Pollen as a potential vector for *Nosema* microsporidia

By
Piper Langdon

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Approved:	Dr. Susan Bjørnson Supervisor Professor
Approved:	Dr. David Dansereau Co-supervisor Senior Lecturer
Approved:	Dr. Genlou Sun Reader Professor
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Abstract

In recent years, pollinators have been experiencing a significant decline. Of particular note are bees such as honeybees (*Apis* spp.) and bumblebees (*Bombus* spp.) which provide essential pollination as both natural and managed pollinators. Among other suggested causes for the decline, such as tracheal mites, microsporidia may be responsible for the increased colony loss seen in bees. Microsporidia are eukaryotic intracellular parasites that are known to infect many invertebrates including bees, in which the pathogen causes many detrimental effects. *Nosema* microsporidia, a genus that includes bee-specific species like *Nosema apis* and *Nosema cerenae* may be of increased importance to the decline of these pollinators. Microsporidia can be transmitted between individuals by direct contact or indirectly via infected feces. In addition to these transmission routes, the collection of raw pollen from flowers by foraging bees may be a further means of transmission. Unpasteurized pollen samples from three sources were examined for the presence of *Nosema* microsporidia. Samples were boiled to release the genetic material from the spores, which was then amplified using primers targeting a ~1230bp region of the SSU rRNA genes of the microsporidia. Amplification of the DNA was inconsistent, with 1/9 of the infected larvae and 5/9 of the infected larvae in pollen giving a band for the presence of *Nosema* microsporidia. Although no extractions from the pollen tested positive for *Nosema* microsporidia, due to the inconsistency of the extraction the presence of the pathogen cannot be determined conclusively. Other extraction methods such as the use of enzymes or chemical treatments may provide more precise results, and thus would allow for the status of pollen as a vector of microsporidia to be known.

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INTRODUCTION

1.1 Bee Decline

Insect pollinators serve an important role in agriculture; however, these insects have been experiencing a decline in recent years (Oldroyd 2007). Pollinator insects include honeybees (*Apis* spp.) and bumblebees (*Bombus* spp.); however, other species such as mason bees (*Osmia* spp.) and leaf-cutting bees (*Megachile* spp.) are also important pollinator species (Artz and Nault 2011). Approximately 35% of global crop production is reliant on pollinators (Klein 2007) that are either part of the natural environment or brought into a region to aid in crop pollination when native pollinators are not abundant (Frier et al. 2016). For example, managed pollinators are essential to the almond industry in California, which relies upon the import of 1.9 million western honeybee, *Apis mellifera* L., colonies into the region each year (Pitts-Singer et al. 2018). Without these managed honeybee colonies, the almond industry of California would not be able to produce the quantity of almonds that it currently does, which is over 80% of the world's almonds (Pitts-Singer et al. 2018). *A. mellifera* is the most common managed pollinator species; however, they are not always effective pollinators and often work better alongside native pollinators (Frier et al. 2016).

Despite their ecological and economic importance, *A. mellifera* have experienced population decline in recent years. While annual colony losses of approximately 10% are considered normal, losses of up to 80-100% of managed *A. mellifera* colonies have been reported in some areas of the United States (Oldroyd 2007), with overall colony losses for the United States averaging around 25-30% per year (Holt and Grozinger 2016).

Not only are managed bees experiencing decline, but native bees are as well. These native pollinators often share an environment and face the same risks that managed pollinators do. Localized populations of native pollinators are facing declines in recent years, the extent to which varies by region (Potts et al. 2010). A 1993 study in California concluded that 75% of native *A. mellifera* hives had been lost, although some regions saw little change in native pollinator populations (Natural Research Council 2006).

The reason for pollinator decline is unknown. Although tracheal mites were suggested as a cause (Natural Research Council 2006), other factors have been suspected to contribute to pollinator decline, including habitat loss, pesticides and pathogens. Microsporidia are known pathogens of many bee species, both managed and native (Koch et al. 2017; Paris et al. 2018).

1.2 Microsporidia as a pathogen of bees

Microsporidia are known to infect many pest and beneficial insect species, including native and managed bees. *Nosema bombycis*, the first described microsporidium, was described in 1857. This pathogen causes pébrine in silkworms, a disease that prevents the larvae from producing silk (Franzen 2008). Other pathogens of the genus *Nosema*, which contains approximately 80 species (Kirk et al. 2008) infect many beneficial insects and cause a wide range of detrimental effects. For example, *Nosema adaliae* has detrimental, although not lethal, effects on the lady beetle *Adalia bipunctata* L., which may be problematic for its successful use in biological control programs (Steele and Bjørnson 2014).

Nosema ceranae and *Nosema apis* are common species of microsporidia that infect bees. The symptoms and environmental tolerances of these two species vary, with

high *N. ceranae* prevalence in temperate climates and *N. apis* prevalence peaking in the colder months. Both species infect honeybee colonies and are thought to be a cause of population decline (Soklič and Gregorc 2016). In 2016, 39-60% of all *A. mellifera* colonies in Canada were infected with *Nosema* microsporidia, with *N. ceranae* occurring three to five times more often than *N. apis* (Emsen et al. 2016). However, *N. ceranae* was not found in *A. mellifera* in Canada until 2008 (Williams et al. 2008), and this shows that microsporidiosis is quickly becoming an increasingly significant problem for these pollinators.

Nosema microsporidia have been detected in wild populations of bees as well. *N. ceranae* was found in the majority of wild *Bombus* spp. populations surveyed in Argentina (Bravi et al. 2019), which shows that wild populations can become infected. Spillover between managed and wild populations of bees may play a role in the spread of microsporidiosis (Koch et al. 2017).

Phylum Microsporidia is comprised of a group of eukaryotic, obligate intracellular pathogens that are highly specialized for infection (Keeling and Fast 2002). Despite being eukaryotic, microsporidia lack many features that are typical of eukaryotic cells. Highly reduced in their morphology and ultrastructure, microsporidia lack mitochondria, peroxisomes, and a stacked Golgi apparatus (Vávra and Larsson 2014). As a group, their 70S ribosomes are prokaryotic in size and they have 16S and 23S subunits but lack a separate 5.8S subunit (Dong et al. 2010).

The phylum Microsporidia contains almost 190 genera, and more than 1300 species. These spore-forming pathogens infect a wide range of invertebrate and vertebrate hosts (Vávra and Lukeš 2013). Microsporidian spores have a thick endospore and

exospore layers that contain chitin, and these layers provide the spore with some protection from UV light and unfavourable environmental conditions such as high temperature (Zeng et al. 2011). The spore is the only stage of the pathogen that is able to survive outside of a host (Vávra and Larsson 2014) and these highly resistant, chitinous spores allow microsporidia to persist in a wide range of environments until a susceptible host is available. Microsporidian spores range in size from 1μm to 30μm, although the majority fall on the smaller end of this size range (Vávra and Larsson 2014).

Microsporidian spores have been shown to remain infectious after complete desiccation, freezing, and after long periods of time have lapsed, with some species remaining viable for at least two years (Didier et al. 2004). Microsporidia can also persist for prolonged periods by infecting host organisms. For example, *Paranosema locustae*, a microsporidian that has been used for biological control of grasshoppers, can spread throughout an ecosystem and remain within localized populations for at least 11 years (Lange and Azzaro 2008).

All microsporidian spores have a polar tube, a unique organelle that is characteristic of the group, and contain an infective stage (‘sporoplam’) of the pathogen. The polar tube is coiled tightly within the microsporidian spore, where it is attached to the anterior region by an anchoring disk (Yang et al. 2018). Infection occurs through the eversion of this polar tube. When conditions are favourable for germination, the polar tube everts and it is through this hollow tube that the infective sporoplasm is transferred into the host cell (Vávra and Larsson 2014). The conditions that initiate germination are dependent on the species of microsporidia and its environment, and include factors such as environmental pH, moisture, and specific ion concentrations (Xu and Weiss 2005).

1.3 Transmission of microsporidia

Pathogens, regardless of whether they are bacteria, fungi, or microsporidia, can pose a threat to pollinator populations. In particular, microsporidia may be responsible for the decline of bees that has been seen in recent years.

Microsporidian spores are transmitted both horizontally (between cohorts) and vertically (from parent to offspring). Although horizontal transmission often occurs through the contamination of food or water, there are other means by which microsporidian spores are transferred horizontally, including through certain bee behaviours, such as self-grooming (Holt and Grozinger 2016). The mode of vertical transmission varies with the species of microsporidia. *N. ceranae* spores are transmitted from male honeybees to the queen during insemination; however, while the infection develops within the queen, the pathogen is not transmitted to her eggs (Roberts et al. 2015). In other insects such as the two-spotted lady beetle, *A. bipunctata*, microsporidia are transmitted vertically (Steele and Bjørnson 2014).

Horizontal transmission is common among insects but the potential for horizontal transmission depends on both the species of microsporidia and the species of the host. The primary means of horizontal transmission in honeybees is through the fecal-oral route, whereby susceptible individuals become infected after they have consumed food or water that has been contaminated with microsporidia-infected feces (Holt and Grozinger 2016). Another potential horizontal transmission route may involve self-grooming, a behaviour which is used by bees to clean themselves. During grooming, bees may ingest spores that have been picked up through casual contact with other individuals or from the environment. Furthermore, transmission of microsporidia from infected individuals to

uninfected ones through trophallaxis (the act of transmitting food or fluids between individuals) is common in social invertebrates (Paris et al. 2018).

In some cases, the symptoms of microsporidiosis aid in the horizontal transmission of the infection. *N. apis* causes diarrhea in the honeybees (Holt and Grozinger 2016), and as spores are released in the feces, diarrhea exacerbates the transmission of microsporidia within a hive. However, the spread of microsporidia is not always encouraged by such symptoms. In contrast to *N. apis*, *N. ceranae* does not cause diarrhea. In the latter case, it is thought that the spores are transmitted primarily via trophallaxis (Smith 2012). Although *N. ceranae* spores are not propagated by diarrhea, fecal-oral transmission may still occur.

The many routes of transmission of microsporidia, combined with the persistence of the spore, allow microsporidia to persist for relatively prolonged periods and to infect a wide range of susceptible hosts. To better understand the transmission of microsporidia, it is important to investigate potential vectors that could carry the pathogen.

1.4 Pollen as a means of vectoring microsporidian pathogens

In addition to the aforementioned methods of transmission, pollen may serve as a potential vector of microsporidia in the local environment. While flowers have been suggested as a potential source of microsporidia for bees (Koch et al. 2017), the pollen of these flowers has not been considered or examined as a potential means of horizontal transmission. An examination of pollen, particularly from flowers that have been visited by insect pollinators, may provide some insights on how microsporidia are transmitted among insect populations in the local environment.

Pollen is an important food source for honeybees, providing them with protein, lipids, and other nutrients. Pollen is collected in the corbiculae (pollen baskets) of worker bees as they forage among flowers (Higes et al. 2008). A single worker bee consumes approximately 3.4 to 4.3 g of pollen per day whereas a 10-frame colony of honeybees consumes 13.4 to 17.8 kg annually (Crailsheim et al. 1992). Pollen collected from flowers is packed into the corbiculae and taken back to the hive. As a result, any pathogens which are present in the pollen are also brought into the hive, where they may infect susceptible individuals.

As eusocial insects, bees show age-based polyethism, whereby younger individuals act as nurse bees to care for the developing brood, while older individuals are workers who forage for food (Robinson 1987). When compared to nurse bees that are confined within the colony, forager honeybees have a higher rate of *N. ceranae* infection, which suggests that foraging bees are encountering spores in the external environment (Jack et al. 2016). However, these foraging honeybees may have a higher rate of microsporidiosis because microsporidia-infected bees begin foraging earlier than uninfected bees, and those infected with *N. ceranae* are twice as likely to forage early (Goblirsch et al. 2013), and not because they have encountered microsporidia in the environment.

In one study, *N. ceranae* was found in 95% of the pollen from honeybee corbiculae, and these spores were infective (Higes et al. 2008). Although it is possible that the pollen became contaminated with *N. ceranae* spores when the pollen was groomed into the corbiculae, it is also possible that the spores were on the pollen before the bee arrived at the flower.

A 2018 study that focused on contamination of commercial bee products, such as honey, royal jelly, and pollen of Brazil, reported that 87.80% and 26.83% of the pollen samples analyzed had *N. ceranae* and *N. apis* spores, respectively (Teixeira et al.). Other pathogens were examined during this study, including the bacterium *Paenibacillus larvae* (the causal agent for American foul brood) and fungus *Ascosphaera apis* (the causal agent of chalkbrood), but the pollen was not examined for other *Nosema* species. Microsporidian spores may be present in pollen, and may be transmitted horizontally when pollen is collected by honeybees in the field.

Microsporidia pose a significant risk to the health and efficacy of insect pollinators; therefore, it is crucial to understand the transmission routes of these pathogens in the natural environment. The objective of this study is to determine whether raw pollen collected by honeybee foragers can serve as a means of transmission of microsporidia. Further understanding of how microsporidia are transmitted among insects in nature will allow us to better understand the transmission routes of microsporidia, and the factors that may promote disease epizootics among insects in nature.

MATERIALS AND METHODS

1.1 Rearing of infected *Adalia bipunctata*

Laboratory-reared *Adalia bipunctata* stock beetles infected with *Nosema adaliae* were used to produce control larvae for the *Nosema* microsporidia. Siblings of the infected larvae that were used as controls had their infection status confirmed by light microscopy. *A. bipunctata* larvae reared for this trial were provided a constant supply of diet, a 50:50 ratio of organic honey and pollen (Lacewing and Ladybug Food, Planet Natural, MT),

and were provided green peach aphids (*Myzus persicae* Sulzer) once a day. Aphids were raised on nasturtium (*Tropaeolum minus* L., Dwarf Jewel Mix, Strokes Seed Ltd., ON). Larvae were provided water daily through a moistened cotton wick. Both mating adult pairs and larvae were reared in environmental chambers (Sanyo 350H) under controlled conditions (16L:8D; 25°C:20°C). Prior to DNA extraction, larvae were starved of aphids for 24 hours to reduce the effect of residual aphid DNA in the DNA extraction.

1.2 Extraction of DNA

Positive and negative controls were prepared by extracting DNA from *A. bipunctata* larvae that had been confirmed to be infected with *N. adaliae* or to be uninfected, respectively. The positive control of *Nosema adaliae* that was obtained from a larva that was cleaned using Monarch® PCR & DNA Cleanup Kit, 5 μg (New England Biolabs, MA). The product was sent to TCAG Genomics (ON) where the pathogen was confirmed to be *Nosema adaliae.* The negative control was obtained from an *A. bipunctata* larva that was not infected by *Nosema adaliae*. The same positive and negative control were used in all trials. In addition to the two controls, DNA from two larvae that were known to be infected with *N. adaliae* was extracted for each trial to provide confirmation that the DNA extraction had worked. The first of the new larvae were treated identically to that of the positive control, whereas the second larva had a pollen grain added to it prior to extraction to simulate the effect of pollen on the extraction. These two extractions were carried out independently for each new trial.

Individual larvae were prepared for extraction by macerating them in 200 uL of sterile water. A 1.5 mL microcentrifuge pestle (Fisherbrand, ON) was used to grind the larval tissue to ensure that microsporidian spores were released into the water.

Samples of pollen were taken from three brands of non-pasteurized (raw) commercial bee pollen, each from separate geographical regions: British Columbia, Canada (BC Buzz, BC), Ontario, Canada (Charlie-Bee, ON), and Greece (Ebion Bee Pollen, Greece). Pollen samples were prepared by adding one pollen grain to a 1.7 mL microcentrifuge tubes (VWR, ON) to which 200 µL of sterile water was added. 250 µL of 10% Chelex solution (100 mesh (dry), Sigma-Aldrich, ON) was added to each sample (larva or pollen). Samples were boiled in a heat block (95°C) for 20 min and were agitated every 5 min by hand. After boiling, the samples were removed from the heat and were subsequently centrifuged for 1 min at 10000 x g to settle the Chelex beads. 50 µL of the supernatant was extracted for PCR amplification.

1.3 PCR Amplification of DNA

The primers SSU-rRNA-fwd (5'-CACCAGGTTGATTCTGCC-3') and SSU-rRNA-rev (5'-TTATGATCCTGCTAATGGTTC-3') were used to amplify a 1233 nucleotide sequence of the microsporidia genome. These primers are specific for *Nosema* microsporidia and target SSU rRNA genes of the microsporidia (Dong et al. 2010). The PCR reaction was composed of 0.5 µL (10µM) of both the forward and reverse primer (Integrated DNA Technologies IDT), 10.5 µL of ddH2O, 12.5 *Taq* polymerase (New England Biolabs, MA), and 1 µL of the template DNA. PCR amplification was carried out in a G-Storm 4822 Thermal Cycler. The PCR was programmed for: initial

denaturation at 95°C (30 sec), followed by 35 cycles of denaturation at 95°C (30 sec), primer annealing at 43°C (45 sec), and primer extension at 68°C (80 sec). There was a final extension at 68°C (5 min). If amplified samples could not be run due to time constraints, the samples were kept in a -20°C freezer until they could be run, usually for 24 hours or less.

1.4 Visualization of Amplified Samples

5 μL of Promega Blue/Orange Loading Dye, 6X (Fisherbrand, ON) was added to each PCR product prior to loading. 20 μL of the PCR product was visualized on 1% agarose (source) in 1x TAE buffer gel, stained with 3μL 10 ng/μL ethidium bromide (source), and was run for 1 h at 100V. Positive and negative control samples were included with each visualization. Samples were run alongside a 100bp DNA ladder (Gel Loading Dye Purple 6X, New England Biolabs). The resulting gel was examined using a Gel Doc™ XR+ image system (BIO RAD) and Image Lab™ software (v6.0.1). Presence of a band at approximately 1230bp indicated that a sample was positive for *Nosema* microsporidia.

RESULTS

For each of the three samples of pollen that were examined (BC, ON, and Greece), three independent analyses were run. In each of the three analyses, eight grains of pollen were examined and ran alongside the controls, resulting in a total of twenty-four pollen extractions being examined for each sample of pollen. Each of the nine trials of amplification and visualization that were performed were organized in the same manner (Figure 1).

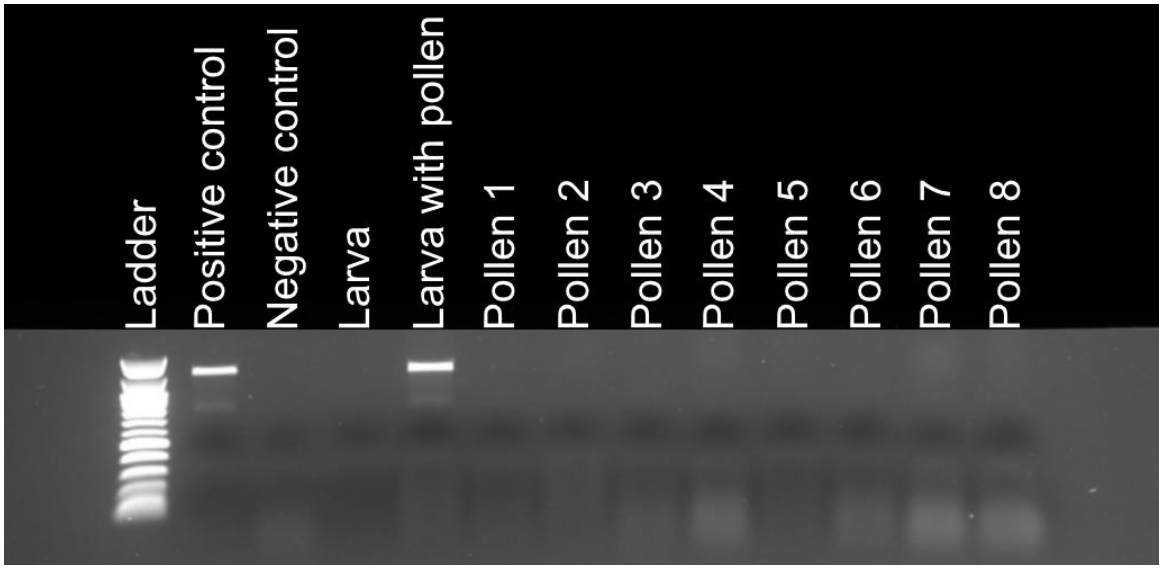

Figure 1. Example of amplified DNA extracted from (raw) unpasteurized bee pollen from three commercial sources, visualized on 1% agarose gel with 3μL 10ng/μL ethidium bromide for the detection of *Nosema* microsporidia.

In all the nine amplifications, both the positive control and negative control worked as intended. Of the nine larvae that were known to be infected with *Nosema adaliae*, only one of the samples tested positive for the presence of the microsporidia. This band was found in one of the three analyses run for pollen sample B (Table 1). Of the nine larvae that were known to be infected with *N. adaliae* and were mixed with pollen, five of the nine samples tested positive for the presence of the microsporidia. While two of the three larvae in the pollen extractions showed bands (pollen sample B and pollen sample C), there was a band in only one of the three amplifications of pollen sample A (Table 1). Of the twenty-four pollen extractions that were examined for each of the three pollen samples (n = 72 pollen extractions), none showed the presence of a band that would indicate *Nosema* microsporidia being detected by the PCR amplification (Table 1).

Table 1. Results of the DNA extraction for the detection of *Nosema* microsporidia in (raw) unpasteurized bee pollen from three commercial sources.

	Larva tested positive	Larva with pollen tested positive	Pollen tested positive
Pollen sample A Source: BC (3 replicates)	0/3	1/3	0/24
Pollen sample B Source: ON (3 replicates)	1/3	2/3	0/24
Pollen sample C Source: GR (3 replicates)	0/3	2/3	0/24
Total	1/9	5/9	0/72

DISCUSSION

Although no bands appeared in the pollen samples that would indicate the presence of *Nosema* microsporidia, the presence of microsporidia in the pollen cannot be ruled out. The analyses returned inconsistent results, with only 1 out of 9 larvae, and 5 out of 9 larvae in pollen analyses giving bands for microsporidia, respectively (Table 1). Because many false negatives were observed among the larva and larva in pollen analyses, it is not improbable that there were false negatives among the pollen grains analysed.

Adalia bipunctata larvae used in the experiment were infected with *Nosema adaliae*. This pathogen is transmitted vertically (Steele and Bjørnson 2014), and sibling larvae were examined and confirmed to be infected with the microsporidian by light microscopy. As a result, the infected larvae used were known to be infected and the lack of bands that would indicate the presence of *Nosema* microsporidia was not due to absence of the microsporidia. Furthermore, due to the inclusion of the infected larva in

pollen analyses, 5 out of 9 of which were extracted and amplified successfully, we can conclude that the inclusion of pollen in the samples did not interfere with the extraction and thus any false negatives were not due to the presence of pollen in the samples.

Additionally, the PCR amplification is not responsible for the false negatives of the larva and larva in pollen analyses. The positive control that was included for each set of analyses gave a band at approximately 1230bp, indicating the presence of *Nosema adaliae*. The positive control produced a band for all nine analyses; therefore, the inconsistent results observed for the larva and larva in pollen analyses were not associated with the PCR amplification. Thus, it is likely that the inconsistent results that were observed were associated with the DNA extraction that was performed prior to amplification.

Although boiling is effective for releasing DNA from microsporidia spores (Ombrouck et al. 1996), in this case, the boiling of samples was not enough to consistently rupture the *N. adaliae* spores. Of the nine extractions from the larvae infected with *N, adaliae*, only a single extraction and subsequent amplification showed the presence of the microsporidium. These larvae were confirmed to be infected by light microscopy, so all of the nine larvae analyzed should have resulted in a band for *Nosema* microsporidia. Consistent results from the larvae would allow for the pollen analyses to interpreted with more certainty. In lieu of boiling the spores to burst them and release the DNA, other methods should be considered as they may give more consistent results in future studies.

Microsporidian spores can be seen under light microscopy at a high magnification (400X total magnification), and this allows for visual confirmation of infection. However,

the use of light microscopy to detect *Nosema* spores has been found to be less reliable than detection using PCR (Bollan et al. 2013), and visual detection of the spores may be a difficult task if samples also contain an abundance of pollen grains and miscellaneous debris. Although microsporidian spores vary in the size (Vávra and Larsson 2014), and size may be used to differentiate species, the identification of microsporidia by spore size alone would not be efficient or reliable. As a result, to effectively determine whether *Nosema* microsporidia are present within pollen, a reliable means of disrupting the spore is essential in studies moving forward. This may include modifications to the boiling method that was used to ensure consistent results.

The use of enzymes may prove reliable for the disruption of the chitinous microsporidian endospore and exospore. Enzymes, including chitinase and proteases (such proteinase K) have been suggested as possible methods to burst the chitinous microsporidian spore (Ghosh and Weiss 2009). Proteases are non-specific enzymes, whereas chitinase specifically targets the chitin of the endospore and exospore. However; both proteases and chitinases are able to lyse microsporidian spores for genetic extraction (Ghosh and Weiss 2009). The extraction method may be improved by using glass beads and vortexing when chitinase or protease are used (Müller et al. 1999). Proteinase K may be combined with phenol-chloroform extraction or ethanol precipitation, which may aid in the extraction of genetic material from the microsporidia (Ghosh and Weiss 2009). The use of proteases or chitinases may provide more consistent and conclusive results with respect to the extraction of the microsporidian genome from the spore.

Chemical treatments, including hot alkali, may be useful for lysing the microsporidian spore because such treatments deproteinate the chitinous endospore and

exospore layers. Unfortunately, hot alkali destroys the sporoplasm within the spore (Yang et al. 2014), and because the primers target the ribosomes, the use of chemical treatments may ultimately prevent the detection of microsporidial DNA.

As boiling to release the genetic material from the microsporidia did not give consistent results in this study, the other methods considered may allow for consistent extraction from the microsporidia. Both enzymes and chemical treatments may be considered as possible routes forward to extract genetic material from the *Nosema* spores.

DNA extraction and PCR amplification failed to show the presence of *Nosema* microsporidia in raw, unpasteurized pollen, but because the methods used provided inconsistent results, one cannot conclude with confidence that *Nosema* microsporidia are not present within raw pollen. *Nosema apis* and *Nosema ceranae* have been detected in pollen in previous studies (Higes et al. 2008; Teixeira et al. 2018), so it is likely that microsporidia were present, but remained undetected, in the raw pollen that was tested in this study.

Nosema microsporidia infect honeybees and other pollinator species, and infections cause sub-lethal and detrimental effects. Although the presence of *Nosema* microsporidia in pollen was not observed in this study, the potential for pollen to act as a vector for transmission deserves future attention. Detecting *Nosema* microsporidia within pollen accurately would allow the prevalence of many species to be known, as well as allow for the transmission route of the pathogen to be better understood. Having further insight into the microsporidia that are present within an environment may allow for action to be taken to lessen the impact of the pathogen on beneficial insects such as pollinators.

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