Microsporidia: an emerging threat to bumblebees?

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Abstract

Microsporidia may be emerging infectious diseases (EIDs) in bumblebees. Two drivers – commercial bumblebees and managed honey bees – have been identified as possible sources of pathogen spillover. In addition, declines in bumblebee populations may have led to lower genetic diversity and subsequent higher susceptibility to infection, enabling microsporidia to increase in prevalence. There is strong evidence for relatively recent increases in the prevalence of *Nosema bombi* in North America. However, the lack of definitive data on spillover by microsporidia, in North America or elsewhere, makes it difficult to identify the causes of such increases. Phylo-genomic studies are urgently needed to identify the global population structure of microsporidia in bumblebees, and thus identify the source of current and future epidemics.

Microsporidia in bumblebees – where? when? why? how?

Microsporidia (see Glossary) were first recorded in bumblebees in 1913 [1,2]. Nearly a century later, they are at the heart of a controversy about the role of **emerging infectious diseases** (EIDs) in driving bumblebee declines [3,4]. Bumblebees are important pollinators across temperate, alpine, and arctic regions for a range of crops and wildflowers [5,6], and so understanding why they are declining is an important question for the sustainability of agriculture and natural ecosystems [7]. A recent study [8] provided intriguing evidence that microsporidia might be involved in these declines. However, determining whether microsporidian infections and disease have become more common in bumblebee populations, with concomitant higher impacts, as the emerging infectious disease hypothesis requires, is complicated by underlying uncertainties about the identity, diversity, and impact of these pathogens. Here, I assess what is known about microsporidia in bumblebees, and identify issues that are holding back our understanding of this host-pathogen interaction.

The diversity of Microsporidia in bumblebees

An array of microsporidia, including *Nosema apis*, *Nosema bombi*, *Nosema ceranae*, *Nosema portugal*, *Nosema thomsoni*, *Tubulinosema pampeana*, and *Vairimorpha* spp., have been shown or suggested to infect bumble bees [1,2,9-12]. Consequently, assessing prevalence, and possible changes in prevalence, requires accurate identification of the infectious agent. Prior to the development of molecular tools [13], nearly all microsporidian infections in bumblebees were identified as *N. bombi* (Table 1). Best practice at the time meant that identifications should have been based on the presence of spores (and other life-stages) in the

Malpighian tubules, the main site of infection identified in the original description of the species [2]. However, many studies fail to detail their screening method for *Nosema* in sufficient detail, and combined with the controversy about whether *N. apis* can infect bumblebees [1,14,15], and the recent discovery of *N. ceranae* and *T. pampeana* infections [9,11], this makes the species identification in many earlier studies uncertain (Table 1, Table 2). Since the development of molecular tools it has been possible to combine dissection and microscopy with molecular screening, to produce definitive accounts of prevalence [e.g., 8,16,17]. However, at the same time the use of molecular screening on its own has resulted in studies that measure the presence and absence of pathogen DNA, without determining if this represents a true infection [10,12,18-20](Table 1, Table 2). As false positives can be generated by pathogen spores that are being vectored, or that have been ingested, or by mis-priming during PCR reactions, interpretation of prevalence based on molecular screening alone is problematic.

In terms of assessing prevalence in the field, *N. bombi* [2,13,21], *N. ceranae* [9,22], and *T. pampeana* [11] are the only microsporidia that have been definitively shown to infect wild bumblebees (Figure 1). Whether *N. bombi* as known today is the same as the original microsporidian described under this name [2], and reported in subsequent microscopy studies, is unlikely to be resolved, although descriptions of tissue specificity make this likely. Hereafter, I will assume that the species identification given by authors for microsporidian infections is accurate, whilst bearing in mind the caveats detailed above.

Impact of microsporidians in bumblebees

One reason that *N. bombi* and *N. ceranae* raise concern as potential causal agents of EIDs is their apparently high virulence. Obviously, an EID with low impact is unlikely to be a driver of host population declines. Whittington and Winston [23] reported the suggestion, by bumblebee suppliers, that *N. bombi* may have been behind the collapse of commercial *B. occidentalis* breeding in the late 1990s. However, in a correlational experiment they found no impact of *N. bombi* on commercially-sourced infected colonies [23], which would appear to contradict this claim. In contrast, recent experimental studies have demonstrated significant negative impacts on individual health and colony-level reproductive fitness by *N. bombi* [24-27], and on individuals by *N. ceranae* [28, but see 19]. All of these studies have been conducted on either *Bombus lucorum* or *Bombus terrestris*, two common Palearctic species, with the latter being one of the main species produced commercially for pollination services

[29]. Interestingly, the virulence of *N. bombi* appears to vary across these two host species [27]. Whether impacts vary across the other ~250 species of bumblebee remains to be determined, but given their wide range in life history, this seems likely. In addition, whether commercial rearing has selected for more virulent strains of the parasite, or changes in tolerance/susceptibility in the host, remains an open question. The impact of *T. pampeana* has yet to be investigated. In addition, how or if impacts at the level of individual colonies translate into changes in inter-annual population dynamics in the field remains unexplored.

Patterns of prevalence of microsporidians in bumblebees

Bumblebees, like other eusocial insects, comprise three classes of individuals – males, queens and workers – and studies have suggested variation across these in the prevalence of *N. bombi* [reviewed by 30]. However, measuring prevalence is not trivial. Queens are available for sampling for a relatively short period after hibernation, and thus spot samples are likely to produce a relatively good measure of prevalence. In contrast, workers and males are produced over a period of months. The seasonal progression of the annual *Nosema* epidemic, both within [31] and among [32] colonies, poses a challenge to generating a meaningful assessment of prevalence in workers or males and making comparisons across species or years. Nevertheless, most studies have focused on workers as they are more abundant, and collecting them puts less pressure on declining bumblebee populations (Figure 2).

The concept of microsporidians as causal agents of EIDs lies behind the question of whether microsporidian infection and disease, in particular *N. bombi* and *N. ceranae*, is increasing in bumblebees. Thorp and Shepherd [www.xerces.org/Pollinator_Red_List/Bees/Bombus_Bombus.pdf] suggested, based on the putatively *Nosema*-driven collapse of commercial breeding for *B. occidentalis*, and concomitant declines in native bumblebee populations in North America, that *N. bombi* might be the causal agent of an EID in North America. This was backed up by reports of high *N. bombi* prevalence in declining bumblebee species in the USA [16]. The basis for similar interpretions of *N. ceranae* rely on an association of its presence in UK bumble bees with its presence in European honey bees [19], and the idea that *N. ceranae* is also driving an EID in the European honey bee, *Apis mellifera* [reviewed in 33].

If commercial distribution and use of bumblebees for pollination is a driver of microsporidian emergence in wild populations (either through **spillover** or **spillback**), one way to assess this

(albeit crudely) is to examine datasets collected pre- and post-commercialisation. *N. bombi*, as identified by microscopy, was present in Europe (Denmark, Switzerland, UK), New Zealand, and North America, with prevalences varying from 0-100% in spring queens, 0-55% in workers, and 0-50% in males, prior to commercialisation [reviewed by 30](Figure 2). Studies post-commercialisation in Europe [34] and North America [8,16,35-38] show similar prevalence ranges in queens and workers (Figure 2). Given that *N. bombi* has largely been suggested to be an EID in North America [www.xerces.org/Pollinator_Red_List/Bees/Bombus_Bombus.pdf], a fairer comparison might be between studies in North America pre- and post-commercial production of bumblebees. Unfortunately, only two studies of *N. bombi* from North America prior to commercialisation exist [39,40]. Interestingly, both report low levels of infection (<5% in [39]).

Can space be a substitute for time? If microsporidians cause EIDs, then they should exibit higher prevalence in areas nearer to the proposed source population (commercial bumble bees for *N. bombi*, managed honey bees for *N. ceranae*). Colla et al. [41] found *N. bombi* in 14% of bumble bees next to a Canadian greenhouse using commercial *B. impatiens*, as opposed to <4% of bees at non-greenhouse sites. However, a second greenhouse site had no infected bees, making this result hard to interpret. In a larger-scale study, Murray et al. [42] showed a gradual decline of *N. bombi* prevalence in male *B. terrestris* as distance from Irish strawberry farms using commercial bumble bees increased (prevalence in workers showed no trend in either direction). This could be interpreted as increased transmission, and thus prevalence of the microsporidian near commercial operations (that is, pathogen spillover or spillback), or alternatively as commercial males from infected colonies exhibiting **philopatry** (although current evidence of male dispersal argues against this [43]). Whitehorn et al. [44] found generally low prevalence of *N. bombi* in bumblebee workers around Scottish fruit farms, irrespective of whether they were using commercial bumblebees or not, a result reflected in a study of fruit farms in England [45]. One reason for these contrasting results may be variation across studies in the time elapsed between the placement of commercial colonies and the sampling of wild bees for prevalence, as transmission is a dynamic and potentially rapid process. Overall, there is no definitive evidence that *N. bombi* infections are higher in areas where managed bumblebees are present. An important, and currently unanswered question here is what the actual levels of microsporidian infection in commercial colonies are. Graystock et al. [46] found both *N. bombi* and *N. ceranae* infections in commercial colonies

ordered between 2011-2012 from three producers in Europe, while Sachman-Ruiz et al [47] found *N. bombi* in commercial colonies in Mexico (Table 2). Since then, at least some producers have responded by improving their in-house screening protocols with the aim of producing disease-free colonies. We currently lack knowledge of microsporidian levels in commercial colonies at a global scale, which is essential for understanding potential current and future EID threats.

Data outside *N. bombi* are scarce. Fürst et al [19] showed that, in areas with relatively low *N. ceranae* prevalence, patterns in bumblebees in the UK match those of honey bees, suggesting spillover from honeybees to bumblebees of this microsporidian. However, this pattern disappeared at higher levels of infection. Finally, Graystock et al. [45] found that *N. ceranae* prevalence increased away from greenhouse sites that were not using commercial bumblebees. No obvious explanation for this pattern exists. Further work is needed to show whether *N. ceranae* actively passes from honey bees to bumblebees in the field, and whether this in turns leads to higher prevalence in bumblebees.

The most obvious way to address whether microsporidia are emerging in bumblebees is to look at time series data, and a study doing this for *N. bombi* has recently been published. Cameron et al. [8] used museum specimens to screen North American bumblebee species that had previously been identified as in sharp decline and with current high prevalence levels of *N. bombi* [16]. They found a significant increase in prevalence of *N. bombi* across five species, occurring in the mid- to late-1990s [8]. This matches the timescale of decline in these species [16], and thus could be argued to be indicative of an EID. However, before drawing such a conclusion, the next question has to be, where did the *N. bombi* infecting these declining hosts come from? And this itself raises the question of genetic variation within *Nosema* species and its local and global distribution.

Genetic variation in *Nosema* spp. in bumblebees

The first study of genetic variation in *N. bombi*, using the rRNA gene, found significant variation (both **SNPs** and **indels**), but no evidence that this variation was partitioned among infections either geographically (across Europe) or across host species [48]. A similar study in North America identified one more allele of the same gene, but again found no partitioning of variation across space or species [17]. In contrast to these studies, which only identified *N. bombi* infections, Li et al. [10] screened bumblebees from across China using the same gene,

and identified *N. bombi, N. ceranae,* four related clusters, and additional *Nosema* spp.. However, in the absence of dissection data, it is not clear which of these represent real infections as opposed to contamination or vectoring. Given the relative frequency of sequences, it seems likely that Chinese bumblebees were infected by *N. bombi*, *N. ceranae*, and four clusters labelled *Nosema* A-D. Again, there was no phylogeographic structure in the presence of these sequences across host species [10]. Further work by Vavilova et al [20] combined these data with new sequences sampled in Western Siberia (no proof of infection status was given), suggesting that sequence clusters A-C identified by [10] are closely related to, or part of *N. ceranae. Nosema D*, and three new clusters unique so far to Western Siberia (WSP1-3) belong to the *N. bombi* clade [20]. In addition to the rRNA gene, this study utilised the *MetAP2* gene to identify sequence variants, stressing the need for a multi-locus approach to *Nosema* phylogeography. Again, due to sampling, there were no clear patterns of variation across host species or geographical location. Cameron et al. [8] used the rRNA gene, combining sequences from across Europe, North America, and China (taken from [10]), and largely confirmed the results of [20], despite the sequences from Western Siberia not being used in their analysis. Interestingly, sequences from European and North American (both museum and modern) isolates fell almost completely into a single clade, which also contained sequences from China. Cameron et al. [8] also conducted a genome-wide analysis using reduced representation genome sequencing. However, this approach still only identified low levels of variation across sequences, and no differentiation across North American and European isolates (with most variation occurring within regions).

What do these genetic data tell us? If we take them at face-value, they provide evidence of distinct lineages within both *N. bombi* and *N. ceranae*, but no evidence of geographical or host-species structure to their distribution. This, in turn, argues either (i) against microsporidia causing EIDs, or (ii) for a recent rapid expansion of, or selective sweep within microsporidian populations across Europe and North America, which could be evidence for the EID hypothesis. However, sampling issues – in terms of geography, host species, and identification of real infections - severely limit the conclusions we can draw. To understand the dynamics of microsporidians in bumblebees, global phylogenomic studies across multiple host species, with a special focus on areas where commercial bumblebees or honey bees are not present, are urgently needed. Genomic data may allow the identification of rapid population expansions, or selective sweeps, both of which would provide evidence that microsporidia are functioning as EIDs. The rapid spread of *N. bombi*-infected *B. terrestris* in

South America may provide a unique opportunity to determine if and how *N. bombi* crosses species barriers and spreads in native bumblebee populations [49].

Concluding remarks

Despite recent studies, too many key questions remain unanswered (see Outstanding Questions). Are commercial bumblebees and honey bees past, present, or future sources of microsporidian infection for wild bumblebee populations? If so, does this rely on spill-over or spill-back dynamics? Has selection in commercial breeding operations selected for higher virulence or transmission in *N. bombi.* Has the commercial movement of bumblebees and honey bees homogenised phylogeographic and host-specific patterns in wild microsporidian populations? How does the virulence of microsporidia vary across host species, and how does this translate into population-level impacts? These questions call for an array of well-designed field and laboratory experiments, as well as global, host-species-rich phylogenomic studies. An integral feature of such studies must be the incorporation of dissection/microscopy techniques to identify the presence of real infections, or the development of molecular tools that differentiate between presence/absence and actual infection. As scientists increasingly turn to molecular methods for measuring prevalence, data quantity cannot be allowed to compromise the essential data quality needed if we are to understand the dynamics of disease in our wild pollinators.

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Figure legends

Figure 1. Timeline of important events in our understanding of microsporidia in bumble bees.

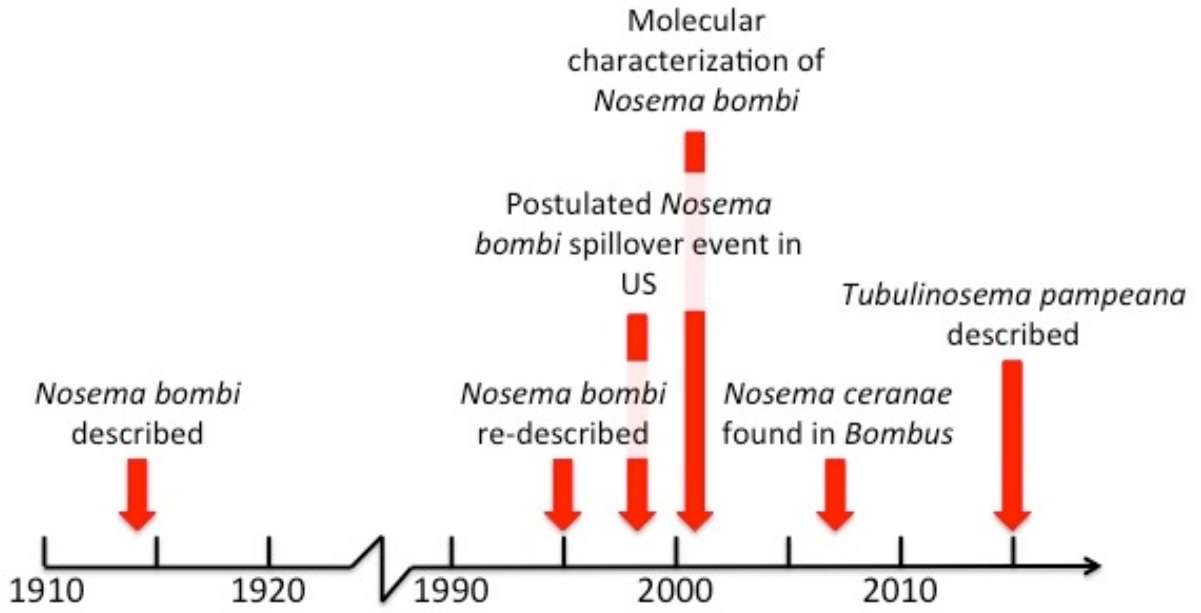

Figure 2. Reports of microsporidian prevalence pre- and post-commercial use of bumble bees. Each bar shows the prevalence range found in a specific study for a specific caste of bumblebee.

424 **Table 1. Records of *Nosema* spp. infection in wild bumblebees[a].**

Year	Country	Microscopy	Molecular	*N. apis*	*N. bombi*	*N. ceranae*	*T. pampeana*	Other	Definitive infection[b]	Reference
2015	Argentina	Yes	Yes	No	-	Yes	-	-	Yes	[50]
2014	USA	No	Yes	-[d]	Yes	-	-	-	No	[51]
2013	Germany	No	Yes	-	Yes	-	-	-	No	[52]
2013	Colombia	No	Yes	No	No	Yes	-	-	No	[53]
2013	Korea	Yes	Yes	No	Yes	No	-	-	Yes	[54]
2009-13	Argentina	Yes	Yes	-	-	-	Yes	-	Yes	[11]
2011-13	USA	No	Yes	-	Yes	-	-	-	No	[38]
2011	USA	Yes	Yes	-	Yes	-	-	-	Yes	[37]
2011	UK	Yes	No	-	Yes	-	-	-	Yes	[34]
2011	UK	Yes	Yes	-	-	Yes	-	-	Yes	[28,45]
2011	UK	No	Yes	Yes	Yes	-	-	-	No	[28,45]
2010	Uruguay	No	Yes	No	No	Yes	-	-	Yes	[55]
2004, 10-12	Chile	No	Yes	No	Yes	No	-	Yes	No	[12]
2011	UK	No	Yes	-	-	Yes	-	-	No	[19]
2010	UK	Yes	No	-	Yes	-	-	-	No	[44]
2010	USA	Yes	Yes	-	Yes	-	-	-	No	[36]
2010	UK	Yes	No	-	Yes	-	-	-	No	[56]
2009	Sweden	Yes	Yes	-	Yes	-	-	-	No	[57]
2008	Ireland	Yes	No	-	Yes	-	-	-	No	[42]
2008	China	No	Yes	-	Yes	Yes	-	Yes	No	[10]
2007-8	Russia	No [c]	Yes	-	Yes	-	-	-	No	[20]
2007-8	USA	Yes	Yes	No identification to species given					No	[58]
2007-9	USA	Yes	Yes	-	Yes	-	-	-	Yes	[16,17]
2006-7	USA	Yes	Yes	-	Yes	-	-	-	Yes	[35]
2006-7	USA	Yes	No	No identification to species level					No	[59]
2005	UK	Yes	No	-	Yes	-	-	-	Yes	[60]
2004-8	Poland, Russia	No	Yes	-	Yes	-	-	-	No	[18]
2004-5	Canada	Yes	No	-	Yes	-	-	-	No	[41]
2003-5	Denmark, Sweden	Yes	No	-	Yes	-	-	Possibly	Yes	[61]
2003	Ireland	Yes	No	-	Yes	-	-	-	Yes	[62]
2002-3, 8	USA	Yes	Yes	-	Yes	-	-	-	Yes	[63]
2001	Switzerland	Yes	No	-	Yes	-	-	-	-	[64]
200?	Denmark, Ireland, Netherlands, Sweden, Switzerland, UK	Yes	Yes	-	Yes	-	-	-	Yes	[48]

1998-9	Switzerland	Yes	No	-	Yes	-	-	-	No	[65]
1996-8	Turkey	Yes	No	-	Yes	-	-	-	No	[66]
199?	New Zealand	Yes	No	-	Yes	-	-	-	Yes	[21]
1987, 2005-8	Argentina	Yes	Yes	-	-	Yes	-	-	Yes	[9]
1986-7	New Zealand	Yes	No	-	Yes	-	-	-	No	[67]
197?	Canada	?	No	-	Yes	-	-	-	?	[39]
1962	Denmark	Yes	No	-	Yes	-	-	-	No	[68]
194?	Canada	Yes	No	-	Yes	-	-	-	Yes	[38]
191?	UK	?	No	-	Yes	-	-	-	?	[69]
191?	UK	Yes	No	-	Yes	-	-	-	Yes	[2]

[a]References were assessed for methodology, pathogen species identified, and whether this identification could be viewed as definitive (based on the methods given). [b]Either correct tissue screened microscopically, or infection shown microscopically and species confirmed molecularly, or determined molecularly and intensity quantified at levels indicative of infections. [c]Microscopy used, but unclear on what specimens and not part of screening. [d]Abbreviations:-, species not screened for; ?, lack of data through inability to access report.

Table 2. Records of *Nosema* spp. infection in commercial bumblebees[a].

Year	Country	Microscopy	Molecular	*N. apis*	*N. bombi*	*N. ceranae*	*T. pampeana*	Other	Definitive infection[b]	Reference
201?	Mexico	No	Yes	-	*Yes*	-	-	-	No	[47]
2011-12	UK	Yes	Yes	No	Yes	Yes	-	-	No	[46]
2011-12	UK	Yes	Yes	No	No	Yes	-	-	Yes	[28,45]
2011-12	UK	No	Yes	Yes	Yes	No	-	-	No	[28,45]
2008	Ireland	Yes	No	-	Yes	-	-	-	No	[42]
2002	Canada	Yes	No	-	Yes	-	-	-	No	[23]
200?	Japan	Yes	No	-	Yes	-	-	-	Yes	[70]

[a]Papers were assessed for methodology, pathogen species identified, and whether this identification could be viewed as definitive (based on the methods given). [b]Either correct tissue screened microscopically, or infection shown microscopically and species confirmed molecularly. [c]Abbreviations: -, species not screened for.