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Review

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Approaches and Challenges to Managing *Nosema* (Microspora: Nosematidae) Parasites in Honey Bee (Hymenoptera: Apidae) Colonies

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Abstract

The microsporidia *Nosema apis* (Zander) and *Nosema ceranae* (Fries) are common intestinal parasites in honey bee (*Apis mellifera* L.) colonies. Though globally prevalent, there are mixed reports of *Nosema* infection costs, with some regions reporting high parasite virulence and colony losses, while others **report** high *Nosema* prevalence but few costs. Basic and applied studies are urgently needed to help beekeepers effectively manage *Nosema* spp., ideally through an integrated pest management approach that allows beekeepers to deploy multiple strategies to control *Nosema* when *Nosema* is likely to cause damage to the colonies, rather than using prophylactic treatments. Beekeepers need practical and affordable technologies that facilitate disease diagnosis and science-backed guidelines that recommend when, if at all, to treat infections. In addition, new treatment methods are needed, as there are several problems associated with the chemical use of fumagillin (the only currently extensively studied, but not globally available treatment) to control *Nosema* parasites. Though selective breeding of *Nosema*-resistant or tolerant bees may offer a long-term, sustainable solution to *Nosema* management, other treatments are needed in the interim. Furthermore, the validation of alternative treatment efficacy in field settings is needed along with toxicology assays to ensure that treatments do not have unintended, adverse effects on honey bees or humans. Finally, given variation in *Nosema* virulence, development of regional management guidelines, rather than universal guidelines, may provide optimal and cost-effective *Nosema* management, though more research is needed before regional plans can be developed.

Key words: *Nosema apis*, *Nosema ceranae*, honey bee, integrated pest management

Despite great efforts, beekeepers lose a large percentage of their colonies every year, with average overwintering losses hovering near 30% in the United States and first reports of total summer losses (2013) nearing 25% (Steinhauer et al. 2014). Several factors contribute to this high mortality rate, including pesticides, parasites, pathogens, and poor nutrition. Many of these stressors act synergistically to undermine bee health (Potts et al. 2010, vanEngelsdorp and Meixner 2010). However, because these stressors are widespread, are difficult to diagnose, and can have long-term sublethal effects, it is frequently hard for beekeepers to know when and why colonies are experiencing stress. Furthermore, the thresholds at which many of these stressors seriously damage colonies are unknown. Management strategies to effectively mitigate these stressors are also not well-developed, and in some cases, the management approach can unintentionally create stress for the colony. In agricultural crop systems, integrated pest management (IPM) or the use of multiple complementary strategies, ranging from pesticide application to release of biocontrol agents, has been widely and successfully used to control disease agents and limit pest damage (Gray et al. 2009). However, due to the current state of research, it is difficult to develop similar IPM approaches for honey bee husbandry.

In this review, we use the prevalent and frequently damaging parasites *Nosema apis* (Zander) and *Nosema ceranae* (Fries) as model disease agents and identify knowledge gaps that hinder development of effective management protocols. There have been several reviews of *Nosema* infections in honey bees in recent years (Fries 1993, Fries 2010, Higes et al. 2010a, Higes et al. 2013a, Araneda et al. 2015). Here, we provide an extensive overview of the biology of *Nosema*–honey bee interactions, discuss the current diagnostic and management options for beekeepers from an IPM perspective, and highlight specific areas where further research is required before IPM-based management strategies can be developed.

Nosema Parasites and Noseomosis

Transmission of *Nosema apis* and *Nosema ceranae*, Microsporidian Parasites of Honey Bees

Nosema apis and *Nosema ceranae* are two globally prevalent parasites of European honey bees (*A. mellifera* L.), with the latter parasite species representing an emerging disease agent (see reviews Fries 1993, Fries 2010, Higes et al. 2010a, 2013a, Araneda et al. 2015). Both *Nosema* spp. belong to a larger group of obligate, spore-forming fungal parasites called microsporidia that generally cause progressive, chronic infections (Keeling 2014). In honey bees, *Nosema* infection appears to primarily be transmitted via fecal–oral exposure when bees clean comb or consume food or water tainted with *Nosema* spores. Infection may also spread through nestmate- or self-grooming, as molecular studies detect high levels of *Nosema* DNA in whole-bee washes (Bourgeois et al. 2012b). In addition, *N. ceranae* DNA was isolated from royal jelly, suggesting that workers may transmit infections to their larval siblings, though it is unclear if parasites were secreted with food by infected workers or if spores originated from external worker or comb surface contamination (Traver and Fell 2012).

Consumed spores are carried through the honey bee digestive tract until currently unidentified cues cause spore germination and intracellular invasion of midgut cells (as reviewed for microsporidia in general in Cali and Takvorian 2014). When a spore germinates, it expels an internally coiled tube called the polar filament. The polar filament subsequently infiltrates a host cell membrane and the spore contents (sporoplasm) are passed into the host cell cytoplasm. Once inside a host cell, the sporoplasm begins to proliferate and the resulting progeny, termed "vegetative states," procure nutrients from host cells (Higes et al. 2007, Paldi et al. 2010). As obligate parasites, microsporidia are completely dependent on their hosts for furnishing the proper environment and energy for reproduction, which is reflected by their compact and simplified genomes and loss or reduction of some internal structures (Williams et al. 2014a). For example, microsporidia lack fully functional mitochondria, cell structures that efficiently produce large quantities of energy molecules (ATP) via oxidative phosphorylation. Instead, microsporidia contain reduced versions of mitochondria (mitosomes) and vegetative states may re-organize host cell mitochondria from which they obtain ATP. Thus, while chronic microsporidian infections may be slow to build, infections are frequently energetically costly for hosts. During intracellular amplification, *Nosema* vegetative states also likely undergo sexual reproduction (Gómez-Moracho et al. 2015a,b). Ultimately, new spores are formed that either infect neighboring midgut cells or are evacuated by the host. If eaten by a new bee, these spores propagate the infection.

Until recently, it was thought that *N. ceranae* might escape from the worker midgut tissue to infest other tissues, while *N. apis* remained confined to reproducing in the midgut (Chen et al. 2009), potentially explaining *N. ceranae*'s alleged greater virulence. Follow-up studies with orally infected workers suggest that both *Nosema* species are limited to reproducing in the midgut (Huang and Solter 2013). However, studies incorporating wash steps have detected *N. ceranae* DNA in queen reproductive tissues in addition to the midgut (Traver and Fell 2012, Roberts et al. 2015), suggesting that *N. ceranae* parasites may have a broader tissue distribution in queens than in workers, potentially due to different routes of exposure (fecal–oral vs. sexual, see below). *Nosema* spp. tissue distribution remains an active area of research, and future studies should incorporate dissection wash steps to rule out molecular contamination and microscopy assays to confirm intracellular parasite presence in tissues.

In addition to transmission via the fecal–oral route, new studies suggest that both parasite species may be sexually transmitted. Spores of *N. apis* (Peng et al. 2015) and DNA from both parasites are found in drone sperm (Roberts et al. 2015). Queens artificially inseminated with *Nosema*-contaminated sperm show reproductive and midgut tissue distribution of *N. ceranae,* with only *N. apis* being detected in gut tissue (Roberts et al. 2015). Though authors acknowledge that these experiments cannot rule out the possibility that queens became infected through self-grooming post-insemination, these studies highlight the need for additional field investigations. Regardless of whether queens acquire infection sexually or orally, and despite molecular evidence of infection of reproductive tissues (spermatheca and ovaries), field studies indicate that infected queens do not vertically transmit *Nosema* spp. to eggs (Roberts et al. 2015).

Global Distribution of *Nosema* spp. in Managed Honey Bee Colonies and Factors Contributing to Heterogeneity in Parasite Prevalence and Virulence

Nosema apis has been subject to epidemiological scrutiny in European honey bees (*A. mellifera*) since the early 20th century (as summarized in Kudo 1920). In contrast, *N. ceranae* is an emerging parasite of *A. mellifera*. *Nosema ceranae* was thought to only naturally infect Asian honey bees (*Apis cerana*) when it was first described in the 1990s (Fries et al. 1996). In 2006, *N. ceranae* was identified in European honey bees in Spain (Higes 2006). Shortly thereafter, studies from Spain found that *N. ceranae* was highly virulent in caged bees (Higes et al. 2007) and infection was correlated with collapse of colonies in the field (Higes 2008). These and other studies suggested that *N. ceranae* was potentially more virulent to *A. mellifera* than *N. apis* and that, globally, *N. ceranae* was replacing *N. apis* (Klee et al. 2007). In particular, *N. ceranae*'s host shift from *A. cerana* to *A. mellifera* was a hypothesized driver behind its alleged greater virulence. Indeed, pathogens that successfully traverse host species barriers are sometimes more damaging to their new hosts than the hosts that they co-evolved with (Weiss 2003). Furthermore, complete genome sequencing of both parasite species (Cornman et al. 2009, Chen et al. 2013) points to some differences in parasite virulence factors (see section *Nosema* spp. Virulence Factors and Host Defense Mechanisms) that may offer *N. ceranae* a competitive advantage over *N. apis*. Given potential links between *N. ceranae* and worldwide declines in honey bee health, scientific literature concentrating on one or both *Nosema* species has rapidly expanded and continues to grow. However, the evolving picture of *N. ceranae* and *N. apis* virulence and culpability in colony losses is complex.

Currently, both species of *Nosema* have a global distribution, but there are temporal and regional variations in prevalence in addition to rates of singly infected and co-infected colonies (see recent publications for examples: Bollan et al. 2013, Szalanski et al. 2013, Shutler et al. 2014, Szalanski et al. 2014). While numerous studies have documented *Nosema* spp. prevalence, fewer longitudinal studies and several cage experiments, primarily in European and American populations and summarized here, have recorded costs of infection in the field.

In Spain, historical samples show increasing *N. ceranae* prevalence (Botías et al. 2012b), and today, *N. ceranae* appears to be both highly prevalent and, in many studies, highly virulent (Higes 2008, Higes et al. 2009, Higes et al. 2010b, Botias et al. 2013, Cepero

et al. 2014, but see Fernández et al. 2012). Other European and North American countries have reported conflicting findings. In Serbia, for example, *N. ceranae* is nearly ubiquitous and the only microsporidian species that infects *A. mellifera*, but it is not associated with colony loss (Stevanovic et al. 2013). Longitudinal studies in Sweden found that *N. apis* was more prevalent than *N. ceranae* and that *N. ceranae* prevalence was not increasing. In Germany, *N. apis* was also more prevalent than *N. ceranae*, and neither species was associated with overwintering mortality (Gisder et al. 2010, Forsgren and Fries 2013). Conversely, a smaller study in Switzerland only detected *N. ceranae,* and found that its presence could seasonally predict colony mortality (Dainat et al. 2012). *Nosema ceranae* was also associated with overwintering death in Belgium, and additional, synergistic negative effects of *Nosema* infection on colony survival were observed when other diseases were present (Ravoet et al. 2013). In keeping with varied reports across Europe, a recently published survey of 621 colonies located in 11 European countries found that *N. apis* and *N. ceranae* distribution and infection intensity seasonally and regionally varied but that colony loss was only linked to disease in some regions and *Nosema* spp. were not reported as a major driver of colony loss across Europe as a whole (Meixner et al. 2014).

In Canada (Ontario), *Nosema* infection was linked to smaller spring adult populations and, though not alone correlated with winter loss, emerged as a significant predictor of colony death in conjunction with other stressors (*Varroa* mite presence, poor food supply, and small bee population; Guzmán-Novoa et al. 2010). Also, a recent survey of colonies in two Canadian provinces found that *N. ceranae* was more prevalent than *N. apis* (Emsen et al. 2015). In the United States, *N. ceranae* is generally more prevalent than *N. apis* but each species' prevalence varies regionally (Szalanski et al. 2013, and as summarized in Chen and Huang 2010) and differs between managed and feral populations (Szalanski et al. 2014). Also, *Nosema* co-infections are more commonly found in collapsed colonies (Cox-Foster et al. 2007, vanEngelsdorp et al. 2009) along with other pathogens. In South America, retrospective molecular analyses have detected early *N. ceranae* infections (1979) in Africanized drones from Brazil (Teixeira et al. 2013), with similar early detections in limited *A. mellifera* samples from Uruguay (Invernizzi et al. 2009). Longitudinal studies are needed to assess the impact of *N. ceranae*'s invasion of South American honey bee populations.

Cage studies conducted in Europe and North America also report heterogeneous findings on *Nosema* species-specific virulence (Higes et al. 2007, Paxton et al. 2007, Forsgren and Fries 2010, Williams et al. 2014b, Huang et al. 2015, Milbrath et al. 2015, Natsopoulou et al. 2015a), where *N. ceranae* has been found to be more virulent, no more virulent, or less virulent than *N. apis*. Mixed infections were sometimes more virulent than infection with either species or less virulent than *N. ceranae* infection alone. Several factors are thought to explain the global variation in reports of *Nosema* spp. distribution and virulence, as discussed in the following text.

Climate and Seasonality Likely Contribute to Nosema spp. *Distribution and Intensity*

Earlier investigations found that *N. ceranae* spores are tolerant of heat and desiccation and that *N. ceranae* infections proliferate at the same rate or faster in bees incubated across a range of temperatures (Fenoy et al. 2009, Martin-Hernandez et al. 2009). In addition, low temperatures were found to inhibit *N. ceranae* germination more than *N. apis* germination (Gisder et al. 2010). These temperature studies, combined with regional surveys that found that *N. ceranae* was not predominant or not increasing in honey bee populations in colder climates, were initially thought to explain why *N. ceranae* appears less damaging and prevalent in cooler, temperate regions such as Germany and Sweden (Gisder et al. 2010, Forsgren and Fries 2013) compared with Spain. However, a recent Canadian study found that *N. ceranae* was more prevalent than *N. apis* in both the surveyed provinces and colonies singly infected with *N. ceranae* had higher spore counts than colonies singly infected with *N. apis*, while co-infected colonies had the greatest infection intensities in one province (Emsen et al. 2015). These findings suggest that *N. ceranae* can outcompete *N. apis*, even in colder, temperate climates. Furthermore, though *N. ceranae* is the most prevalent *Nosema* species found in Spain, the geographic distribution of both *Nosema* spp. varies, indicating that local climate or other conditions shape *Nosema* spp. establishment (Martín-Hernández et al. 2012).

How seasonality and local climate are linked to *Nosema* species prevalence and virulence requires future investigation. Historically, *N. apis* levels have been reported to maximally peak in the spring, with a small peak in the fall (Fries 1993). In Spain, however, *N. ceranae* prevalence is seasonally stable (Higes et al. 2010a), but studies elsewhere have reported that *N. ceranae* colony levels fluctuate or cycle (Gisder et al. 2010, Traver et al. 2012, Stevanovic et al. 2013), hinting that parasite levels are seasonally driven or linked to temperature (Chen et al. 2012). In a regional example, as earlier described, *Nosema* and other biotic stressors were surveyed in apiaries from 11 European countries. The authors found that colonies located in more northern apiaries tended to have higher spore loads (Meixner et al. 2014). In keeping with hypothesized explanations of *Nosema* seasonal fluctuations, the authors proposed that colder climates limited worker ability to defecate outside the colony, leading to greater infection levels, especially in the spring and fall. Notably, in this study, infection did not always correlate with colony loss.

Differences in Virulence Between N. ceranae *Strains May Contribute to Global Trends, but Additional Studies Are Needed*

In accordance with its recent invasion of *A. mellifera* populations, molecular analyses of *N. ceranae* isolates from distinct geographical regions do not significantly segregate based on origin, suggesting that variation in host populations may be a more important factor in explaining global virulence trends than variation among *Nosema* strains (Dussaubat et al. 2013b, Gómez-Moracho et al. 2015b). Indeed, a high degree of *Nosema* sequence variability has been noted within honey bee colonies and even within the same bee (Gómez-Moracho et al. 2015b). Evidence of recombination in *N. ceranae* in addition to the parasite's ability to maintain within-host genetic diversity likely have contributed to the successful spread of *N. ceranae* within *A. mellifera* and between other bees species (Hatjina et al. 2011, Gomez-Moracho et al. 2014, Van der Zee et al. 2014, Gómez-Moracho et al. 2015c). Widespread genetic variability in conjunction with recombination could increase *N. ceranae*'s capacity to evolve resistance to treatment methods or lead to local strain adaption. For example, *N. ceranae* strains in *A. cerana* populations distributed throughout China group based on geographic origin and cluster separately from *N. ceranae* strains derived from *A. mellifera* populations (Li et al. 2012).

Variation in Host Bee Population Susceptibility May Contribute to Global Parasite Virulence Trends

Though it is early yet to conclude that variation in *A. mellifera* populations rather than *N. ceranae* strains predominantly governs

global virulence trends, new research offers support for this hypothesis. For example, when *N. ceranae* isolates from France and Spain were compared, genetic variation among isolates could not be significantly linked with parasite origin (Dussaubat et al. 2013b). Furthermore, honey bees from the *Apis mellifera iberiensis* subspecies found in Spain were equally susceptible to infection from both isolates across all measured parameters (mortality, spore production, midgut lesions). In a similar study, *A. m. iberiensis* was equally susceptible to infection with *N. ceranae* isolates from the Netherlands and Spain (though there was a nonsignificant trend for greater survival in cohorts infected with *N. ceranae* from the Netherlands; Van der Zee et al. 2014). Again, molecular analyses did not segregate *N. ceranae* isolates based on geographic origin. However, not all studies offer strong support for bee strain contributing to geographic virulence differences (Villa et al. 2013). Interestingly, when three *A. mellifera* taxa from two different regions (North Mediterranean, Near and Middle East) were infected with the same *N. ceranae* isolate, source colony rather than geographic origin emerged as the most important factor modulating host performance (Fontbonne et al. 2013). Additional studies have shown that some strains of bees are more tolerant or resistant to infection, giving rise to selective breeding programs (see section Colony Management Practices). Further studies are needed to determine the respective roles of *Nosema* isolates and host strains in global virulence patterns.

Parasite-Specific Developmental Trajectories and Nosema *Interspecies Competition Likely Contribute to Global Disease Prevalence Patterns*

In single-species infections, *N. ceranae* tends to produce greater numbers of spores than *N. apis*, hinting at a competitive advantage for *N. ceranae* (e.g., Huang and Solter 2013, Williams et al. 2014b, Milbrath et al. 2015. But also see Forsgren and Fries 2010, where there was no difference in parasite spore loads in single-species infections, though the incubation period was shorter, and see Natsopoulou et al. 2015c, where single infections did not differ in intensity). However, the minimum infective dose for *N. ceranae* may require more spores than *N. apis* (Huang et al. 2015) in some populations of honey bees.

In mixed infections, the order of microsporidian species exposure matters. In simultaneously acquired mixed infections, *N. ceranae* does not appear to be more competitive than *N. apis* (Forsgren and Fries 2010, Milbrath et al. 2015, Natsopoulou et al. 2015c), though Williams et al. (2014b) found indirect evidence of interspecies parasite competition. Alternatively, prior infection with one *Nosema* species dampens reproduction of a subsequently acquired infection of the other species (Natsopoulou et al. 2015c). This mutual repression determined by infection order of *Nosema* spp., however, is not symmetric: initial infection with *N. ceranae* hampers *N. apis* reproduction more than initial infection with *N. apis* inhibits later *N. ceranae* reproduction. Indeed, the authors hypothesize that the greater competitive advantage of *N. ceranae* in sequential mixed infections may explain *N. ceranae*'s predominance in some regions. Unbalanced effects of infection order have also been found for *N. ceranae* and deformed wing virus, where *N. ceranae* shows a strong competitive advantage if administered as the primary infection in cage studies (Doublet et al. 2015). Finally, potential differences in virulence factors between *N. apis* and *N. ceranae* that may contribute to differential pathogen success are discussed in section *Nosema* spp. Virulence Factors and Host Defense Mechanisms.

The Presence of Other Abiotic and Biotic Stressors Can Affect Nosema *Establishment and Host Survival*

For example, studies have shown that pesticide exposure can act synergistically with *Nosema* infection to increase disease prevalence, intensity, or mortality (Alaux et al. 2010b, Pettis et al. 2012, Wu et al. 2012). Other chemicals deliberately placed in colonies to control *Nosema* may increase pathogen loads in the long term (see section Chemical Treatments). Finally presence of other pathogens and parasites (e.g. *Varroa* mities, chalkbrood, *Crithidia mellificae*, chronic bee paralysis virus) can interact with other temporal (season) or hive conditions such as low food stores, leading to greater *Nosema* prevalence or intensity (Guzmán-Novoa et al. 2010, Hedtke et al. 2011, Ravoet et al. 2013, Toplak et al. 2013).

Differences in Experimental Design Affect Study Findings (Fries et al. 2013)

Differences in study methodologies across laboratories could contribute to differences in virulence findings. For example, adult worker susceptibility to infection changes with age (Roberts and Hughes 2014). Furthermore, with a limited number of laboratories examining *Nosema* distribution and virulence, it can be difficult to obtain independent, regional corroboration of study findings.

Nosema spp. Pathology in Honey Bees

Consequences for Individual Workers and Honey Bee Colonies

Infected honey bee workers are energetically deprived, exhibit precocious foraging (Woyciechowski and Moroń 2009, Dussaubat et al. 2013a, Goblirsch et al. 2013), and are more likely to die prematurely (as reviewed in Higes et al. 2010a). *Nosema* replication appears to be a key proximate driver of the energetic costs of infection. While reproducing within host midgut cells, *Nosema* parasites cause tissue damage and use host ATP energy molecules (Paldi et al. 2010, Dussaubat et al. 2012; see section Transmission of *Nosema apis* and *Nosema ceranae,* Microsporidian Parasites of Honey Bees). Thus, host digestion is likely hindered while host resources are redirected to support parasite replication, indirectly and directly depriving the host of sustenance and molecular fuel. Indeed, if permitted, *N. ceranae*-infected workers will consume extra food, and if food access is acutely restricted, workers will starve faster than uninfected siblings (Mayack and Naug 2009). Additional studies have documented nutritional and metabolic abnormalities and changes in feeding behavior in both *N. apis*- and *N. ceranae*-infected workers (Wang and Moeller 1970, Moffett and Lawson 1975, Naug and Gibbs 2009, Mayack and Naug 2010, Martín-Hernández et al. 2011). Furthermore, molecular and metabolomics studies indicate global changes in infected workers' metabolic profiles (Aliferis et al. 2012, Holt et al. 2013).

The energetic costs associated with *Nosema* infection likely contribute to workers' behavioral symptoms of infection, including accelerated maturation from nursing and brood care to foraging behavior (see Page Jr and Peng 2001 for a review of honey bee biology). Normally, behavioral maturation rates are governed by workers' internal nutritional and hormonal status, which are also sensitive to diverse colony cues (Ament et al. 2010). Comparative molecular studies of gene expression in worker fat body tissue suggest that energetic costs of *Nosema* infection may "starve" workers, preventing them from either reaching or maintaining the nutrient-rich physiology and attendant molecular profiles associated with nursing (Holt et al. 2013). Interlinked changes in worker nutritional and hormonal status (likely involving the insulin signaling pathway and the vitellogenin–juvenile hormone axis) as a result of energy

deprivation may subsequently promote foraging behavior and physiology. However, a recent study has found evidence that *N. ceranae* infection, rather than the resulting impacts of infection on worker energetic state, alters the conserved, energetically sensitive octopamine pathway, which also has been linked to foraging behavior (Mayack et al. 2015). In honey bees, many diverse stressors are known to cause precocious foraging (Even et al. 2012). Taken together, these studies raise interesting questions about whether precocious foraging is the direct result of *Nosema* parasite infection, an indirect result of energetic stress or a general host stress response, with similar changes in regulatory pathways responding to *Nosema* infection as to other stressors. For example, common genes are regulated in worker brain tissue following exposure to ectoparasites (*Varroa* mites) and endoparasites (*N. ceranae*; McDonnell et al. 2013). However, brain tissue responses in parasitized individuals more closely resemble each other than those of foragers, potentially indicating that disease-specific molecular mechanisms underlie precocious foraging, at least in the brains of parasitized individuals. Indeed, some elements of *Nosema*-induced foraging appear disease-specific (as opposed to stress-specific), as foraging patterns differ between *N. ceranae* infected workers and workers given a sterile wound (Alaux et al. 2014). Also, *N. apis*- and *N. ceranae*-infected foragers display different foraging frequencies and productivities compared with healthy foragers (Naug 2014, Lach et al. 2015). Finally, while workers infected with *N. ceranae* or deformed wing virus exhibit accelerated behavioral maturation, the pace of change is greater for deformed wing virus-infected workers than for *N. ceranae*-infected workers (Natsopoulou et al. 2015b). Collectively, these studies suggest that different stressors, while promoting early foraging, do not necessarily promote behavioral and physiological states in diseased individuals that are comparable with "normal foragers," and some aspects of precocious foraging are likely driven by disease-specific molecular etiologies.

For *Nosema*-infected workers, premature foraging contributes to premature death, which undermines colony stability. Foraging is the terminal vocation for workers, and individuals with shorter life expectancies due to infection or another stressor are more likely to undertake foraging tasks (Woyciechowski and Moroń 2009, Kuszewska and Woyciechowski 2013). As foraging is energetically intensive and dangerous, colonies minimize resource losses when short-lived individuals undertake hazardous foraging tasks. Infected foragers also suffer greater extrinsic mortality than healthy foragers, as infection is associated with disorientation and other metabolic and behavioral abnormalities (Kralj and Fuchs 2010, Alaux et al. 2014, Naug 2014, Wolf et al. 2014). Consequently, infected foragers' contributions to colony fitness are lower than that of healthy foragers. For example, healthy foragers appear more efficient at gathering resources than *N. ceranae*-infected foragers (Naug 2014), and *N. apis*-infected foragers are less likely to collect pollen (Lach et al. 2015). Moreover, harmonic radar tracking studies show that infected foragers take longer rests and are less likely to return to the colony during homing experiments (Wolf et al. 2014). Thus, colonies that are not killed by infection with either *Nosema* spp. still suffer costs: they are slower to grow, have smaller adult populations relative to brood area, and produce less honey (Fries 1993, Botias et al. 2013, Villa et al. 2013).

However, when a colony does succumb to infection, its failure in part likely arises from imbalances in worker division of labor leading to population declines. As infected workers forage precociously and therefore die prematurely, younger workers are compelled by colony cues to fill the foraging void, perpetuating a cycle of early adult death. Indeed, simulation models suggest that precocious foraging and early forager death are linked with colony failure (Barron 2015, Perry et al. 2015). Colonies unable to compensate for resources invested in workers that die early (and therefore also contribute less to their colony's fitness), may dwindle until the weakened colony succumbs to Noseomosis or another stressor (as summarized in Higes et al. 2010a). Interestingly, infected workers produce higher levels of a pheromone (ethyl oleate) that slows worker behavioral maturation (Dussaubat et al. 2010). Though changes in pheromone levels could disrupt normal colony homeostasis, excessive ethyl oleate production could cautiously be interpreted as a colony attempt to slow behavioral maturation of healthy workers to help infected colonies maintain a balanced nurse:forager ratio.

Consequences for Adult Queens, Drones, and Immature Castes

Few studies have characterized Noseomosis in queens, drones, and immature host stages. Briefly, infection in adult queens and drones, as in workers, results in aberrant physiology and metabolic costs (Alaux et al. 2011, Retschnig et al. 2014, Peng et al. 2015). *Nosema apis*-infected queens are more likely to be superceded (Furgala 1962), while *N. ceranae* changes queen pheromone profile (Alaux et al. 2011). Additional studies are needed to determine if *N. ceranae* also precipitates queen replacement, though one study did not find a correlation (Villa et al. 2013). Infected drones have smaller body masses, hinting at metabolic costs, shortened life expectancies, and lower fertility (Retschnig et al. 2014, Peng et al. 2015).

Molecular studies have uncovered *Nosema* infections in pupal drones (Traver and Fell 2011), in larval and pupal workers (Rodríguez et al. 2014), and in larval queens (Traver and Fell 2012), suggesting that all castes can become infected during development. Experiments directly testing infection in larval workers found not only that worker larvae can become infected but also that early infection reduced adult longevity (Eiri et al. 2015). Additional studies in field settings are needed, as *Nosema* incidence and prevalence in immature stages, as well as disease etiology and ramifications for colony health, remain largely uncharacterized.

Nosema spp. Virulence Factors and Host Defense Mechanisms

Overall, virulence factors that enable *Nosema* spp. to successfully invade host midgut tissue are poorly understood. The insect midgut is lined with the peritrophic membrane (PM), a protective mucosal film secreted by intestinal cells (Terra 2001). How *Nosema* traverses or subverts the honey bee PM is poorly understood. However, recent studies suggest that *N. ceranae* impairs local host immune defenses in midgut tissue, such as apoptosis (Higes et al. 2013b, Kurze et al. 2015). Apoptosis, or programmed cell death, can serve as a general immune strategy where host cells systematically self-destruct to undermine parasite reproduction. Yet, infected bees do exhibit higher levels of proteins related to oxidative stress in midgut tissue, indicating that bees may defend themselves by producing reactive oxygen species (ROS; Dussaubat et al. 2012, Vidau et al. 2014). ROS production is a conserved, nonspecific immune response, and ROS molecules can be highly toxic to both parasite and host cells. Whether *N. apis* similarly manipulates host apoptosis or induces similar ROS responses remains to be confirmed.

Once successfully established within a host cell, *Nosema* parasites use several molecular virulence factors to acquire host molecules. Indeed, comparative genomic analyses of *N. ceranae* and *N. apis* provide a better understanding of how these parasites have metabolically adapted to import essential nutrients and energy from host cells (Cornman et al. 2009, Chen et al. 2013). Both *Nosema*

species contain a number of genes coding for energy transport and biosynthesis. However, these genes are more highly represented in *N. ceranae,* indicating that *N. ceranae* may be better able to obtain host ATP and other molecules. These genetic resources may confer a survival advantage to *N. ceranae* and promote successful competition with *N. apis* (Chen et al. 2013).

Nosema spp. also appear to modify systemic expression of canonical worker immune genes, though changes are often transient and particular to the *Nosema* species of infection, incubation period, host tissue examined, and other factors incorporated in experiment design (Antunez et al. 2009, Chaimanee et al. 2012, Schwarz and Evans 2013). However, expression of members of the canonical Toll signaling pathway is altered in infected workers (Holt et al. 2013) and some Toll pathway genes are upregulated in drones from a *N. ceranae*-tolerant honey bee strain (Huang et al. 2012), pointing to the Toll signaling pathway's likely involvement in host defense against microsporidia. Interestingly, *N. ceranae*-tolerant bees may also be less susceptible to apoptosis inhibition (Kurze et al. 2015). Also, other conserved immune factors–pathways including the recognition protein *Dscam*, the immune response receptor (*Imd*) for the Imd signaling pathway, and some AMPs (antimicrobioal peptides) are temporally upregulated in *N. ceranae*-infected workers (Schwarz and Evans 2013). Additional molecular studies have identified other genome regions and noncanonical immune genes in fat body tissue that modulate worker response to infection (Holt et al. 2013, Huang et al. 2014). Part of the challenge of dissecting host immune response is that worker immune, hormonal, metabolic, and nutritional statuses are interlinked. Thus, some changes in immune function may be a byproduct of disease costs or of a generalized stress response (Holt et al. 2013). Furthermore, host age and timing of exposure interact. For example, older bees survive incipient infection with *N. ceranae* better than younger bees, even though older workers also produce higher spore loads (Roberts and Hughes 2014). Additional comparative analysis of *N. apis* and *N. ceranae* genomes shows that protein sequences involved in responding to stress and endogenous stimuli were significantly more represented in *N ceranae* than *N. apis*, which may indicate that *N. ceranae* has a superior capacity to cope with host immune defenses, potentially contributing to *N. ceranae*'s dominance in some geographic regions (Chen et al. 2013).

Changes in individuals' behavior following infection influences parasite growth trajectories and likelihood of transmission. For example, recent choice tests found that infected workers prefer honey with greater antimicrobial activity, and that consumption of favored honey could reduce *N. ceranae* pathogen loads in cage trials (Gherman et al. 2014). Thus, self-medication through selective diet may be one way that individuals repress infection, but field studies are needed. *Nosema ceranae*-infected bees also prefer warmer temperatures and are more likely to be found in the center of the colony (Campbell et al. 2010). Authors suggest that workers suffering from *Noseomosis* may inherently prefer warmer temperatures because their ability to thermoregulate is potentially restricted by infection costs. Alternatively, as *N. ceranae* develops better at warmer temperatures, this thermotactic predilection could cautiously be interpreted as a host–parasite manipulation to enhance parasite reproduction. Regardless, congregation of infected workers in certain hive regions likely influences parasite transmission. For example, cage experiments investigating permutations in diseased and susceptible host density, with workers or drones serving as the initial source of *N. ceranae* infection, found not only that *N. ceranae* transmission exhibited some density-dependent properties, but also that drones transmitted *N. ceranae* at higher rates than workers (Roberts and Hughes 2015). The number of spores produced by individual workers and drones also varied with initial infection density and caste. Together, these finding suggest that multiple factors regulate *Nosema* transmission within the complex and dynamic context of colonies.

Changes in social interactions (or lack thereof) also contribute to disease dynamics. Interestingly, workers may perceive if nestmates have been exposed to an immune challenge and treat bacteria-injected nestmates more aggressively (Richard et al. 2012). However, recent studies show that though both *Nosema* parasites alter worker cuticular hydrocarbon profiles, healthy workers do not treat *Nosema*-infected workers differently from uninfected controls (McDonnell et al. 2013, Murray et al. 2015). Therefore, workers harboring infections escape social persecution, which may have consequences for disease transmission.

Finally, precocious foraging could serve as a general social immune response. As previously discussed, infected individuals have shorter life expectancies, and thus optimize their contribution to colony fitness by performing the riskiest task of foraging, sparing their healthy siblings with longer life expectancies. By leaving the colony, infected workers may further reduce chances of in-hive transmission, and as previously mentioned, infected workers may be disoriented or less likely to return to their natal colony (see section *Nosema* spp. Pathology in Honey Bees). However, this disorientation might benefit the parasite if disoriented bees show a greater tendency to drift to neighboring colonies.

Methods for Diagnosing Nosema Infections in Colonies

Without laboratory assistance, the majority of beekeepers do not have the ability to determine if their colonies are infected with *Nosema*, let alone determine infection species or severity. Accurately diagnosing *Nosema* infection is difficult because there are few obvious clinical symptoms, and those that may be present (e.g., diarrhea for *N. apis*) are not necessarily unique to microsporidian infection (Bailey and Ball 1991). Thus, while beekeepers may speculate that their colonies are infected with *Nosema*, national self-reports of colony loss attributable to *Nosema* infection may not accurately reflect morbidity and mortality due to microsporidia.

Light Microscopy and Molecular Techniques

Currently available diagnostic techniques comprise light microscopy and molecular tools and have recently been reviewed in Fries et al. (2013). These tools come with the obvious limitation that they can be expensive and time-consuming. Molecular techniques in particular are not accessible to beekeepers. Using light microscopy, beekeepers can confirm *Nosema* presence (Fig. 1) and estimate infection intensity by conducting spore counts with a hemocytometer, but unfortunately, light microscopy cannot easily distinguish between *Nosema* species, which may be important for effective disease treatment. Beekeepers in the United States and Canada may access microscopy services for free (aside from shipping costs) by sending samples to the U.S. Department of Agriculture for spore detection and quantification (but not species identification; USDA 2015).

At the moment, only molecular techniques can determine both infection intensity and species of infection (Fries et al. 2013). Furthermore, molecular techniques are more sensitive than light microscopy and can detect infection at very low levels, as they amplify DNA from both vegetative and spore parasite states (vegetative states, because they lack thick spore walls and refractive properties,

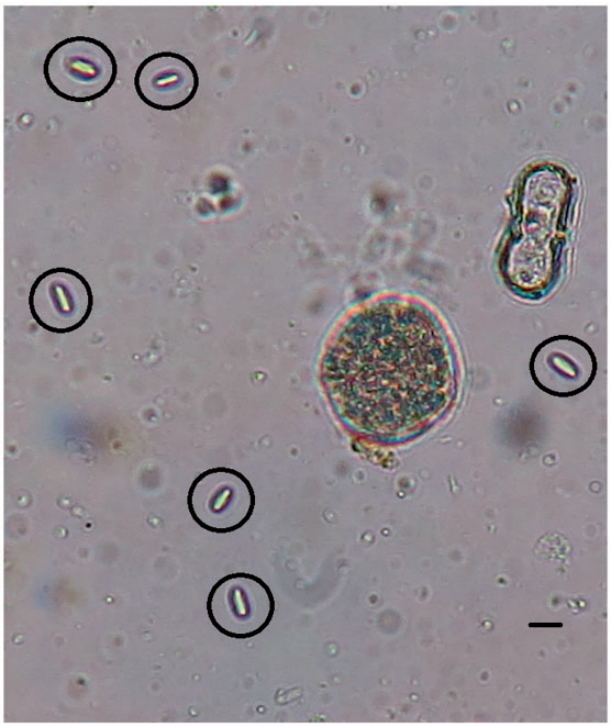

Fig. 1. *Nosema* spp. spores in whole abdomen homogenates. Light-refracting, *Nosema* spp. spores are circled in the image (400×). The bar is approximately 5 um in length. Other artifacts (pollen, tissue debris) are visible.

are not easily detected using light microscopy). However, several alternative and more accessible tools can be developed, which would greatly improve the efficiency and cost of *Nosema* diagnosis (see following sections).

ELISA (Enzyme-Linked Immunosorbent Assay) Test

Recently, Aronstein and collaborators adapted the enzyme-linked immunosorbent assay (ELISA) technique to detect *N. ceranae* infections (Aronstein et al. 2011, 2013). In an ELISA, an antibody that is specific for a protein or hormone of interest is coupled to a dye, and thus it is possible to determine if a target protein or hormone is present and visually estimate its levels. Using specialized equipment to measure the concentration of the dye (a spectrophotometer) and a standard curve with known quantities of material, it is possible to quantify the amount of the target molecule (or spores). Alternatively, a simple color change can be used to document presence or absence of the target molecule, as in the case of pregnancy tests, which measure levels of the human chorionic gonadotropin hormone in urine. Aronstein and collaborators developed antibodies that specifically bind to the spore wall protein (SWP32) of *N. ceranae*, and incorporated these in an ELISA. This method was validated with quantitative polymerase chain reaction (qPCR), and not surprisingly, both ELISA and qPCR methods were shown to be more sensitive than microscopy.

The published ELISA method requires expensive equipment. However, this methodology could readily be adapted to produce dipstick tests that could be easily used by beekeepers in the field. To test a colony, sample workers would be collected and macerated. A drop of the sample would then be applied to the dipstick. If *N. ceranae* spores are present, the dipstick would change color. Infection intensity could be estimated by comparing color results with a reference card provided by the manufacturer (Aronstein et al. 2013). Such technology eventually may offer beekeepers an inexpensive, fast, and easy way to detect and quantify *N. ceranae* spores in colonies. Assessments of other biotic health threats also could be incorporated if specific antibodies are developed.

Measuring Acoustic or Odor Signatures of Colony Stress

New acoustic technology may offer novel means of diagnosing *Nosema* infection as well as other colony disorders. For example, an Australia-based research team has piloted an acoustic system for detecting *Varroa* presence in colonies (Qandour et al. 2014). This system posits that honey bee colonies, when exposed to different stressors, produce unique sound waves that can be diagnostic of specific problems (e.g., *Varroa* presence). Similar technologies are being developed in the United States, where researchers have submitted a patent for the "Honey bee acoustic recording and analysis system for monitoring hive health," which could potentially link the presence of volatile toxicants or disease odors in colonies to acoustic signals of distress (Bromenshenk et al. 2009). However, these technologies are in development and future peer-reviewed studies are needed to validate these systems.

An analogous diagnostic method would allow beekeepers to identify colony stressors based on chemical odorants. Recent studies show it is possible to capture and analyze pheromone signatures from bees on a Langstroth colony frame (Carroll and Duehl 2012), and if future studies correlate chemical levels or presence with *Nosema* disease, volatile profiles may be used as diagnostic tools. For example, *Varroa* mite-parasitized pupae (Nazzi et al. 2004) and chalkbrood infected-larvae (Swanson et al. 2009) produce unique volatile cues. "Electronic noses" have diverse applications in other industries, and there is ongoing research to create sensors to monitor food quality, environmental contaminants, and human diseases (see Röck et al. 2008 for review), and thus this technology may be adapted for honey bee management as well.

Though acoustic and odorant technologies are either in development or remain hypothetical, if proven through independent tests, such systems would greatly advance beekeepers' ability to diagnose diseases. Such technology might in theory be minimally invasive and far less time-consuming, as it would obdurate the need to collect and process bee samples. Second, these systems could be harnessed to diagnose many different problems and potentially even diagnose severity. Third and finally, if manufacturing costs are not prohibitive, this technology could offer long-term cost-savings. However, given the diversity of odors in the colony and variation associated with genetic and environmental factors (Graham, 2015), identifying disease-specific markers is likely to be challenging.

Challenges With Sampling and Establishing Treatment Thresholds

Under the IPM paradigm, farmers monitor crop damage incurred by pests and intervene only when pest levels reach a predetermined economic threshold (ET), where intervention is necessary to prevent additional crop damages that would be more costly than taking action to control pest activity (EIL: economic injury level); Gray et al. 2009). At this time, there is no consensus on either an ET or EIL for *Nosema*. Workers can carry up to tens of millions of spores, but, as previously noted, parasite presence is not a guarantee of disease symptoms at the colony level (Bailey and Ball 1991, Higes et al. 2010a). Before management guidelines can be developed for *Nosema* treatment, further research is desperately needed to first

establish the best practices, taking time and costs into account, for sampling colonies and quantifying *Nosema* intensity. Second, additional studies are needed to link *Nosema* levels with colony damage so treatment guidelines can be established. Achieving both of these goals will be challenging, as *Nosema* prevalence and virulence may vary geographically and temporally.

Sampling Colonies for *Nosema* Infection

Standard guidelines for sampling colonies for *Nosema* are outlined in Fries et al. (2013). Briefly, as *Nosema* spp. disease progression is chronic and infected workers forage precociously, it is clear that foragers are the best population of bees to sample to maximize detection sensitivity. However, within the same colony, infection levels can vary dramatically between foragers, so larger sample sizes are needed if samples are to be screened via light microscopy. Using a light microscope, approximately 60 bees should be included in a pooled sample to increase detection sensitivity (95% confidence of identifying a 5% infection prevalence in the colony; Fries 1997). Alternatively, some studies suggest that determining the percent of infected bees within a sample is a better gauge of infection intensity. This latter method is time-intensive and impractical (as reviewed in Fries et al. 2013). If samples are to be screened with molecular techniques, additional research is needed to determine appropriate sample sizes, as molecular techniques offer higher levels of sensitivity than microscopy.

Collecting the requisite number of foragers from the colony entrance can be time-consuming, especially if a large number of colonies must be sampled. If foragers cannot be collected, it is possible to sample workers from outer frames of top supers where older bees are more likely to reside, but this may increase sample heterogeneity (Fries et al. 2013). Unfortunately, sampling dead bees may actually underestimate the prevalence and intensity of infection (Stevanovic et al. 2013). However, collecting worker fecal matter from colonies allows diagnosis of *Nosema* spp. and potentially infection intensity (Copley et al. 2012, Stevanovic et al. 2013). If further developed and validated, these methods may circumvent time-intensive bee collections in addition to eliminating the need to kill colony members.

Establishing when to sample colonies also presents a challenge. Forager spore loads can fluctuate dramatically from one week to the next and even within the same day (Meana et al. 2010, Botías et al. 2012a). Also, as previously described, *N. apis* and *N. ceranae* colony levels may seasonally fluctuate (see section Global Distribution of *Nosema* spp. in Managed Honey Bee Colonies and Factors Contributing to Heterogeneity in Parasite Prevalence and Virulence).

Setting ETs and EILs

Setting ETs and EILs for *Nosema* spp. is especially challenging, as there is global variation in reports of *Nosema* virulence. Damage incurred by infestation may vary with climate, bee subspecies, the presence of other hive stressors, and potentially by *Nosema* strain (see section Global Distribution of *Nosema* spp. in Managed Honey Bee Colonies and Factors Contributing to Heterogeneity in Parasite Prevalence and Virulence). Studies to date have used spore counts or parasite DNA copy number to estimate parasite burden in individuals and colonies. However, parasite burden does not always directly correlate with morbidity and mortality. For example, protein-rich diets may enhance both worker longevity and parasite reproduction (see section Colony Management Practices). Furthermore, different populations of honey bees may be more tolerant of or resistant to infection (see sections Global Distribution of *Nosema* spp. in Managed Honey Bee Colonies and Factors Contributing to Heterogeneity in Parasite Prevalence and Virulence and Colony Management Practices). Tolerance traits allow hosts to carry parasite burdens without suffering the same infection costs as less tolerant individuals with commensurate infection levels. Resistance traits allow hosts to actively suppress infections, effectively reducing the number of parasites they carry (Schneider and Ayres 2008). Studies are desperately needed to determine at what point infection levels threaten colony survival, and how local conditions and host population traits may regionally affect thresholds for EILs. Given variation in *Nosema* virulence (see section Global Distribution of *Nosema* spp. in Managed Honey Bee Colonies and Factors Contributing to Heterogeneity in Parasite Prevalence and Virulence), researchers developing IPM-based management guidelines should consider how broadly these guidelines can be generalized.

Currently Available and Potential Future Nosema Treatments

Chemical Treatments

Chemical treatments against Noseomosis include fumagillin, application of bacteria-derived (surfactins, organic acids) or plant-derived (essential oils [EOs] and other plant products) compounds with fungicidal activity, and potentially the future use of genetic products (RNA interference, RNAi). These compounds may relieve heavy infestations in the short term, and some treatments may cross taxonomic boundaries to counter other honey bee pathogens, including bacteria and viruses. However, chemical treatments also have several drawbacks. These compounds inhibit active infections within bee midgut cells but will not kill spores contaminating colonies. After a treatment wears off, future applications may be necessary to prevent re-infection of the same colonies from these residual spores. Repeated application of the same treatment may select for resistant *Nosema* strains and be costly to beekeepers. These compounds may also have unintended, negative off-target effects for bees or humans exposed to treatments. Finally, only the effectiveness of fumagillin has been formally tested in multiple cage and field trials, and, as will be discussed below, there are still several unresolved issues regarding use of fumagillin products in hives. The effectiveness of plant- or bacteria-derived products and RNAi has primarily been studied in cage trials, with limited field studies to date. Clinical use of these chemicals must be thoroughly evaluated to ensure safety to both bees and humans in addition to efficacy in the field. Investigating chemical efficacy in the laboratory and then field represents a significant investment of time and resources. However, new screening technology, which measures chemicals' ability to inhibit *Nosema* replication in cell culture, may accelerate identification of candidate compounds for treating microsporidian parasites (Gisder and Genersch 2015).

Fumagillin

A recent publication has reviewed the pros and cons of fumagillin formulations (e.g. Fumagilin B; Medivet Pharmaceuticals Ltd, High River, Alberta, Canada) use against *Nosema* parasites in honey bees (van den Heever et al. 2014). Briefly, in 1949, fumagillin was obtained from the fungus *Aspergillus fumigatus* and discovered to have far-reaching antimicrobial properties. It has historically been deployed against *N. apis* in the commercial dicyclohexylamine (DCH) salt formulation, which is dissolved in sugar water. Both cage and field assays demonstrate that fumagillin application can control *Nosema* spp. infection with limited or no recorded negative effects

on bees and, in some cases, positive effects on colony survival and productivity (as summarized in van den Heever et al. 2014). However, *N. ceranae* infections can reemerge in colonies within 6 months of treatment, presumably due to lingering spores contaminating colonies or new infections introduced by drifting workers (Williams et al. 2008). The rate at which *Nosema* spp. infections reoccur is partly governed by how rapidly the fumagillin treatment is consumed by the colony and how quickly the chemical breaks down. Fumagillin is degraded by both heat and UV light exposure. Thus, chemical storage conditions and climate may affect the duration of *Nosema* control achieved. Furthermore, timing of fumagillin application (relative to honey removal) is restricted because fumagillin is toxic to humans.

Fumagillin targets a conserved protein (methionine aminopeptidase type 2) that is present in *Nosema* spp., bees, and humans (van den Heever et al. 2014). Due to fumagillin's nonspecificity and thus potential for human toxicity, its use is banned in the European Union (EU) barring "exceptional circumstances" and its handling must be monitored by a veterinarian. Where its employment is legal, fumagillin cannot be fed to colonies before a nectar flow, as some of the product may be sequestered in honey stores that will be taken for human consumption.

Recent research has highlighted potential problems associated with the expected degradation of fumagillin within colonies in addition to negative, off-target effects (Huang et al. 2013b). Cage studies showed that at decreasing concentrations of fumagillin (approximating fumagillin degradation over time in colonies), *N. apis* and *N. ceranae* are eventually able to begin reproducing. At low fumagillin concentrations, *N. ceranae* levels actually surpass those achieved in control workers that are never exposed to fumagillin. The authors cautiously suggest that excess rebound in *N. ceranae* but not *N. apis* populations at low fumagillin concentration may in part explain why *N. ceranae* appears to be supplanting *N. apis* in some regions. Worryingly, these findings also suggest that fumagillin may relieve *N. ceranae* infection in the short term but ultimately intensify disease. Furthermore, fumagillin alters protein production in worker midguts at treatment concentrations that do not repress either *Nosema* spp., suggesting that workers accrue off-target effects without gaining protection against *Nosema* infection as fumagillin degrades. However, these cage studies await validation from field trials.

Another important (and until recently) overlooked consideration of commercially prepared fumagillin is that individual components of the formulation may degrade at different rates (van den Heever et al. 2015). In the marketed salt formulation, fumagillin is the negative ion, while DCH is the positive ion. DCH alone is toxic to rats and can cause chromosomal changes in human cell cultures (summarized in van den Heever et al. 2014). Moreover, DCH is far more temperature-stable and degrades more slowly than fumagillin, which has implications for DCH's persistence in sequestered colony honey (van den Heever et al. 2015). Thus, given fumagillin's ubiquitous usage where legal, field studies are needed to determine if current recommendations and risk assessments for fumagillin use against *Nosema* spp. must be reconsidered.

Bacterial Metabolites

Antimicrobial molecules called surfactins produced by bacteria may also be used to treat *Nosema*. Surfactins have unique properties that allow them to create pores in cell membranes and the resulting perforations are lethal for targeted cells (Seydlová and Svobodová 2008). Biomedical research has identified many potential antifungal, antiviral, antitumor, and antibacterial therapies for surfactin use in humans (Seydlová and Svobodová 2008). Likewise, surfactin treatment (or other bacterially produced compounds) alone or in conjunction with EO application (see next section) inhibits growth of honey bee pathogens, including *Paenibacillus larvae*, the destructive bacterial cause of American Foulbrood (AFB), and *Ascosphaera apis*, the fungal agent of chalkbrood in laboratory growth assays (Sabaté et al. 2009, Sabaté et al. 2012). Similarly, studies have found evidence that surfactins can lower *Nosema* infection titers in the field. Feeding colonies surfactins produced by *Bacillus subtilis* bacteria isolated from honey samples reduces *N. ceranae* spore counts in inoculated workers (Porrini et al. 2010). In addition, organic (lactic) acids isolated from *Lactobacillus johnsonii* bacteria reduce spore loads in the field in conjunction with fumagillin (Maggi et al. 2013). These bacteria-derived organic acids did not cause acute mortality over 72 h, and workers in treated colonies had greater fat stores and lower spore loads than control colonies. In both these experiments, however, infected workers receiving treatment still carried thousands to several million spores. Thus, while some aspects of these results are promising, spore counts remain high even with treatment. Further development and testing is required to make sure these treatments alleviate disease costs without harming bees and that they are labor- and cost-efficient (e.g., *Nosema* control is achieved after a low number of treatments).

Essential Oils and Other Natural Compounds

EOs may represent another class of anti-microsporidian substances. EOs are aromatic blends of 20–60 components isolated from plants, with different plant species and even tissues yielding different oils (see Bakkali et al. 2008 for a review). EOs aid in plant defense against bacterial, viral, or fungal infection. EOs, especially menthol and thymol, are incorporated in a number of beekeeping products (e.g., Apiguard; Vita (Europe) Limited, Basingstoke, Hampshire, UK) used to control *Varroa* mites and serve as an alternative to manufactured chemicals such as tau-fluvalinate (Apistan; Wellmark International, Schaumburg, IL), coumaphos (Checkmite+; Bayer Healthcare LLC, Mississauga, Ontario, Canada), and amitraz (Apivar; Veto-pharma, New York, NY). These EO formulations are placed in colonies and release volatiles that create a toxic environment for mites.

Various EOs have also been shown to be effective at reducing *Nosema* spore loads. Feeding caged bees thymol suppresses *Nosema* reproduction while leaving worker life span unaffected or even extended (Maistrello et al. 2008, Costa et al. 2010). However, as in the case of bacterial metabolites, thymol application in cages significantly reduced spore counts but did not eliminate *Nosema* infection. For example, after 25 days of feeding workers thymol-laced syrup or control syrup, thymol-fed workers had an average of 60.2 ± 9.2 million spores, while control workers had an average of 118.1 ± 15.8 million spores (Costa et al. 2010). Importantly, field studies are needed to determine if thymol treatment reduces *Nosema* loads sufficiently to prevent the negative effects of *Nosema* infection. Several other plant extracts have also been tested for antimicrosporidian activity in bees [e.g., wormwood (*Artemisia absinthium*), garlic (*Allium sativum*), sweet bay (*Laurus nobilis*) yerba mate (*Ilex paraguariensis*), beet root (*Beta vulgaris*), oak bark (marketed as Nosevit Plus; completebee.com; distributed by Dadant & Sons, Hamilton, IL)] and lemongrass and spearmint oil (marketed as Honey-B-Healthy; Honey-B-Healthy, Inc, Cumberland, MD; Botías et al. 2013a, Porrini et al. 2011a, Rhoades 2011) with variable results.

Translating the results of these cage studies to the field is challenging. First, some extracts are not palatable to bees, and thus, there may be poor consumption of EO-supplemented food in the field when alternative food sources are available. Ensuring that bees have high, oral doses of these extracts may be difficult, expensive, and labor-intensive in colonies. Second, even if treatments reduce spore counts, it is essential to determine if EO treatment actually improves worker longevity and colony survival. Finally and importantly, additional research is needed to determine if EOs inflict unintended, sublethal costs in honey bees. For example, EOs can have cytotoxic effects on invertebrates, including mollusks and insect larvae (Bakkali et al. 2008). In bees, volatile exposure in a laboratory setting causes changes in expression of genes related to detoxification, immunity and behavioral maturation (Boncristiani et al. 2012). Furthermore, 24 hours of volatile exposure to thymol in a colony setting resulted in detectable levels of thymol in worker brains and altered worker phototactic behavior (Bergougnoux et al. 2013, Carayon et al. 2013). As oral or volatile exposure could result in different toxicity effects, additional studies are needed to characterize EO mechanisms related to *Nosema* control in honey bees under field-treatment conditions.

There are several possible mechanisms by which EO compounds may reduce *Nosema* spore loads. EOs penetrate mitochondrial membranes, causing a break-down of mitochondria function and release of toxic ROS, ultimately causing cell death (summarized in Bakkali et al. 2008). As discussed earlier, microsporidia siphon energy stores (ATP) from their honey bee hosts' mitochondria (see section Transmission of *Nosema apis* and *Nosema ceranae*, Microsporidian Parasites of Honey Bees), and thus, EO disruption of the mitochondria may limit microsporidian growth and reproduction. In addition, if EOs cause host cell death via ROS-mediated apoptosis, any associated, immature *Nosema* vegetative states would also die. Finally, ROS release serves as a basic invertebrate immune defense. Therefore, if EOs promote ROS release, honey bee defenses may be enhanced. Indeed, molecular studies suggest that enzymes involved in ROS production or oxidative stress are upregulated in gut tissue of workers infected with *N. ceranae* (see section *Nosema* spp. Virulence Factors and Host Defense Mechanisms). However, all of these mechanisms may damage both *Nosema* parasites and the host cells, and thus, there may be sublethal effects of EO treatment that could be mitigated if EOs were only used when necessary, as opposed to prophylactically.

Despite relatively little scientific investigation, EO formulations and other naturally derived or inspired compounds are available for use in colonies. These formulations may generally appeal to beekeepers, as organic farming practices are gaining mainstream interest and organic bee products may command a premium on the market. Also, as fumagillin formulations are banned in the EU, plant-derived products may serve as alternative therapies. As these formulations gain popularity, it is imperative that scientific research validate treatment efficacy and investigate potential negative effects on colonies.

RNA Interference (RNAi)

The comparative genome analysis of *N. ceranae* and *N. apis* led to the identification of parasite-specific genetic elements that are potentially related to virulence, which could be harnessed for developing RNAi-based therapeutics against *Nosema* diseases (Chen et al. 2013). Such RNAi technology would exploit antiviral defense mechanisms found in honey bees (as reviewed in Brutscher et al. 2015). By synthesizing and subsequently feeding bees double-stranded RNA (dsRNA) for a target gene sequence, it is possible to dramatically reduce RNA levels of the target gene. A previous study evaluating the feasibility of RNAi for controlling *N. ceranae* showed that ingestion of dsRNA homolog specific for *Nosema* ADP–ATP transporter reduced parasite load and potentially reduced host hunger levels, as indicated by host responsiveness to sugar water (Paldi et al. 2010). Similarly, RNAi has been successfully deployed to reduce levels of viruses in laboratory experiments (Maori et al. 2009, Liu et al. 2010, Desai et al. 2012, though field studies using treated colonies unfortunately did not evaluate effects on viral titer, Hunter et al. 2010) and to increase mortality of *Varroa* mites that are parasitizing treated hosts (Garbian et al. 2012). These results provide evidence that RNAi holds therapeutic potential for the treatment of *Nosema* parasites and other pathogens and parasites in honey bees. RNAi offers the advantage of target specificity, as dsRNA sequences would be unique to bee parasites, though previous studies have demonstrated off-target effects when longer dsRNA sequences are used (Nunes et al. 2013). Thus, follow-up studies would be needed to ensure that dsRNA exposure does not negatively affect molecular processes in all honey bee castes and life stages. Furthermore, dsRNA presumably would be administered orally, and studies are needed to determine how frequently treatments would need to be applied. Routinely feeding colonies large quantities of dsRNA might be costly and time-consuming.

Equipment Management and Sterilization Methods

Providing workers with new foundation is another way to limit infection (Bailey and Ball 1991). Because combs may accumulate spores over time, providing workers with fresh living-space substrate can reduce infection incidence. In addition, new foundation may also reduce other parasite and pesticide burdens within colonies. Unfortunately, maintaining enough undrawn combs to circulate through colonies represents an extra expense for beekeepers, as they will need to buy, store, and manually cycle combs. In addition, if workers are required to draw fresh comb, this may reduce colony productivity.

Another alternative or complementary strategy to chemically treating vegetative growth of *Nosema* parasites is to inactivate environmental spores by sterilizing hive equipment. Heating colony equipment (hive bodies and undrawn comb) to 120°F for 24 hours reduces subsequent *N. apis* infections (Cantwell and Shimanuki 1970) and theoretically heating colony equipment to high temperatures could also kill *N. ceranae* spores. Other sterilization methods include UV exposure, which can kill spores of both *Nosema* spp., and gamma radiation, which has been successfully shown to deactivate *N. apis* spores in liquid suspension (Katznelson and Robb 1962).

Naturally, all these sterilization methods pose some logistical challenges. For example, baking hive equipment to kill spores may be impractical, as wax from drawn frames will melt and heating facilities are required. Gamma radiation, however, has been previously used on a large scale in Australia to sterilize hive bodies and frames contaminated with *Bacillus larvae*, the highly virulent agent of AFB (Katznelson and Robb 1962, Hornitzky 1994). As gamma radiation does not damage hive equipment (materials are only heated ~3°C), it is perhaps the only viable, current method for hive sterilization. In sufficient doses, gamma radiation has the added and large benefit of eradicating other bee pathogens, including fungi, viruses, and bacteria. On the other hand, use of gamma radiation requires that bees are first removed from equipment before hive bodies can be sterilized. Also, materials must be transported to a

radiation facility, which can incur additional costs. As hive bodies and frames are expensive, gamma radiation can be cost-effective (as summarized in Hornitzky 1994). For example, some Australian states sponsored gamma radiation treatment for AFB eradication. Whether such programs would be effective in other countries depends on facility availability.

Colony Management Practices

In addition to chemical treatments and hive sterilization techniques, beekeepers may use colony management practices to mitigate Noseomosis, including selective breeding, queen replacement, and potentially nutrient supplementation.

Selective Breeding or Queen Replacement

Human-mediated selection may produce resistant or tolerant honey bee breeds. For example, Danish beekeepers have selected for a *N. ceranae*-tolerant strain of honey bees and genetic mapping has been used to identify chromosomal regions that underpin resilience to *Nosema* (Huang et al. 2012, Huang et al. 2014). Other studies provide further evidence of genetic resistance in sampled Russian subspecies (but not Italian) honey bees (Bourgeois et al. 2012a). In Uruguay, there are also reports that Africanized honey bees have more natural resistance to *N. ceranae* as well as black queen cell virus infections than Italian honey bees (Mendoza et al. 2014). These studies suggest that there is enough genetic variability in host populations to choose bee strains that are either tolerant of or resistant to Noseomosis.

Selecting for *Nosema* resistant or tolerant bees offers many long-term benefits. These bees would require fewer chemical applications to control *Nosema* infestations, saving beekeepers both time and money and reducing the chances of *Nosema* parasites developing resistance to available treatments. However, selective breeding programs drawing upon state-of-the-art scientific techniques can require intense resource commitments (Niño and Cameron Jasper 2015). For example, the Danish selection program was conducted over 20 years, started with 500 colonies, and required annual screening for *Nosema* and replacing the queens of susceptible colonies (Huang et al. 2013a). Another important consideration when breeding *Nosema*-resilient bees is whether the selected strains can withstand other colony stressors. For example, if selected colonies are always treated for *Varroa*, the resulting bees might be *Nosema*-resilient but susceptible to mite infestation. An ideal goal of a long-term breeding program might be to combine traits of bees that are resistant to unique stressors. For example, such a program might aim to combine traits from *Varroa*-resistant bees (Rinderer et al. 2010) with *Nosema*-resistant or -tolerant bees.

Selective breeding represents a long-term strategy for *Nosema* management and would be a large task for an individual beekeeper to undertake. However, inducing queen replacement may serve as a short-term measure that beekeepers can use with other tactics to control *Nosema*. Honey bee queens generally live 1–3 yr but their fecundity declines over their lifetime (Page Jr and Peng 2001). As *Nosema* causes premature worker death, queen fertility is important, as lost workers must be replaced. Researchers tested whether forcing *Nosema*-infested colonies to rear new and potentially more fertile queens could ultimately reduce *N. ceranae* and *N. apis* infestation (Botías et al. 2012c). Queen replacement did reduce the percentage of parasitized foragers in colonies. However, there were a number of short-term, negative effects, including a reduction in the number of adult bees and, in some cases, lower food stores from reduced foraging rates. Breaking the brood cycle with forced queen replacement might be beneficial in controlling brood parasites, including *Varroa* mites, but the reduced food stores may render the colony more sensitive to other stressors. Thus, beekeepers must use this strategy carefully, during peak blooming periods with abundant nutritional resources, which would allow the colony to recover.

Nutrient Supplementation

Improving overall colony nutrition may help bees cope with multiple abiotic and biotic stressors, including *Nosema* spp. Honey bees derive all requisite nutrients from consumption of nectar and pollen. Nectar serves as an important source of carbohydrates and other micronutrients, while pollen provides protein, fats (including essential sterols), vitamins, and minerals. As honey bees are generalists, diverse (multifloral) nectar and pollen promotes colony health (Vaudo et al. 2015).

Multiple studies have underscored the role of pollen (or protein supplements) in adult worker health and longevity (as reviewed in Huang 2012, DeGrandi-Hoffman and Chen 2015). For example, caged workers fed on polyfloral pollen diets exhibit higher constitutive levels of glucose oxidase, an enzyme with antimicrobial properties, compared with workers fed on monofloral diets (Alaux et al. 2010a). In addition, caged pollen-fed or protein-supplement-fed workers had lower titers of naturally acquired deformed wing virus infections than workers fed on sugar water alone, suggesting that protein augments worker ability to suppress viral infection (DeGrandi-Hoffman et al. 2010). Moreover, pollen ingestion activates detoxification molecular pathways in caged adult workers (Schmehl et al. 2014). These pathways also help workers process ingested pesticides, and thus, diets incorporating pollen can improve worker survival after pesticide exposure.

Several cage experiments have highlighted a complex relationship between *Nosema* parasitism, worker nutrition, and severity of *Noseomosis* symptoms. In short, when caged workers were fed protein diets (pollen, beebread, or other protein or vitamin supplement) as opposed to sugar water alone, protein access generally increased the longevity of caged workers and the *Nosema* spore loads in infected workers (Rinderer and Dell Elliott 1977, Porrini et al. 2011b, Basualdo et al. 2014, Zheng et al. 2014). Degree of life span extension as well as spore production varied with pollen–protein supplement. For example, both increased pollen diversity and pollen quality (protein content) improved survival of workers infected with *N. ceranae* (Di Pasquale et al. 2013). Meanwhile, in a separate experiment, *N. ceranae* spore loads were greater in bees fed on some commercial protein diets compared with wildflower pollen and other commercial diets, though potential seasonal effects were noted (Fleming et al. 2015). These studies suggest that good nutrition improves workers' ability to tolerate rather than resist (actively suppress) infection (Schneider and Ayres 2008).

These cage experiments have interesting implications for colony health. Results suggest that supplementing colonies with polyfloral or high protein content pollen might improve worker longevity even in the presence of *Nosema*. However, pollen supplementation might also raise spore counts, potentially increasing parasite transmission within and between hives. Unfortunately, few studies have examined the relationship between pollen availability and outcomes of *Nosema* infection in the field. One study assessed the effects of *N. apis* and pollen supplementation on individual worker longevity in the field and found that worker longevity can be strongly affected by colony context and does not always reflect predictions based on cage studies (Mattila and Otis 2006). However, when colony level, as opposed to individual performance, is assessed, field studies show

that colonies given access to rapini forage versus protein supplement have lower levels of *Nosema* and black queen cell virus (DeGrandi-Hoffman et al. 2015). Clearly, adequate nutrition is essential to colony health and productivity, but additional field studies are needed to determine when nutritional supplementation in the field can ameliorate *Nosema* infections in addition to the best type of nutritional supplementation to provide.

Summary and Future Directions

Here, we discussed the current state of research on Noseomosis etiology, diagnosis, and management and summarized challenges to creating IPM-based management strategies for *Nosema* spp. in honey bee colonies. First, beekeepers need science-backed recommendations for when and how to sample for *Nosema* infection and accessible and time-saving methods for detecting parasites. Molecular technology such as dipstick tests that yield both estimates of infection loads and identification of *Nosema* spp. would provide an affordable diagnostic method to beekeepers. Next, beekeepers need science-based directives for when to treat *Nosema* infections. Given the global variability in reports of damage inflicted by *Nosema* parasites and the inconstant nature of spore loads in colony samples, establishing treatment thresholds will require intensive field studies to establish the *Nosema* infection thresholds that lead to negative impacts at the colony level. Furthermore, novel interventions for Noseomosis management are needed. Fumagillin is currently the only well-studied treatment that reduces spore loads in colonies, but new research indicates that it may have problematic long-term effects on parasite populations and unintended health consequences for bees (and humans). Furthermore, fumagillin use is banned in some regions. New molecular-based therapies such as RNAi or microbe- or plant-derived compounds may suppress *Nosema* reproduction while limiting the risks to both bees and humans. However, these new treatment options are in the early stages of development and require rigorous tests of efficacy and investigation of nontarget effects in the field before recommendations can be made. A challenge posed by all chemical interventions (existing or hypothetical) to-date is that only parasite vegetative states are targeted, meaning that *Nosema* diseases may re-emerge from reservoir spores contaminating colony equipment after treatments wear off. Thus, the number of times treatments must be applied to effectively suppress infection must be factored into cost-effectiveness analyses and management plans to reduce the chances of selection for treatment-resistant *Nosema* strains. Gamma radiation could be used to deactivate spores contaminating colony equipment; however, this management option presents logistical challenges. Finally, due to worldwide heterogeneity in disease prevalence and virulence, future treatment recommendations may not be universally applicable. If pursued in the future, creation of regionally appropriate management guidelines will require intense research efforts and resource commitments but may avert unnecessary treatments where *Nosema* spp. are prevalent but not associated with colony losses.

Preventative measures to manage *Nosema* infection include honey bee breeding programs. If successfully implemented at large scale, selective breeding could help bees to cope with multiple stressors and would reduce the overall need to treat colonies, providing long-term cost-savings. However, comprehensive selective breeding would require a large initial input of resources and continued government and scientific oversight to maintain funding and ensure efficacy. Other management efforts to improve honey bee nutrition through landscape diversification will likely yield additional returns by benefiting both managed and native pollinator populations that face many of the same or similar stressors imposed on honey bees.

Knowledge gained through characterizing *Nosema* infection in honey bees and successful development and implementation of management practices may serve as a template for managing other honey bee diseases, including those caused by other emerging, recently classified intestinal parasites. Indeed, recent reports indicate that the trypanosomal parasite *Lotmaria passim* is globally distributed in European honey bees and may be linked to colony loss (Schwarz et al. 2015). Improved *Nosema* control may also benefit wild pollinators by reducing pathogen spillover or providing means to help control microsporidia in other pollinator populations (Graystock et al. 2013, Furst et al. 2014, Arbulo et al. 2015). Finally, honey bees and their microsporidian parasites may serve as a model disease system for human microsporidiosis (Fayer and Santin-Duran 2014), and knowledge gained through honey bee pathology studies may be translated to human medical practices.

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