nest pests (Eickwort, 1990). Others have an uncertain status in nests. *Pneumolaelaps* species seem to be obligate specialists in bumble bee nests, and might be best classified as kleptoparasites that consume only the freshly collected pollen intended for larvae, although they have been observed feeding on injured bees (Hunter & Husband, 1973; Royce & Krantz, 1989). On the whole, the ecologies of mites are understudied, and totally unknown for some bumble bee associates, like the *Cerophagus* spp. (O'Connor, 1992).

Of greater concern is the obligate endoparasitic mite, *Locustacarus buchneri*. This bumble bee tracheal mite is an internal parasite inhabiting the airways and abdominal air sacs of adult bees (Husband & Sinha, 1970). It has been reported to lead to lethargy and reduced foraging (Husband & Sinha, 1970) and infected male bumble bees brought into the laboratory have reduced longevity (Otterstatter & Whidden, 2004). In North America, it seems to be more common in early-emerging species, such as *B. bimaculatus, B. perplexus,* and *B. vagans* (MacFarlane et al., 1995), although not all early-season species are affected (*e.g., B. mixtus* in Canada; (Otterstatter & Whidden, 2004)). Bees are infected as 3rd instar larvae, female mites overwinter within new queens, and populations build quickly and spread throughout the colony (Yoneda, Furuta, Kanbe, et al., 2008). Colonies infected with *L. buchneri* have been purchased from commercial sources (Otterstatter et al., 2005; Yoneda, Furuta, Tsuchida, et al., 2008), and

there has been great concern that commercial trafficking of bumble bees will carry this parasite into novel hosts (Goka et al., 2001). The mite is widely distributed in the Northern Hemisphere, and has also been detected in Korea (Keum et al., 2021), Argentina (Plischuk et al., 2013), and in New Zealand, where it was introduced along with its bumble bee hosts (Macfarlane, 1975). Rearing companies have taken actions to attempt to control this mite, likely in response to early concerns (Goka et al., 2001). At present, the consensus is that mites seem well-controlled in colonies sold commercially (Meeus et al., 2011), and in European surveys, even phoretic mites were absent until colonies were deployed in the field (Rożej et al., 2012).

Dipterans

Apocephalus borealis is a parasitic phorid fly widely distributed throughout North America (Brown, 1993). Females oviposit one or more eggs into the body of the host and larvae feed upon the host's tissues until pupation. Mature larvae leave the host's body between the head and pronotum prior to pupation, often decapitating the host in the process (Core et al., 2012). Although there are few host records for this species, it has been recorded as a parasite of not only bumble bees (*B. bifarius, B. californicus, B. flavifrons, B. melanopygus, B. occidentalis* and *B. vosnesenskii*), but also black widow spiders (*Latrodectus mactans*), yellowjacket wasps (*Vespula* spp.), and most recently, honey bees (*A. mellifera*) (Brown, 1993; Core et al., 2012; Otterstatter et al., 2002). In honey bees, phorid parasitism causes aberrant behavior, such as flying at night and nest abandonment (Core et al., 2012). Parasitism of bees seems seasonal, with peak rates observed in late summer (Core et al., 2012; Otterstatter et al., 2002). In addition, both adults and larvae tested positive for *Vairimorpha ceranae* and Deformed Wing Virus using molecular tests, suggesting that the flies have the potential to vector these pathogens among species (Core et al., 2012).

Bumble bees are also prey to parasitism by conopid flies. As with phorid parasites, conopid females oviposit into adult bees, and their larvae are endoparasites. Although more

than one egg may be laid, only one larva will advance to pupation in a single host (Schmid-Hempel & Schmid-Hempel, 1989). Larvae initially consume hemolymph, then move to the fat body, ovaries, and other vital organs, killing the host as they mature (Abdalla et al., 2014). Pupation takes place inside the dead host, and some bumble bee hosts have been shown to bury themselves in soil just prior to the parasite's pupation (Malfi et al., 2014). Again, little is known about the host ranges of these flies, but in North America, there are at least five species that have been documented to attack *Bombus* spp. Most Conopid parasites of *Bombus* in North America are in the genus *Physocephala.* One record of *Zodion obliquefasciatum* from a *B. auricomus* host (Frison, 1917) was apparently misidentified (Frison, 1926), but there are two additional records of *Zodion* sp. from Canada that have not been verified (Macfarlane & Pengelly, 1974). *Physocephala burgessi* has been found parasitizing *B. pensylvanicus sonorus*; *P. marginata* has been recovered from *B. fervidus* and *B. nevadensis*; *P. sagittaria* has been recorded in *B. auricomus* and *B. pensylvanicus; P. texana* has been found parasitizing *B. bifarius*, *B. californicus*, *B. flavifrons*, and *B. occidentalis*; *P. tibialis* has been recovered from *B. bimaculatus*, *B. griseocollis*, *B. impatiens,* (Freeman, 1966; Gibson et al., 2014; Malfi et al., 2014) and *B. vagans* (Richardson et al. 2016). *Physocephala* are not restricted to bumble bee hosts, however. *Physocephala texana* has been recorded parasitizing honey bees (*A. mellifera*), *Nomia melanderi,* and sand wasps (*Bembix* spp.), and *P. marginata* has been recovered from *A. mellifera* and *Megachile mendica* as well (Gibson et al., 2014; Parsons, 1948).

Sarcophagid flies have been infrequently reported as parasites of bumble bee adults and larvae, but as most are primarily scavengers; their status as true parasitoids has been questioned (Dahlem & Downes, 1996). North American records of sarcophagid flies thought to have parasitized bumble bees include *Boettcheria litorosa* (also as *Sarcophaga litorosa*)*, Liosarcophaga sarracenioides* (as *Sarcophaga sarracenioides or S. tuberosa sarracenioides*)*, Brachicoma* spp. (*Brachycoma* [sic] *sarcophagina,*), *Helicobia morionella* (also as *Sarcophaga morionella*) (Frison, 1926; MacFarlane et al., 1995; MacFarlane & Pengelly, 1977; Macfarlane

& Pengelly, 1974; Ryckman, 1953; Stone et al., 1965). In Ontario, a collection of 385 wild adult bumble bees yielded 3.3% with an endoparasitic sarcophagid larva (MacFarlane & Pengelly, 1977). In a captive *B. fervidus* nest, 78% of the cocoons held immatures parasitized by sarcophagid flies, but the parasitic nature of these is less certain (MacFarlane & Pengelly, 1977). Frison and Plath both experienced large numbers of Sarcophagids in their captive rearing experiments (Townsend, 1936), but very little has been recorded on the relationship between the flies and bumble bees in recent years, and outbreaks have not been reported in modern rearing facilities. Ryckman (1953) reported rearing *Boettcharia litorosa* and *H. morionella* from adult bumble bees, but there have not been more recent reports of this relationship. *Helicobia morionella* are more commonly reported as facultative parasitoids of gastropods (Coupland & Barker, 2004; Stegmaier, 1972). Members of the Sarcophagid tribe Miltogrammini are associated with hymenoptera nests, and primarily considered to be kleptoparasites who feed and develop on the provisions provided to brood (Shewell, 1989). One European species in this tribe, *Senotainia tricuspis,* has been recorded as an endoparasite of bumble bees, but it is more commonly associated with honey bees (Bailey & Ball, 1991). Larvae of the bumble bee mimic syrphid fly *Volucella bombylans* have also been recorded as pests of weak nests, but these organisms are scavengers and are not thought to feed on healthy larvae (Gabritschevsky, 1926; Hobbs, 1967; Monfared et al., 2013). Because of the mechanisms by which most dipteran parasites of bumble bees locate and parasitize the hosts, the risk of dipterans in rearing facilities is relatively low.

Hymenopterans

Braconid wasps in the genus *Syntretus* are known as parasites of adult queen, worker and male bumble bees in Europe (Alford, 1969; Schmid-Hempel et al., 1990). Although less work has been conducted on wasp parasitoids of bumble bees in North America, 2% of spring-caught queens were parasitized by wasps assumed to be *Syntretus* in Virginia (Goldblatt & Fell,

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1984), and 3% of *B. vosnesenskii* queens from the West were parasitized with wasp larvae assumed to be *S. splendidus* (Mullins et al., 2020). *Syntretus* wasps oviposit in adult bumble bee hosts while the bees are foraging or resting away from the nest, depositing multiple eggs (mean number of wasps per bee = 23.2) into the membrane between head and prothorax (Alford, 1969). Larvae live in the host for three to four weeks, before exiting the host as fifth-instar larvae via the membrane between the second and third metasomal segments. Successful pupation seems to depend on the presence of soil (Alford, 1969), thus these insects are unlikely to establish as pests of captive-reared bumble bees. In England, *Syntretus* parasitization occurs in late May and early June (Alford, 1969), suggesting that early-emerging bumble bees may avoid this threat. Parasitization of queens is likely to have the greatest impact on bumble bee populations. The ovaries of parasitized queens atrophy and such queens will eventually stop laying eggs, and nests with parasitized queens may be characterized by having pupae but no new brood (Alford, 1969). About 7% of wild-caught *B. pratorum* queens in Ireland were infected with *Syntreus,* and all died before initiating colonies (Rutrecht & Brown, 2008). However, parasitized workers continue to forage until shortly before their deaths, suggesting that parasitization of this caste has little effect on the growth and health of the colony (Alford, 1969).

Bumble bees are also vulnerable to parasitization by Eulophid wasps in the genus *Melittobia.* Unlike *Syntretus,* which are endoparasites of adult hosts, the *Melittobia* are idiobiont ectoparasites of immature stages (Dahms, 1984a; González et al., 2004). Prior to oviposition on the exterior of the host's cuticle, *Melittobia* females pierce the cuticle, subduing the host, providing the adult wasp with food in the form of hemolymph, and in some cases, inhibiting the development of the host (González et al., 2004). In *B. terrestris, Melittobia* can only develop on pupae and prepupae (Kwon. *et al.,* 2012b). These wasps have a high reproductive capacity, with 200–600 offspring reared on each host (de Wael et al., 1995; Whitfield & Cameron, 1993). Fecundity with *B. terrestris* hosts averaged about 48 per mated female wasp under experimental conditions (Kwon et al., 2012a). The *Melittobia* have a wide host range,

particularly in the aculeate Hymenoptera and including many species of commercially reared

bees: bumble bees, honey bees, and the alfalfa leafcutting bee, *Megachile rotundata* (Dahms,

1984a). With such high fecundity and six to eight generations per year, *Melittobia* infestations

can greatly impact colony health (de Wael et al., 1995). Infestations of *Melittobia* have caused

economic damage in rearing facilities of both leaf cutting bees and bumble bees (Dahms,

1984a; de Wael et al., 1995; Holm & Skou, 1972; Kwon et al., 2012a). Due to their wide host

range, small size and cryptic habits, wasps in this genus are particularly susceptible to

anthropogenic introductions through commercial trade, and this has been reported for two

species, *M. acasta* and *M. australica* (Matthews et al., 2009). Populations of *Melittobia* spp. can

increase rapidly in artificial rearing conditions due to their gregarious nature, their cryptic habit of

remaining on pupal hosts inside of sealed cells, and the rapid development time of the parasite,

all which can result in severe damage to a colony and ultimately colony failure (González et al.,

2004; Kwon et al., 2012b; Matthews et al., 2009). *Melittobia* are difficult to identify to species

and may have wide host ranges, thus many parasite-host records are likely to be inaccurate

(Dahms, 1984a). Some *M. chalybii* records, including those in North American bumble bees, are

likely mis-identified and should be attributed to *M. acasta,* but it is generally accepted that this

parasite can develop on a wide range of hosts, at least under laboratory conditions (González et

al., 2004; González & Matthews, 2005; Husband & Brown, 1976; LaSalle, 1994). Other records

may be of *Melittobia* as a hyperparasite, parasitizing other parasitic insects inhabiting bumble

bee nests, such as flies (*e.g.,* sarcophagid pupa in *B. vagans* nest: (Husband & Brown, 1976))

or even parasitizing moths in nests. Further inquiry and better taxonomic treatment are

necessary to clarify host-parasite relationships in this group (Matthews et al., 2009; Whitfield &

Cameron, 1993).

Congeners of bumble bees of the subgenus *Psithyrus* are obligate social parasites of

bumble bees, with about 30 species worldwide (Williams, 2008). They have evolved a number

of morphological, social, and behavioral adaptations that reflect their social parasitism, with the

loss of corbiculae, an enhanced stinging apparatus, thicker integument, and the loss of a worker

caste the most prominent characteristics that distinguish this group (Plath, 1922). Female

Psithyrus invade a nest, kill or dominate the rightful queen, and use the food-gathering and

nursing labor of the usurped queen's workers to rear her own offspring. Many *Psithyrus* are

host-specific, occupying the nests of one or a few host bumble bee species (Williams, 2008).

This host specificity is additionally supported by evidence that some parasites share chemical

profiles of their host species that may allow them to overcome host defenses (Martin et al.,

2010). Once colonies are deployed in the field, they may come under attack by *Psithyrus*

invaders, but these social parasites would not be an issue in captive rearing (Strange et al.,

2014). The *Psithyrus* are susceptible to the same parasites as their social cousins (*e.g., S.

bombi,* (McCorquodale et al., 1998)), and may vector some of these into nests as they attempt

to invade. Recently, Koch et al. (2021) demonstrated that *Psithyrus* invasions can be prevented

by use of a fabricated plastic excluder affixed to the nest entrance, providing protection for field

deployed colonies. Their mode of parasitism, however, makes them highly unlikely to impact

rearing facilities.

Coleopterans

Originating from sub-Saharan Africa, the invasive small hive beetle, *Aethina tumida*

(Nitidulidae)*,* is a pest of honey bee hives that has the potential to cause destruction to bumble

bee colonies as well (Ambrose et al., 2000). The beetles feed on wax, pollen, honey, eggs, and

larvae, and can foul food stores through fermentation by associated yeasts (Cuthbertson et al.,

2013). Small hive beetles are capable fliers and may disperse over several kilometers

(Neumann & Elzen, 2004). They can locate bumble bee colonies in field conditions and are

attracted to both worker and pollen odors (Spiewok & Neumann, 2006). Experimentally infested

bumble bee colonies sustained large amounts of damage to the comb and had fewer live bees

than a control, indicating that small hive beetle infestation can be devastating to colonies

(Ambrose et al., 2000). Bumble bees do show defensive behaviors that help thwart the establishment of small hive beetles within colonies, including egg removal and stinging larvae to death (Hoffmann et al., 2008), but the beetles are cryptic and oviposit in crevices that are often out of the reach of their host bees (Cuthbertson et al., 2013). Because the larvae require soil in which to pupate (Cuthbertson et al., 2013), there is little chance of the beetle becoming a pest in most rearing facilities, but they may pose issues once colonies are deployed in the field (Spiewok & Neumann, 2006). The beetle may also vector DWV between colonies, since the virus has been shown to replicate in the beetle (Eyer et al., 2009).

Beetles in the genus *Antherophagus* (Cryptophagidae) are phoretic on bumble bees, hitching a ride back to the nest by attaching themselves to the mouthparts or leg of the foraging bee (Chavarria, 1994; Parks, 2016; Wheeler, 1919). Once back in the nest, the beetles feed and rear their young on nest detritus and are not thought to be detrimental to the colony (Frison, 1921). Five species are known from North America, but the genus is widespread, also occurring in South America, Europe, and Asia (Bousquet, 1989). Because bees encounter these beetles while free foraging on flowers, and the beetles are merely nest scavengers, they are not presumed to be an issue in commercial rearing.

Lepidopterans

A number of moths in the family Pyralidae are known as pests of bumble bee nests, targeting nest products including wax and pollen, and in some cases, bee larvae. The bee moth *Aphomia sociella* originates from Europe, but is now adventive and widespread throughout North America, specializing on the nests of the aculeate Hymenoptera (Solis & Metz, 2008). Infestations by this moth can be devastating to bumble bee nests, as the larvae destroy the comb and consume the brood (Frison, 1926; Goulson et al., 2002). Although it has been described as a specialist predator of bumble bees (Goulson et al., 2002), thriving populations of the moth have been discovered in the nests of Vespid wasps, as well as in mouse and bird

nests (Solis & Metz, 2008). Aboveground, artificial bumble bee nests may be more easily located by the moth than natural, subterranean ones (Goulson et al., 2002). *Vitula edmandsii,* the American wax moth, may also be an occasional pest of honey bee hive products (Milum, 1953). In a mixed apiary with both honey bee and bumble bee colonies, most bumble bee nests were infested with *V. edmandsii* but no honey bee hives contained this pest (Whitfield & Cameron, 1993). The larvae of *V. edmandsii* feed upon wax, pollen, and other nest materials, but are not known to feed directly upon living larvae (Frison, 1926). Its western counterpart, the dried-fruit moth *V. serratilineella,* is also known as a pest of *Megachile rotundata,* but because these two moth species have often been considered as one species, it is difficult to discern whether *V. serratilineella* has been associated with bumble bees (Richards, 1984; Sattler, 1988; Scholtens & Solis, 2015).

The greater wax moth *Galleria mellonella* is a well-known pest in honey bee apiaries. Although the greater wax moth has been successfully reared on bumble bee nests (Oertel, 1963) and found in field-deployed colonies of *B. impatiens*, bumble bee nests remained free of this pest even when placed in an apiary containing heavily infested honey bee hives (Whitfield & Cameron, 1993). This pest can be quite destructive in bumble bee colonies, and heavy infestations can lead to rapid colony declines. The lesser wax moth *Achroia grisella* is a similar pest in honey bee hives, but has not been reported in bumble bee colonies (Milum, 1940) and seems to be an issue only in very weak honey bee hives (Williams, 1997). The invasive Indian meal moth *Plodia interpunctella* is a stored product pest with worldwide distribution (Williams, 1997). With six to eight generations per year, populations of this pest can be quite large, and are highly destructive to colonies in captive rearing facilities (An et al., 2007). Unlike the wax moths discussed previously, the Indian meal moth does not feed on wax, but rather develops on high-protein pollen stores and dead brood and adults (Williams, 1997). Moth eggs are sometimes transported into rearing facilities on pollen acquired from honey bees (Kwon et al., 2003). The Mediterranean flour moth, *Ephestia kuehniella,* is a similar pyralid with a worldwide

distribution, but it is thought to feed only on pollen provisions in the nest (Milum, 1940; Schmid-Hempel, 2001).

2) Hive products and associated risks

<u>Pollen</u>

Pollen, the primary food for the development of bee larvae, can be a source of exposure to pathogens and pesticides for commercially raised bumble bees. Pollen is frequently contaminated with pathogens (Chen et al., 2006; Gilliam et al., 1988; Higes et al., 2008) and detritus, and may be contaminated with pesticides or other environmental contaminants (Chauzat et al., 2006; Mullin et al., 2010). Recent work (Graystock et al., 2015; Pereira et al., 2019; Singh et al., 2010) has demonstrated the potential role of pollen in moving pathogens from species to species. There are no regulations in place governing sanitary practices associated with use of pollen by commercial bumble bee rearing facilities despite the acknowledged threat of pollen in spreading pathogens within and among species (Gilliam et al., 1988; Graystock et al., 2016) and recognizing that more than two-hundred tons of honey bee-collected pollen are used annually for bumble bee rearing worldwide (Velthuis & van Doorn, 2006).

Several treatments to reduce the spread of pathogens through pollen have been investigated including irradiation (Graystock et al., 2015; Graystock et al., 2016; Hidalgo et al., 2020; Meeus et al., 2014; Yook et al., 1998), ozone (Graystock et al., 2016; Yook et al., 1998), pulsed light (Naughton et al., 2017) and ethylene oxide fumigation (Strange, unpublished data). Irradiation of pollen at levels from 5 kGy to 16.9 kGy has been shown to eliminate or reduce many pathogens and their infectivity. At lower levels (5 kGy to 7.5 kGy), fungi, coliform and aerobic bacteria, yeasts, and molds were not detected after irradiation (Hidalgo et al., 2020), with little effect on pollen nutrition or structure (Yook et al., 1998). At higher levels of irradiation

(16.9 kGy), Deformed Wing virus, Israeli Acute Paralysis virus, Sacbrood virus, and *V. ceranae* were all removed, while *C. bombi*, *Ascosphaera,* Black Queen Cell virus, and Chronic Bee Paralysis virus were only partly inactivated (Graystock et al., 2016; Simone-Finstrom et al., 2018). *Apicystis bombi* remained infectious after irradiation but infections were reduced by about half (Graystock et al., 2016). These results show promise to reduce negative impacts on bumble bees with these pollen treatments, but there are concerns about potential adverse effects on the nutritional value of irradiated pollen (Graystock et al., 2016; Meeus et al., 2014) and potential negative effects on the gut microbiome (Klinger et al., 2019; Meeus et al., 2014). Notwithstanding, some commercial rearing facilities routinely use irradiated pollen with no known negative effects on bumble bee rearing or performance (Graystock et al., 2016; Meeus et al., 2014). Other possible pollen treatments to reduce pathogens in pollen include ozone (Graystock et al., 2016; Yook et al., 1998) and pulsed light treatments (Naughton et al., 2017). Compared to irradiation, ozone treatment was deemed less effective (Graystock et al., 2016; Yook et al., 1998), which may be related to the poor distribution of ozone within the pollen samples. Pulsed light was shown to be effective at inactivating *C. bombi* in pollen samples in a single study (Naughton et al., 2017). In addition to these approaches, preliminary work conducted by J. Strange and colleagues show promising results for treatment with ethylene oxide fumigation, but not e-beam irradiation (unpublished data). In all cases, significantly more work is required to identify treatment conditions that effectively eliminate pathogens while maintaining nutritional content.

Another potential solution to issues associated with both pathogen and pesticide contamination of pollen is the development of a commercially available pollen substitute. Commercial bumble bee rearing facilities and research programs alike could benefit from a pathogen- and pesticide-free pollen substitute. Use of a pollen substitute would eliminate a source of experimental variability (*i.e.*, varying composition of pollen batches). While pollen substitutes for honey bees are well established (Haydak & Dietz, 1965; Mattila & Otis, 2006), to

date, only two publications have investigated potential pollen substitutes for bumble bees (Bortolotti et al., 2020; Graystock et al., 2016). While results from these studies demonstrate significant progress, much work is needed before a suitable pollen substitute will be available for widespread use.

Wax

Wax is integral in the structure of bumble bee colonies, being produced by queens and workers throughout the colony cycle. While wax is biologically critical to colony growth, it is known that it can serve as a reservoir for pathogens and environmental contaminants in honey bee colonies (Flores et al., 2005; Fries, 1988; Shimanuki & Knox, 1991; Wu et al., 2011). The degree to which this is a problem in bumble bee colonies is not well understood and we consider this an area of severe data deficiency. However, as wax is not reused in production facilities, it poses little risk for horizontal transfer of pathogens in commercial bumble bees and thus is a low priority for study. However, we acknowledge that wax will remain in nest boxes that have been disposed of and may represent a source of infectivity after the colony is no longer in production. Proper cleaning and/or disposal of used equipment should mitigate any risks of wax vectoring disease in rearing facilities.

3) Detection, identification, and quantification

General techniques used to detect and quantify pathogens

Detection of bumble bee parasites falls into two major categories: molecular methods or visual methods. Most parasite detection is destructive, requiring that bees be killed prior to examination. However, mature or transmitting infections of some parasites, including *S. bombi, Vairimorpha* spp., *Crithidia* spp., and *A. bombi*, can be visually detected in feces, a non-lethal

technique (Jones & Brown, 2014). For some parasites, quantification of individual parasites in feces provides an accurate estimation of the intensity of the established infection, *e.g.*, for *Crithidia* (Sadd, 2011). However, such a relationship has not been verified for all observable parasites detectable in the feces, and false negatives may occur during early stages of infection. In addition, low numbers of parasite transmission stages may represent false positives, where transmission stages, *e.g.*, environmentally resistant extracellular *Vairimorpha* spores, are just passing through and are not from established infections. This presents an issue for any analysis where gut tissue is included and is a potential issue in both visual and molecular detection approaches. However, in closed systems, such as rearing facilities, detection of parasites and pathogens in the feces will likely represent actual infections. Although tissues of the head and mesosoma can be infected, all known parasites can be detected by examination of the tissues and hemocoel of the metasoma. Different parasites are typically detected using different techniques, but these are often complementary. Larger organisms are visible with light microscopy during dissection under low magnification (10–40x). This is often followed by an examination of slide-mounted tissues or homogenates at higher magnification (400x) to detect smaller organisms. Finally, molecular methods can be used to detect, identify, and quantify parasites of all sizes from tissue extractions.

Before the development of molecular detection techniques, visual detection with light microscopy was the predominant mode of screening for internal bumble bee parasites. Light microscopy allows for the detection of parasites at 400x magnification, encompassing a broad diversity of organisms. To this day, microscopy continues to be employed in the detection and quantification of bumble bee parasites via the count of spores or cells using a hemocytometer (Fries et al., 2013). Some of the strengths of light microscopy include that it is low-cost, requires little training to employ, and most importantly, it can detect active infections through tissue pathology. However, there is a risk for false negatives as low-level or early stage infections can be missed, suggesting that traditional light microscopy may underestimate parasite prevalence

(Blaker et al., 2014). False positives are also possible, especially for less-experienced researchers who are not fully aware of target parasite morphologies. In addition, many pathogens are tissue specific, thus requiring the correct tissue to be examined for diagnosis (Schmid-Hempel, 1998). However, the primary benefit of visual detection is the ability to diagnose disease and disease intensity, rather than just the presence of a potential disease-causing organism. In all cases it is preferable that known positive samples be observed under the set up being used, to ensure accurate identification and verify the ability of the setup to detect parasites and pathogens of interest. For example, *Crithidia* spp. require phase contrast microscopy for good visualization. However, even then, detection by observers unfamiliar with cell morphology will be aided by using fresh samples where some cells will be motile.

Polymerase Chain Reaction (PCR), developed in 1985, is the most commonly employed molecular technique for DNA amplification, and it has been used to great effect to detect parasites in both bumble bees (Blaker et al., 2014; Cordes et al., 2012; Huang et al., 2015; Koch & Strange, 2012) and humans (Yang & Rothman, 2004). This method uses short oligonucleotides, primers, that are designed to hybridize with known genetic regions within the genomes of targeted organisms. Samples that fail to amplify are diagnosed as negative, and samples that successfully amplify are diagnosed as positive for the targeted parasite. Including control regions that amplify bee DNA or cDNA in PCR is a common quality control measure used to guard against false negatives that can come about through poor specimen handling, nucleotide extraction or bad reactions. Positive controls should also be included in PCR to ensure viability of reactions. Similarly, the use of negative controls that contain no DNA template can help guard against false positives that usually stem from laboratory contaminants. With the development of primers that do not interfere with one another during thermal cycling, PCR can be multiplexed for the detection of multiple pathogens simultaneously (Huang et al., 2015; Procop, 2007; Tripodi et al., 2018). One of the strengths of PCR is that it can be used to detect presence or absence of parasites at very low intensities or in small sample volumes.

Quantitative PCR (qPCR) goes a step further, amplifying and detecting the target sequence simultaneously and, if properly calibrated, yielding a quantitative measure of infection intensity. For screening RNA viruses, reverse transcriptase PCR (RT-PCR) is used, which converts RNA to its complementary DNA strand (cDNA), which is then used as template in PCR (de Miranda et al., 2013). Standardized protocols for PCR-based detection of a variety of common bee pathogens have recently been released (de Miranda et al., 2021).

In a double-blind methods comparison, PCR was found to have an overall higher sensitivity for detecting human-pathogenic microsporidia compared to traditional light microscopy, though both methods proved effective (Rinder et al., 1998). Likewise, (Blaker et al., 2014) found significantly higher sensitivity for detecting microsporidia in bumble bees than light microscopy detected. However, increased sensitivity is not always desirable. PCR methods do not distinguish between exposure and infection, and dead or inactivated parasites may still yield positive results. Such sensitive methods can diagnose samples as positive, regardless of the true infection status within the host, thus positive PCR results should be interpreted with this caveat in mind (Brown, 2017). PCR, qPCR, and RT-PCR assays can be designed to use either species-specific or broad-range primers that can detect multiple members of a targeted taxon, depending on the desired identification level (Graystock et al., 2020; Mullins et al., 2020; Procop, 2007; Yang & Rothman, 2004). While broad-range primers allow for the discovery of new organisms within a targeted taxon, one of the major drawbacks of all primer-based detection techniques is that the researcher will only detect organisms or groups that are being targeted, and that detection is limited to parasites for which sequence data are available. However, post-amplification analysis of PCR products through DNA sequencing can be used to identify parasites to species, generate additional data, and conduct analyses of strain differences that can be useful in understanding disease dynamics (Cameron et al., 2016).

Current advances in molecular technologies, known as next-generation sequencing (NGS) platforms, are beginning to allow for pathogen screening and sequencing through

exploratory metagenomics (Gerth & Hurst, 2017; Runckel et al., 2011). Exploratory work with

the RNA-Seq platform recently detected a number of known bumble bee associated organisms

in two bumble bee species, as well as two undescribed viruses in *O. cornuta* (Schoonvaere et

al., 2016). However, the success of these NGS techniques depends on the existence of

reference databases, such as well-curated sequence deposits, knowledge of the pathology and

natural history of the symbionts detected and identified, as well as the technical ability to

process, analyze, and interpret the data (de Magalhães et al., 2010). As the use of these

methods increases, and databases of pathogen sequences expand, NGS could provide

unexplored levels of pathogen screening abilities for bumble bee research. However, despite

their significant value in these regards, NGS approaches would currently be unfeasible for a

rapid and high-throughput clean stock screening program, where targeted visual or molecular

approaches of known parasites and pathogens of concern will be more effective.

Viruses

Because of their small size (typically 20-30 nm, (James & Zengzhi, 2012)), viruses are

not visible with basic microscopy and are primarily detected through molecular methods (de

Miranda et al., 2013). Using RT-PCR, specific primers can be employed to determine the

presence of a virus, and the viral load can be quantified using calibrated qRT-PCR (*e.g.,*

McMahon et al., 2015). In addition, it is possible to run a multiplex RT-PCR and screen for

multiple RNA viruses simultaneously (Chen et al., 2004). However, detecting the presence of a

virus is not equivalent to detecting a viral infection. An advantage to the structure of many ss-

RNA viruses is that it is possible to screen for their complementary strand, which, if found,

indicates active replication within the host (de Miranda et al., 2013; Mazzei et al., 2014). This is

not possible for DNA-based parasites.

Bacteria

Not all bacteria can be cultured on standard media (Přidal, 2001; Shrivastava, 1982) and in addition, while some can be easily viewed using standard microscopy approaches, the delineation of bacterial pathogens is difficult. Therefore, molecular methods are commonly used for detection of bacteria, such as *Spiroplasma apis* and *S. melliferum* (Meeus et al., 2012). Often, culture-based and molecular methods are used in conjunction with one another in order to determine physical and chemical characteristics, experiment with inoculation and host specificity, and resolve taxonomic issues (Kwong et al., 2014; Kwong & Moran, 2013; Praet et al., 2017).

Protozoans

The infective spore stage of neogregarines and the motile stages of trypanosomatids can be detected through microscopic examination of tissues, tissue homogenates, or fecal samples at 400x. However, these organisms have complex life cycles with cryptic vegetative growth phases that can be easily missed by microscopy, making molecular detection methods more reliable. The gross morphology of some protozoans makes their identification to broad groups rather simple, but discerning species morphologically is impossible under typical magnification. Morphological differences that separate species can be seen with scanning electron microscopy and other specialized equipment (Liu et al., 1974; Schmid-Hempel & Tognazzo, 2010). *Crithidia* spp. are quite small, typically less than 10 µm long in all stages, and some stages are highly motile and visible when alive (Schmid-Hempel & Tognazzo, 2010). It is important to note that while *C. bombi* has three distinct morphological stages (amastigote: spherical form with no visible flagellum, choanomastigote: pear-like shape surrounding flagellar pocket, and promastigote: large cells with long flagellum; (Logan et al., 2005; Ruiz-González & Brown, 2006)), the vast majority of screening efforts via microscopy focus on the promastigote stage, potentially under-reporting infections of the other morphological stages. Spores of neogregarines are larger, 21–27 µm, and are easily visible at 400x (Liu et al., 1974). Infections

can be quantified by counting spores in a hemocytometer (Human et al., 2013). Broad-range

primers have been developed to detect trypanosomes, including *Crithidia* spp., as well as

neogregarines, including *Apicystis bombi* (Meeus et al., 2009; Mullins et al., 2020; Schmid-

Hempel & Tognazzo, 2010). In preliminary screening, a broad-range primer may be used, then

positives can be sequenced and identified (Gallot-Lavallée et al., 2016). Broadly screening and

sequencing positive samples may maximize the probability of detecting potential pathogens, for

groups that are likely to contain unexpected or undescribed species, such as *Crithidia*. A similar

approach uses species-specific primers coupled with broad-range primers, allowing for the

detection of unexpected species (Stevanovic et al., 2016; Szalanski et al., 2016; Tripodi et al.,

2018).

<u>Fungi</u>

Similar to other spore-producing pathogens, visual detection of microsporidian spores at

400x is common and spore intensities can be assessed in slide-mounted tissues, homogenized

gut samples, or feces smeared onto a hemocytometer (Human et al., 2013). The infective

spores are the most readily distinguishable life stage of the microsporidia, as vegetative

intracellular growth is cryptic and often undetectable by microscopy; however, methanol fixation

and Giemsa staining can reveal these growth stages within tissue (Fries et al., 2013). Spores of

most bee-infecting microsporidia species are highly refractive and approximately 5 µm long.

Levels of infection are quantified or categorized (*i.e.*, low infection when <2 spores, moderate

when 2-20 spores, and high infection >20 spores/ visual field) based on repeated spore counts

for multiple visual fields per smear viewed at 400x (Cordes et al., 2012; Human et al., 2013).

Distinguishing different species or even genera of microsporidia using light microscopy can

prove difficult as the gross morphology of spores is similar across the group, although species-

specific tissue pathology has been noted (Plischuk et al., 2015). PCR has higher resolution for

detecting and distinguishing different microsporidia species, and species-specific primers have

been developed for *V. apis, V. ceranae,* and *V. bombi* (Blaker et al., 2014; Erler et al., 2012; Graystock et al., 2020; Klee et al., 2006). Microscopy and PCR are often used in combination to maximize probability of detection while also assessing presence and intensity of sporulating infections, and are therefore complementary approaches (Blaker et al., 2014; Calhoun et al., 2021).

Entomopathogenic fungi with hyphal growth, such as chalkbrood (*Ascosphera* spp.), are uncommon in bumble bees and usually detected visually, based on the presence of hyphae in the abdominal cavity and the tissues of the alimentary tract (Kissinger et al., 2011; MacFarlane, 1976; Maxfield-Taylor et al., 2015). Chalkbrood produces visible hyphae that cover the bee carcass in late stages of infection, but this pathology has only been seen in larval infections of non-*Bombus* bees (Schmid-Hempel, 1998). Detection in bumble bees could include visual inspection via microscopy at low magnification (10–40x), examination of slide-mounted tissues at higher magnification (200–400x), culturing and isolation for morphological identification of reproductive structures, as well as molecular screening using broad-range or specific primer pairs (James & Skinner, 2005; MacFarlane, 1976; Maxfield-Taylor et al., 2015).

Nematodes

Due to their relatively large size, bumble-bee associated nematodes can be detected during dissections of the metasoma at low magnification (10–40x). *Sphaerularia bombi* is the most commonly encountered nematode parasite in bumble bees, although it is primarily restricted to queens (Alford, 1975; MacFarlane et al., 1995). The 8–20 mm long cucumber-like inverted uterus of a mature female in the abdomen is readily identified through dissection (Alford, 1969; Plischuk & Lange, 2012). Juveniles and eggs of *S. bombi* can also be detected in the feces of bees and quantified via a hemocytometer (Jones & Brown, 2014). Mermithids are rarely recorded parasitizing bumble bees, but are often large (*e.g.,* 46 mm in length) and easily detected during dissections at low magnification (Rao et al., 2017). The parasitic stages of

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mermithids lack the morphological characters to distinguish species, thus molecular characterization is recommended (Kubo et al., 2016; Tripodi & Strange, 2018).

Acarines

Tracheal mite presence is determined through visual examination of the metasomal air sacs under a dissecting microscope (Kissinger et al., 2011; Otterstatter & Whidden, 2004). Adult females are nearly spherical, about 450–550 μm across and are the most readily detectable stage, although eggs, males, and larviform females are typically 50–200 μm and usually apparent at low magnification as well (Husband & Sinha, 1970). Primers have also been developed for PCR-based detection of tracheal mites (Arismendi et al., 2016; Goka et al., 2001).

Mites on the exterior of bumble bees are not thought to pose a problem but can be detected upon visual examination of the thorax, propodeum, and tergites under low magnification (Kissinger et al., 2011). They can be common. A survey of 11 *Bombus* species in 15 sites in Ontario, Canada turned up 33 mite species, almost half of which are obligate to bumble bees, although not to particular species (Haas et al., 2019). Queens had the highest incidence, followed by males and then workers. The abundance and species richness of mites increased with local bee abundance. Surveys for mites in other bumble bee communities would be useful.

Dipterans

Detection of dipteran parasitoids has primarily occurred via visual techniques during dissection, but some can be reared to adulthood if allowed to remain in the body cavity while the flies complete their development (conopids: several months; phorids: 3 weeks: (Otterstatter et al., 2002)). Mature conopid larvae and pupae can be detected in the abdominal cavity of host bees without magnification due to the large size of the larvae (Malfi et al., 2014). Typically, however, dipteran larvae are detected during dissection at low magnification (10–40x) to ensure

detection of early instar larvae. Dipteran endoparasites must maintain a connection to the tracheal system of the host bee for respiration, so they are often associated with the abdominal air sacs. Identification of dipteran larvae can be challenging for non-specialists, and there are few keys available that can allow for genus or species identification, although family-level identification is relatively simple (McAlpine et al., 1981). Conopid larvae, pupae, and eggs, as well as adults, can be identified to genus using the keys developed by Smith & Peterson (1989), although many genera have been added to the family since the development of the larval key in the 1960s. Many adult species of conopids in North America can be identified by the keys of (Camras, 1996; Camras & Hurd, 1957). Adult phorids can be identified to genus with the key of (Peterson, 1989), and species within *Apocephalus* (*Mesophora*) can be identified with the key of (Brown, 1993). Phorid-specific PCR primers have been developed to detect molecularly internal parasites of bees. Detection of dipteran nest pests and ectoparasites of larval bumble bees, such as the Sarcophagids, would require inspection of the nests and opening nest cells. Family-level identification can be conducted with the adult and larval keys presented in McAlpine, et al. (1981), although lower-level classification would require specialized keys.

Hymenopterans

Syntretus wasps can be detected through dissection of adult bees to observe larvae or rearing larvae to adulthood in the carcass of adult hosts. The wasp larvae range in length from 1.8-4.3 mm, with the pupae measuring 2.2-3.1 mm long (Alford, 1968). Adult wasps found in and around bumble bee nests can be identified to genus using the key of Wharton, et al. (1997). Little work has been conducted on this genus in North America, therefore if found, identification to species is unlikely.

Melittobia wasps are small (1.0-1.5 mm) and the larvae develop cryptically within the pupal cells of their bumble bee hosts. Therefore, nest inspections using microscopy are generally used for detection, although simple visual inspection is adequate when large

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outbreaks of the wasps occur and adults are flying (Matthews et al., 2009). A key to genera of the subfamily Tetrastichinae and a list of North America species are available in LaSalle (1994), although *Bombus* are notably absent from the accompanying appendix of host lists. Keys for separating species of *Melittobia* are provided by Dahms (1984b). Identification to species is somewhat possible with adult wasps, particularly males, although there has been much taxonomic confusion in the genus and expert identification is warranted.

The nest parasite bumble bee species within the subgenus *Psithyrus* can easily be identified from adults using subgenus- and species-level keys for bumble bees (Koch et al., 2012; Mitchell, 1962; Thorp et al., 1983; Williams et al., 2014).

Coleopterans

Small hive beetles and *Antherophagus* beetles can be detected upon visual inspection of nests and nest debris. Descriptions of the distinguishing features of all life stages of small hive beetles may be found in (Neumann et al., 2013), along with molecular identification, nest inspection, and trapping techniques that can be easily modified for screening bumble bee colonies. Identification of *Antherophagus* to genus can be achieved with the key included in Bousquet (1989), but identification to species is unlikely with existing keys.

Lepidopterans

Nest-fouling moths can be detected upon visual inspection of nests and rearing facilities and through trap monitoring. Multiple means of monitoring the stored-product pest, *P. interpunctella,* are available, including sticky traps with and without pheromone attractants (Mohandass et al., 2007) and UV light traps. Once established, moths often destroy the nest entirely and thus early detection is essential for maintaining colony health (Kwon et al., 2003). There are nearly 5,000 species of pyralid moths and identification to species can be challenging (Solis, 2007). Adults of *Aphomia* spp. in North America can be identified in Solis & Metz (2008).

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The larvae of *Ephestia kuehniella* and *P. interpunctella,* can be identified with the key provided by Solis (2006).

4) The Clean Stock Program

<u>Uncertainty and known risks</u>

In many cases, the effects of parasites on individual and colony health, stability, and growth are unknown or at best, only partly known. Often, negative effects of these organisms at the colony level will only become apparent when colonies or individuals are experiencing other stressors (Brown et al., 2003). Because much of the experimental work documenting the effects of parasite infection has been in single species (largely either *B. impatiens* or *B. terrestris*), it is unclear how pathogenicity in one host translates to other species across the genus (Cameron & Sadd, 2020). Because there is little support in the literature for a safe level of most parasite-host systems, we recommend that a zero tolerance policy for parasite infections be implemented in commercial operations, such that any colony exhibiting symptoms or suspected symptoms be isolated and screened to determine the causal agent. Colonies exhibiting symptoms should never be shipped for commercial sale. Moreover, we recommend regular testing for different parasites to pinpoint any infections before symptoms appear and spread within the rearing facility, and before colonies are shipped for commercial operation where the managed bees could contaminate shared flowers leading to spillover into the community of wild bees.

Our understanding of pathology varies by parasite group, as do the screening techniques available. Virus levels that cause pathology remain largely unknown for bumble bees. For example, deformed wing virus has been found in bumble bees numerous times when no pathology is clearly evident (Dolezal et al., 2016; Gusachenko et al., 2020; Levitt et al., 2013; Li et al., 2011; McMahon et al., 2015; Singh et al., 2010). Likewise black queen cell virus seems

to be widely distributed among bumble bee species (Peng et al., 2011; Radzevičiūtė et al., 2017; Reynaldi et al., 2013; Sachman-Ruiz et al., 2015; Singh et al., 2010), but a specific pathology in commercial or research colonies of bumble bees is unknown. Complete elimination of viruses in rearing facilities is unlikely; however, reduction of viral load for all known viruses is important to produce disease-free bees. Pollen used in the rearing process, thus, should be brought in virus-free or sterilized appropriately (Simone-Finstrom et al., 2018) before bees are exposed to it.

We recognize that large-scale commercial rearing of biological organisms for agricultural use comes with a variety of risks, both known and unknown. Risk mitigation is most successful when risks are enumerated prior to appearing and managed. However, not all risks to commercial bumble bee production can be known *a priori.* There are known risks about which little is known, and there are undoubtedly unknown or uninterpreted risks. For example, producers know several management techniques to ensure year-round production of bumble bees for commercial pollination service (e.g., Röseler, 1985). The research and conservation community knows which species are used, where they can be shipped, and several of the management strategies employed. We also know that we do not know other proprietary business and management strategies these companies employ, such as the number of colonies that are shipped annually, nor sanitation and sterilization strategies for bee feed, nesting material, and equipment. These are exactly the items that need to be discussed to ensure clean stock production. Further, there are likely issues related to health and sanitation of which we are unaware, particularly for emergent diseases. The protection of the proprietary nature of these production processes places wild pollinators and crop producers at some risk and the historical reluctance of commercial bumble bee producers to share this information may in fact be creating concern in the conservation, regulatory, and scientific communities where it is not needed. Thus channels for communication among producers, consumers, conservationists,

regulators, and scientists should be cultivated so that commerce can proceed and the health of wild populations of bees can be ensured simultaneously.

Known risks to wild and managed pollinators include the escape of managed bumble bee species from containment and pathogen spread among bee communities. It is now well known and documented that commercial bumble bees escape from containment and establish in new regions (Matsumura et al., 2004; Morales et al., 2013; Roig-Alsina & Aizen, 1996) including in North America (Looney et al., 2019; Ratti & Colla, 2010) and South America (Aizen et al., 2018). The degree to which alien commercial bees outcompete conspecifics is not yet fully understood (Ings et al., 2006), but concerns exist (Aizen et al., 2018), and evidence suggests that genetic introgression (Kondo et al., 2009) and transport of parasites and spillover onto wild bees can occur (Alger et al., 2019; Colla et al., 2006; Goka et al., 2001; Maharramov et al., 2013; Purkiss & Lach, 2019; Schmid-Hempel et al., 2014). Despite knowing that these risks exist, the degree to which they might impact agriculture in various parts of North America with differing climates, cultural practices, and agricultural systems is unknown. Further, while we have some knowledge of the impacts that commercial bumble bees have on native bumble bees, there is little knowledge of the impacts these bees might have on honey bees or other managed and wild pollinators. Ensuring both clean stock protocols and access to production and sales records would lessen the degree of uncertainty in this system and would allow for robust contact tracing and containment should releases or disease outbreaks occur.

A clean stock certification program would decrease the levels of uncertainty that exist around commercial bumble bee health. To address these issues and mitigate risk to native bees and commercial honey bee and bumble bee pollinators, a strong commitment by commercial producers to broader pollinator health is needed. We are heartened by the cooperative approach taken in the North American Pollinator Protection Campaign Imperiled Bombus Task Force by members of the industry and want to increase this dialogue. A voluntary and transparent clean stock program that emphasizes the common interests of commercial

producers and the pollinator conservation community would address many of the concerns surrounding bumble bee health. For example, 1.) processes of pollen sterilization could be made available, 2.) sanitation processes could be published, 3.) records of shipments could be made available, 4.) a culture of openness should be cultivated in areas of business operations that impact the community health of bees, and 5.) a voluntary annual inspection of rearing facilities by a clean stock certification group would also accomplish the goals for transparency, disease suppression, and wild bee conservation. Industry standards developed for vertebrate livestock such as the Animal Disease Traceability General Standards (Animal and Plant Health Inspection Service, 2019) and the NLRAD System Standards (Animal and Plant Health Inspection Service, 2020) could serve as models for the industry and conservation and scientific partners to construct a clean stock certification and tracking program.

Clean stock program components

A clean stock program to ensure healthy colonies are available to support agricultural needs, while simultaneously protecting wild and managed bee populations, should include the following Best Management Practices:

1) Screening and detection

2) Quarantine and isolation

3) Sanitation and prevention

4) Treatment

5) Forensic (tracing) capacity

The following controls are critical processes for the success of a clean stock program to prevent the spread of disease within facilities and to prevent spillover of disease-causing agents to wild bee communities. Producers seeking certification should maintain a written, publicly accessible protocol of processes related to production of clean stock, including a strong commitment to

transparency in production processes. Employees should receive annual training in disease prevention and containment. Companies seeking certification should have an annual review and/or inspection of facilities to ensure compliance.

<u>Screening and Detection</u>

Screening for disease in rearing facilities would ideally involve a two-tiered system that is integrated into a system of quarantine and isolation. The first tier is visual inspection of colonies throughout the rearing facility that is aimed at detecting symptomatic infections. The second tier is testing asymptomatic colonies to detect latent disease spread before symptoms appear. The first tier would involve all colonies that are maintained in the production facility and would occur regularly when colonies are fed, moved from starter to full colony boxes, and before shipment or transfer to gyne production lines. Visual screening for symptoms would include, but not be limited to, looking for lethargic bees, trembling or shivering, deformed wings or legs, unusual patterns of defecation and odd odors, failure to thrive, ejected larvae, and other unusual behavior. Identification of symptoms of infection in the first tier should be followed up using appropriate visual or molecular approaches, as outlined previously, to verify causative agents.

The second tier of testing would employ random testing to detect the presence of pathogens that are not yet inducing symptoms. The exact program for testing could vary, but at a minimum should include testing a random subset of all colonies in a rearing facility (Huang et al., 2015) using non-specific Trypanosomatid (*e.g.*, *Crithidia* spp.) and Microsporidian (*e.g.*, *Vairimorpha* spp.) PCR primers, as well as primers specific for *Apicystis bombi*. Bumble bee colonies that test positive for Trypanosomatid or Microsporidian pathogens using general primers should be examined microscopically and with species specific primers in subsequent PCR reactions to verify the causative agent. Colonies should be selected randomly from throughout the rearing facility with colonies of various ages being inspected weekly. A stratified sampling scheme should be employed to select equal numbers of colonies that are two weeks

from shipment, and a month from shipment, etc. Colony-level tests should include one non-callow worker or male bumble bee from each colony designated for testing each week. Broad testing of many colonies is desired for the random tests, but in-depth testing of colonies with disease symptoms is covered below. When a positive test result occurs, tests on individual colony samples should proceed within 24 hours and colonies with individual positive results should be moved to isolation until destroyed. If an outbreak is detected, testing frequency and intensity should be intensified in spatially and/or lineage associated colonies for a one month period. The random stratified testing should be supplemented with a pre-sale test of all colonies one week prior to shipping; a pooled testing strategy could be used to test for these pathogens and would allow for this high coverage sampling with low costs.

Quarantine and isolation

Any new stock brought into the facility should undergo a period of quarantine and testing before integration of that stock into the rearing operation. New queen stock should be kept isolated from main production lines until a full cycle of offspring is produced, observed for two weeks for symptoms, and be tested for the primary disease agents using PCR detection protocols. Longer periods of isolation would increase confidence in disease-free status, but may not be necessary. New colonies that are brought into rearing facilities should first be tested with PCR by subsampling 5% of adult bees and then be observed for two weeks and retested before integration into main production areas.

For main production and breeding lines in production, a colony with any disease symptoms should immediately be isolated from other colonies and tested for known pathologies. Ten workers from colonies with symptoms should be used for detection of known pathogens of concern. Recognizing that abiotic factors can cause pathology, colonies isolated with symptoms could be returned to production assuming that the causative agent for symptoms is determined and two weeks elapse from the resolution of symptoms and detectable infection. Colonies or

individuals that are placed in isolation that test negative for known pathogens should likewise be held for two weeks after resolution of symptoms to ensure that a novel pathogen is not involved.

If workers from the colonies test positive for *V. bombi*, *V. ceranae*, *C. bombi*, *C. expoeki* or *A. bombi*, they should not be sold and should be destroyed to prevent disease spread within the facility. Destruction by freezing is recommended and a sample of ten workers and a portion of the brood comb and pollen should be held frozen for forensic purposes (see below). Infected biological and related material should be kept isolated at all times and disposed of in accordance with local biohazard regulations. At a minimum, material should be contained in two sealed plastic bags, one inside the other, until incineration or fumigation.

Sanitation and prevention

Reducing pathogen levels in facilities should be accomplished through production controls including facility construction and materials management. For example, wild bumble bees should be prohibited from entering a production facility using double entrance doors, screening of ventilation ducts and maintaining positive air pressure in the buildings. Bees that are brought in intentionally to augment production or breeding stock should be processed through a self-imposed company quarantine. These bees must remain separate from production colonies until health can be verified. Equipment and shelving in facilities should be constructed of material that can be easily cleaned and sterilized or is disposable. Construction is ideally concrete and steel with floor drains for ease in cleaning. Colonies in production should be housed in plastic boxes that are either new shipped from the facility at sale or that have been cleaned and sterilized. Other nesting materials (cotton, wax, pollen, etc.) should not be reused.

To reduce pathogen exposure from feeding, food sources should be carefully controlled. Carbohydrate sources are proprietary; importantly, however, we do not know of any production facility that utilizes unsterilized sugar sources for feeding. Unpasteurized honey should not be used, but rather mixtures of sucrose, glucose, and fructose may be manufactured to optimize

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production and antimicrobial preservatives added. In addition to sterile sugar sources, some companies choose to sterilize pollen before it enters the facility. To date, gamma irradiation has been shown to reduce the viability of pathogens in pollen (Graystock et al., 2016) and does not severely reduce nutritive value (Yook et al., 1998), and any sterilization method that significantly reduces pathogen loads in bee feed would be a best management practice under a clean stock program. Equipment used for feeding should either not be shared among colonies or cleaned or sterilized between use in individual colonies. Processes that minimize the need for moving equipment between colonies are optimal.

Currently, best disease management and pest control strategies involve cultural control of outbreaks in rearing facilities. Primary importance should be focused on rapid identification of disease outbreaks, proper disposal of infected hives, and thorough equipment cleaning practices to reduce disease transmission between colonies (Huang et al., 2015). Hand sanitation of facility workers moving from colony to colony is also necessary to reduce pathogen transmission.

Treatments

Treatment is generally implemented when prevention fails. Currently, very few treatments are readily available for the control and management of pathogens and pests within bumble bee colonies. Therefore, at this time we recommend destroying any infected colonies to reduce the likelihood of an outbreak in the rearing facility and wherever the bees are shipped. However, we provide a list of known or potential preventative measures and possible treatments as these may become useful tools in the future.

Monitoring and treatment methods exist for insect pests that infest rearing facilities. *Bacillus thuringiensis* can be used to control for wax moths (*Galleria mellonella* and *Achroia grisella*) (Burges & Bailey, 1968) and Indian meal moths (*Plodia interpunctella*) (McGaughey, 1976). Bait and pheromone traps also exist for monitoring and controlling wax moths and Indian

meal moths. Fruit flies can also be a nuisance in bumble bee rearing facilities; fly paper and bait traps are used to control them as outbreaks occur.

Chemical and biological controls of microbial pathogens in rearing facilities are not well developed. While fumagillin is used as a treatment for *V. apis* in honey bees, it is not effective against *V. bombi* in bumble bee colonies (Whittington & Winston, 2003); thus, sanitation of equipment and isolation of infected colonies is necessary when *V. bombi* is detected in rearing facilities. Research has revealed several promising treatments for potential development. Several studies have found that secondary metabolites found in nectar (particularly alkaloids) can help reduce parasite loads (specifically *C. bombi*) in bumble bees and nectar containing alkaloids is preferentially chosen by bees if they are infected (Baracchi et al., 2015; Biller et al., 2015; Manson et al., 2010; Richardson et al., 2015). These studies have also demonstrated that secondary metabolites can present some negative side effects to the bees, as well. Moreover, there is growing evidence that sunflower pollen consistently reduces *C. bombi* infections in *B. impatiens*, both in the lab and in the field (Fowler et al., 2020; Giacomini et al., 2018; Locascio et al., 2019), further highlighting the potential medicinal properties of different pollen types. Further testing of these treatments is needed to determine their effectiveness as medicine for bumble bee colonies.

Forensic and reporting capacity

Forensic capacity has several components, including the ability to identify the causative agents of disease, the sources of outbreaks, and tracing contact of contagious individuals with healthy bees. Because disease can present after the colonies have shipped from rearing facilities, tracking records should be maintained with unique colony identifiers for two years after shipment. Rearing operations should maintain a database of these shipments including date shipped, destination, shipping origins and inspection data, and other information that could assist in tracing a disease outbreak to its origin.

Samples of diseased material from rearing facilities should be kept frozen at -20°C for a period of two years after the colony is destroyed. As described above, a sample of 10 bees (if available) and a portion of the brood comb and pollen from diseased colonies should be stored frozen and made available to research and regulatory groups requesting access. The remaining material from diseased nests should be destroyed, preferably through incineration, or fumigation and containment in two sealed plastic bags before disposal at a landfill.

We recommend that a clean stock program includes a database of shipment information that is maintained in-house by commercial producers and made accessible to federal, provincial, and state regulatory agencies (*e.g.*, USDA-APHIS, CFIA). Ideally, the database would be tri-national and include Canada, Mexico and the USA and could provide summary data on colony production numbers and shipment destinations to the public upon request. Data should be available quickly, protect the privacy of the production companies and end users, and be detailed enough to address problems as they arise.

5) Concluding recommendations

This white paper is, in part, a response to the current lack of knowledge of industry standards among the broader bee conservation and scientific community that has arisen from past reticence to share production and shipping details. We emphasize the need to align bumble bee commercial practices with pollinator health goals stated in the National Strategy on Pollinators in the Pollinator Research Action Plan (2015) and biosecurity goals stated in the National Strategy for Biosurveillance (2012). Because previous declines in bumble bee populations in North America occurred after commercial disease outbreaks (Flanders et al. 2003), and *Vairimorpha bombi* has been potentially linked to decline status (Cameron et al. 2011), which implicates the commercial population crashes, it is critical to build trust in the system among producers, end users, and the conservation community. Adoption of a clean stock program, based on the best available science, adaptable to new threats, responsive to

changes in data, and with greater information flow, would be a strong step to recovering trust among communities and toward meeting the goals for agricultural biosecurity outlined in the National Strategy for Biosurveillance (2012).

We acknowledge that the implementation of a clean stock program could occur at many levels: municipal, state, federal, or international; however, it is most likely and perhaps most manageable for industry standards to be adopted with a third-party oversight or certification of the process, such as the dairy certifications by the Farmers Assuring Responsible Management program. Whereas many agriculturally produced products including plant material (e.g., Certified Seed programs, Federal Seed Act) and livestock (e.g., Animal Disease Traceability) are subject to federal regulations, the production, sale, and transport of pollinators has largely avoided regulation in North America. While movement of bees across international borders allows for certain regulatory requirements, there is not a unified set of state regulations in the United States or among Canadian provinces, so industry standards could alleviate the need for additional regulation. In addition to adopting disease and pest control measures, reporting of sales and distribution numbers on a state-by-state (province-by-province) basis would allow regulators, wildlife managers, and scientists to respond appropriately to disease outbreaks in wild populations around commercial facilities. Additionally, a clean stock program could help ensure the future health of commercial bumble bee populations in rearing facilities and avoid the collapse of commercial populations. The assurances of clean stock to conservation organizations and government wildlife managers are especially critical to states and provinces with declining bumble bee populations, such as *B. affinis,* the Rusty Patched Bumble Bee, where Endangered Species Act protections might complicate sales of bumble bees.

List of priority actions:

1. **A meeting with USDA, the lead authors, and representatives of the North American Pollinator Protection Campaign (NAPPC) including commercial bumble bee production representatives, bee conservation community partners, bumble bee scientists, and potentially agency representatives from Canada, and Mexico to discuss common interests and needs and to develop components of a bumble bee clean stock certification and oversight. The agenda for this meeting would include:**

   a. **Standards of clean stock certification program**

   b. **Shipment reporting and tracking**

   c. **Program management, implementation, and oversight**

   d. **Mitigating impacts to federally listed at-risk species, including identifying potentially deleterious pathogens and parasites**

2. *An effort coordinated by the North American Pollinator Protection Campaign* **(NAPPC) (aligned with its Imperiled Bombus Task Force) to discuss critical research needed to support disease detection and treatment for an economically and ecologically sustainable clean stock program.**

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Appendix A: Symbiont list identifying potentially deleterious symbionts of concern for clean stock and commercial bumble bee rearing

The list has been sorted into two groupings based on the concern of the symbiont's presence in rearing operations: Priority/Of Concern and High Uncertainty but Potential, with select evidence presented in the main text summarized here. It is important to note that this is likely not a fully inclusive list, given current unknowns, and new symbionts of concern are likely to arise. Any clean stock program should visually monitor for problematic symptoms in individuals and colonies and verify causative agents.

Priority/Of Concern

Symbiont	Evidence
Acari	
Locustacurus buchneri Stammer; Podapolipidae	Reduced foraging, lethargy (Husband & Sinha, 1970); Reduced longevity (Otterstatter & Whidden, 2004)
Insecta	
Aethina tumida Murray; Coleoptera: Nitidulidae	Considerable damage of colony structure from small initial infestations (Ambrose et al., 2000); Soil requirements for pupation (Cuthbertson et al., 2013) may limit outbreaks but also infestation of commercial colonies in field (Spiewok & Neumann, 2006)
Melittobia spp. Westwood; Hymenoptera: Eulophidae	High reproductive capacity (Whitfield & Cameron, 1993); Moved via commerce (Matthews et al., 2009); High economic damage in rearing facilities (de Wael et al., 1995)
Plodia interpunctella Hübner; Lepidoptera: Pyralidae	Feeds on pollen stores, dead individuals, and other nest material (Williams, 1997); Rapid population growth and destructive outbreaks in rearing facilities (An et al., 2007)
Protozoa	
Apicystis bombi Liu, MacFarlane, and Pengelly; Ophrocystidae	High virulence (Jones & Brown, 2014; Rutrecht & Brown, 2008); Potential for spillover (Graystock, Yates, Evison et al., 2013)
Crithidia bombi Lipa and Triggiani; Trypanosomatidae	Virulence context dependent, but may be high (Sadd & Barribeau, 2013); Easily transmitted and has been common in commercial colonies (Graystock, Yates, Evison et al., 2013; Murray et al., 2013); High spillover potential (Colla et al., 2006)

Crithidia expoeki Schmid-Hempel and Tognazzo; Trypanosomatidae	Limited study, but assumed like *C. bombi*
Fungi	
Vairimorpha (Nosema) bombi Fantham and Porter; Microsporidia: Nosematidae	High virulence (Otti & Schmid-Hempel, 2007; Otti & Schmid-Hempel, 2008); Found in commercial distributed colonies (Graystock, Yates, Evison et al., 2013; Murray et al., 2013)
Vairimorpha (Nosema) ceranae Higes et al.; Microsporidia: Nosematidae	Bumble bees susceptible, increases mortality (Graystock, Yates, Darvill et al., 2013)
Viruses	
Deformed wing virus (DWV); Iflaviridae	Replication in bumble bees (Levitt et al., 2013; Li et al., 2011); Pathology in commercially reared bumble bees (Genersch et al., 2006)
AKI-complex: Kashmir bee virus (KBV); *Acute Bee Paralysis Virus* (ABPV), *Kashmir Bee Virus* (KBV), *Israeli Acute Paralysis Virus* (IAPV) Dicistroviridae	Infective to bumble bees, long-lasting infectivity (Bailey & Gibbs, 1964); Can be common in bumble bees (McMahon et al., 2015); Virulence may be dose and transmission route dependent, but can be high (Meeus et al., 2014; Niu et al., 2016; Wang et al., 2018)

High Uncertainty but Potential

Symbiont	Evidence
Insecta	
Aphomia sociella Linnaeus; Lepidoptera: Pyralidae	Feed on nest material and brood (Frison, 1926; Goulson et al., 2002) on pollen provisions (Schmid-Hempel, 2001); Considered similar outbreak potential as *Plodia interpunctella*.
Ephestia kuehniella Zeller; Lepidoptera: Pyralidae	Feeds on pollen provisions (Schmid-Hempel, 2001); Considered similar outbreak potential as *Plodia interpunctella*.
Galleria mellonella Linnaeus; Lepidoptera: Pyralidae	Can be destructive (Oertel, 1963); Favors honey bee colonies (Whitfield & Cameron, 1993)
Vitula spp. Ragonot; Lepidoptera: Pyralidae	Feed on wax, pollen and other nest materials (Frison, 1926); Infest bumble bee colonies (Whitfield & Cameron, 1993)
Bacteria	
Spiroplasma apis Mouches et al.; Mollicutes: Spiroplasmataceae	Disease association in honey bees and presence of bacteria in bumble bees indicates pathogenic potential, but not verified (Clark et al., 1985; Meeus et al., 2012)

Spiroplasma melliferum Clark et al.; Mollicutes: Spiroplamataceae	Associated with disease in honey bees and presence of bacteria in bumble bees indicates pathogenic potential, but not verified (Clark et al., 1985; Meeus et al., 2012)
Viruses	
Black queen cell virus (BQCV); *Chronic Bee Paralysis Virus* (CBPV); *Cloudy Wing Virus* (CWV); *Sacbrood virus* (SBV); *Slow Bee Paralysis Virus* (SBPV); Other "honey bee" RNA viruses	Found in bumble bees, including in commercial colonies (Choi et al., 2010; McMahon et al., 2015; Peng et al., 2011; Sachman-Ruiz et al., 2015; Singh et al., 2010); Need study to further understand occurrence and virulence in bumble bees (Tehel et al., 2016)
Fungi	
Tubulinosema pampeana Plischuk et al.; Microsporidia; Tubulinosematidae	Detected in bumble bees, affecting adipose tissue (Plischuk et al., 2015, 2017); Currently further pathology and effects unknown