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Review

Nosema ceranae in *Apis mellifera*: a 12 years postdetection *perspective*

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Summary

Nosema ceranae **is a hot topic in honey bee health as reflected by numerous papers published every year. This review presents an update of the knowledge generated in the last 12 years in the field of *N. ceranae* research, addressing the routes of transmission, population structure and genetic diversity. This includes description of how the infection modifies the honey bee's metabolism, the immune response and other vital functions. The effects on individual honey bees will have a direct impact on the colony by leading to losses in the adult's population. The absence of clear clinical signs could keep the infection unnoticed by the beekeeper for long periods. The influence of the environmental conditions, beekeeping practices, bee genetics and the interaction with pesticides and other pathogens will have a direct influence on the prognosis of the disease. This review is approached from the point of view of the Mediterranean countries where the professional beekeeping has a high representation and where this pathogen is reported as an important threat.**

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Introduction

Two different microsporidia affect the honey bee (*Apis mellifera L.*) causing nosemosis: the historical well known *Nosema apis*, responsible for nosemosis type A and *Nosema ceranae*, responsible for nosemosis type C (Higes *et al.*, 2010a). Both microsporidia are obligate intracellular eukaryotic parasites, nowadays classified as fungi (Adl *et al.*, 2005). These species differ in spore morphology (Ptaszynska *et al.*, 2014), genome size (Cornman *et al.*, 2009; Chen *et al.*, 2013; Pelin *et al.*, 2015), ability to adapt to temperature, both in terms of spore production (Higes *et al.*, 2010b) and survival (Higes *et al.*, 2007; Martín-Hernández *et al.*, 2007), and effects on the host (Martín-Hernández *et al.*, 2011; van der Zee *et al.*, 2014). Their pathological effects in the field are also different. Nosemosis type A is characterised by the presence of faecal spots inside and outside the hive, weak crawling bees, reduced honey yield, increased winter mortality and a slow build-up in spring (Fries, 1993). Conversely, nosemosis type C has been associated with reduced honey production, weakness and increased colony mortality (Higes *et al.*, 2008a,a; Paxton, 2010; Botías *et al.*, 2013), in most of cases in

the absence of other signs associated with nosemosis type A. Recently, a new species of *Nosema*, phylogenetically related to *N. apis* and named *Nosema neumanni* was identified in Uganda (Chemurot *et al*., 2017). So far, no specific clinical signs have been associated to this new microsporidia and, therefore, this species has not been yet reported to any disease.

N. apis was initially identified in Australia, North America and Europe, but it has now been reported on every continent (Furgala and Mussen, 1990). There is considerable variation in the prevalence in the different countries, probably related to the scale and time of sampling. For example, Farrar (1947) found high prevalence in queens analysed in 'package bees' and Doull (1961) observed that *N. apis* was present in all hives at all sampling dates in Southern Australia. Colony surveys of the past century show that the prevalence of *N. apis* tended to be higher in the later years, which is most likely due to the improvements in monitoring over time. Concerning *N. ceranae*, it was first described in the Asian honey bee (*Apis cerana*) in the 1990s (Fries *et al*., 1996) and later detected almost simultaneously in honey bees in Europe and Asia (Higes *et al*., 2006; Huang *et al*., 2007) and later in honey bees worldwide becoming a globally distributed pathogen (Higes *et al*., 2006; 2010a; Huang *et al*., 2007; Fries, 2010; Medici *et al*., 2012). Currently, *N. ceranae* is considered a pathogen causing important colony losses, especially given its sharply enlarged geographical range in recent years (Klee *et al*., 2007; Martín-Hernández *et al*., 2007). Regarding *N. neumanii*, no information about its distribution or prevalence has been reported so far.

In the last decade, detection of *N. ceranae* infection in honey bees has increased worldwide and most specifically in Southern European countries (Stevanovic *et al*., 2011). By contrast, in northern European countries, *N. apis* is still predominant over *N. ceranae* (Forsgren and Fries, 2013; Blažytė-Čereškienė *et al*., 2016). The first description of *N. ceranae* (Fries *et al*., 1996) did not include information about its impact on Asian honey bee health. In the last years, the knowledge of this parasite has increased exponentially. As an example, in 2010 there were 83 published papers focusing in this microsporidia species and currently there are more than 400 (Source: Scopus). However, despite this progress, it is still a challenge for scientists working in the fields of apiculture and insect pathology to carry out research on *Nosema* for several reasons:

(i) The range and prevalence of *N. ceranae* has increased significantly in the past decade, with different consequences in Northern and Southern temperate areas;
(ii) *Nosema* species can only be confirmed using molecular tools;
(iii) The clinical signs of *N. apis* and *N. ceranae* infection are distinct;
(iv) *N. ceranae* infection is detectable in both healthy and declining honey bee colonies, and thus, its overall contribution to honey bee losses is debatable;
(v) The impact of the newly described *N. neumanni* on colony health is still unknown, as is its potential effect on honey bees or its geographical distribution.

The aim of this review is to provide a state-of-the-art in the main field of *N. ceranae* research, focusing on its routes of transmission, its effect on the prevalence of *N. apis*, its population structure and genetic diversity, and its effect on honey bees at both the individual and colony levels.

First detection and dispersion of an emergent parasite in honey bees

N. ceranae was first detected in *A. cerana* at the end of the XXst Century (Fries *et al*., 1996), and then in *A. mellifera* in Taiwan and Spain in the early XXIst Century (Higes *et al*., 2006; Huang *et al*., 2007). After some initial doubts, *N. ceranae* is now considered a predominant infective agent of *A. mellifera* that is related to high colony losses in the Mediterranean countries (Higes *et al*., 2008a; Bacandritsos *et al*., 2010; Hatjina *et al*., 2011; Soroker *et al*., 2011; Lodesani *et al*., 2014). Indeed, the detection of *N. ceranae* in Spain did not occur by chance but rather was a response to the demands of professional beekeepers. In 2004, there was a high number of requests for pathogen analysis to the Official Honey Bee Laboratory at Marchamalo (Spain) due to colony losses, with well-experienced beekeepers reporting only empty hives or very weak colonies. The prevalence of *N. ceranae* in those colonies was close to 90% almost all year round, from 2004 to 2006 (Martín-Hernández *et al*., 2007).

The original host of *N. ceranae* is unknown but it is generally presumed to be *A. cerana*, from which it was first isolated in 1996 (Fries *et al*., 1996). However, recent analyses of historical samples detected *N. ceranae* in the Asian *A. cerana* and *A. dorsata*, in workers from Taiwan, as early as 1968, and in *A. mellifera*, in workers from the USA (Traver and Fell, 2015) and Brazil (Teixeira *et al*., 2013), as early as 1975 and 1979 respectively. After its initial detection in 2005 (Higes *et al*., 2006; Huang *et al*., 2007), in 2007 *N. ceranae* was reported in the USA, Brazil, China, Vietnam and eight other EU countries (Klee *et al*., 2007; Paxton *et al*.,

2007). More recently, the pandemia was verified as the pathogen crossed geographic boundaries, being detected in honey bee colonies of numerous countries such as Canada (Williams *et al*., 2008), Australia, (Giersch *et al*., 2009), Uruguay, (Invernizzi *et al*., 2009), Japan (Yoshiyama and Kimura, 2011), Chile (Martinez *et al*., 2012), Jordan (Haddad, 2014) or Saudi Arabia (Ansari *et al*., 2017).

A clear difference with respect to *N. apis* is that *N. ceranae* is present in different pollinators, such as bumble bee species (Table 1), which can also spread the infection back to commercial honey bees (Li *et al*., 2012). For example, the richness of parasites in wild bumble bees increases in the proximity of commercially reared honey bees, which seems to be related to a spill over of infectious diseases from domestic livestock to wild populations (Graystock *et al*., 2014). *N. ceranae* infections in commercial bumble bees were found to reduce their survival and also to produce a sublethal effect on the sucrose response threshold (Graystock *et al*., 2013), which might represent a threat to these important pollinators. The recent detection of this parasite in solitary bee species confirms the wide dispersion of the parasite in wild bees (Ravoet *et al*., 2014). Indeed, it appears that the international movement of honey bee queens, colonies and products can intensify the spread of this pathogen.

The situation in Spain was recently replicated in Iran (Nabian *et al*., 2011), where an increasing number of bee samples were sent to the laboratories from colonies with no clear clinical signs, although the most beekeepers noticed rapid dying off of colonies in winter. The analysis of those samples allowed the first detection of *N. ceranae* in Iran. Although Africa is considered to be virtually *N. ceranae*-free (Strauss *et al*., 2013; Muli *et al*., 2014), this parasite has been reported in *A. mellifera intermissa* from Algeria (Higes *et al*., 2009b) and in *A. mellifera adansonii* from Benin (Cornelissen *et al*., 2011). However, in the nearby Ghana, neither *N. apis* nor *N. ceranae* were detected (Llorens-Picher *et al*., 2018). Migratory bee eating birds like *Merops apiaster* may play an important role in the spread of this pathogen across continents (e.g., from Northern Africa to Southern Europe). These birds can regurgitate pellets that contain infective spores over the hives after eating infected honey bee foragers (Higes *et al*., 2008b), since apiaries are usually stop-over sites on migratory pathways of these birds used year after year (Valera *et al*., 2017).

How is *Nosema* transmitted?

Nosema is transmitted through the ingestion of spores via contaminated water or food, through the exchange of food between bees or when they perform their cleaning duties. The median infective dose for *N. apis* has been described to be 94.3 spores per bee (Fries, 1988) whereas for *N. ceranae* it was established in 149 spores per bee, although the minimum dose capable of causing a detectable infection was 1.28 spores (McGowan *et al*., 2016). When the spores enter to the bee's ventriculus, they extrude a polar filament through which the sporoplasm is transferred into the epithelial cells of the host. Once the parasite multiplies and develops within the host-cell cytoplasm, the spores can be led into the gut lumen, where they may be excreted or they may infect additional epithelial cells (Fig. 1). The presence of empty spores inside the parasitized epithelium was considered evidence that autoinfection is a common feature in the life cycle of these pathogens (Fries *et al*., 1996; Higes *et al*., 2007; 2009a), causing extensive and even total destruction of the ventricular epithelial layer. Indeed, although it was thought that *N. ceranae* was only able to infect adult bees, it was also found in prepupae of *A. mellifera* under laboratory conditions (Eiri *et al*., 2015) and in drone pupae from naturally infected apiaries (Traver and Fell, 2011) demonstrating the infectivity of this microsporidium in bee breeding and displaying a range of pathological problems in the subsequent adults (Ben-Vau and Nieh, 2017).

The large increase in the detection of *N. ceranae* worldwide is in part due to the specificity of the molecular techniques that enable *N. ceranae* to be differentiated from *N. apis*, as well as to the more intense commercial exchange between beekeepers over recent years. In Japan, for example, tens of thousands of mated queens are imported every year (Yoshiyama and Kimura, 2011) and there is an increasing use of honey bees as pollinators for greenhouse crops that have led to an increase in the abundance and prevalence of *N. ceranae* in *A. mellifera* (Zhu *et al*., 2014).

Once introduced into a country, the migratory movements between different climatic regions related to honey harvesting and associated to beekeeping practices (e.g., migrations) enhance the potential for contact between apiaries. Thus, new colonies can easily be infected, for example, through the sharing of food resources, and even through the robbery of sick hives. Royal jelly, pollen and honey may also be sources of spores (Cox-Foster *et al*., 2007; Higes *et al*., 2008c; Giersch *et al*., 2009). The recent report that *Nosema* parasites can be transmitted via insemination as a secondary mode of transmission (Peng *et al*., 2015; Roberts *et al*., 2015) is striking and it means that infection by this parasite should be considered in mating stations. This probably also occurs in bumble bees, where *N. bombi* spores have previously been reported in the semen of males (Otti and Schmid-Hempel, 2007) and

Table 1. World distribution of *Nosema ceranae* across hosts of the Apidae and Vespidae families.

Host species	Putative subspecies	Country	Earliest reported sampling year	References
Apidae				
Eastern honey bees				
Genus *Apis*				
cerana		Vietnam	1968	Traver and Fell (2015)
		China	< 1996	Fries and colleagues (1996)
		South Korea	1996	Botías and colleagues (2012a)
		Indonesia	2004	Botías and colleagues (2012a)
		Solomon Islands	2008	Botías and colleagues (2012a)
		Thailand	2008	Chaimanee and colleagues (2010)
florea		Thailand	2008	Chaimanee and colleagues (2010)
dorsata		Vietnam	1968	Traver and Fell (2015)
		Thailand	2008	Chaimanee and colleagues (2010)
koschevnikovi		Indonesia	2004	Botías and colleagues (2012a)
Western honey bee				
mellifera	*ligustica*	Italy	1993	Ferroglio and colleagues (2013)
	mellifera/C-lineage	Poland	1994	Gajda (2016)
	mellifera/C-lineage	Finland	1998	Paxton and colleagues (2007)
	carnica	Serbia	2000	Stevanovic and colleagues (2010)
	mellifera/C-lineage	France	2002	Chauzat and colleagues (2007)
	carnica	Germany	2003	Martín-Hernández and colleagues (2007)
	mellifera	Denmark	2004	Klee and colleagues (2007)
	iberiensis	Spain	2004	Higes and colleagues (2006)
	mellifera/C-lineage	UK	2007	Bollan and colleagues (2013), Budge and colleagues (2015)
	macedonica, *cecropia*	Greece	2005, 2009	Klee and colleagues (2007), Bacandritsos and colleagues (2010)
	carnica	Bosnia and Herzegovina	2006	Stevanovic and colleagues (2011)
	macedonica	FYROM	2006	Stevanovic and colleagues (2011)
	carnica	Hungary	2006	Tapaszti and colleagues (2009)
	carnica	Montenegro	2006	Stevanovic and colleagues (2011)
	mellifera/C-lineage	Sweden	2006	Klee and colleagues (2007)
	mellifera/*carnica*	Switzerland	2006	Martín-Hernández and colleagues (2007)
	carnica	Bosnia and Herzegovina	2008	Santrac and colleagues (2010)
	carnica	Croatia	2009	Gajger and colleagues (2010)
	C-lineage	Turkey	2005	Whitaker and colleagues (2011)
	Hybrids*	Egypt	2011	El-Shemy and colleagues (2012)
	meda	Iran	2011	Razmaraii and colleagues (2013)
	syriaca	Jordan	2014	Haddad (2014)
	*jemenitica**	Saudi Arabia	2015	Ansari and colleagues (2017)
	*intermissa**, *sahariensis**	Algeria	2008	Higes and colleagues (2009), Adjlane and colleagues (2015)
	*adansoni**, *jemenitica**	Benin	2009	Cornelissen and colleagues (2011)
	C-lineage	Taiwan	2005	Huang and colleagues (2007)
	C-lineage	Vietnam	2006	Klee and colleagues (2007)
	C-lineage	Solomon Islands	2008	Botías and colleagues (2012a,b)
	C-lineage	Thailand	2008	Chaimanee and colleagues 2010
	C-lineage	China	?	Liu and colleagues 2008
	C-lineage	Japan	2009	Yoshiyama and Kimura (2011)
	C-lineage (EHB)	USA	1975	Traver and Fell (2015)
	A-lineage (AHB)	Brazil	1979	Teixeira and colleagues (2013)
	A-lineage (AHB)	Uruguay	1990	Invernizzi and colleagues (2009)
	A-lineage (AHB)*	Mexico	1995	Guerrero-Molina and colleagues (2016)
	C-lineage (EHB)	Canada	2006	Williams and colleagues (2008)
	A-lineage (AHB)*	Costa Rica	2006	Calderón and colleagues (2008)
	C-lineage (EHB)	Argentina	2008	Medici and colleagues (2012)
	C-lineage (EHB)	Chile	2010	Martinez and colleagues (2012)
	C-lineage (EHB)	Australia	2007	Giersch and colleagues (2009)
	C-lineage (EHB)	Norfolk Island, Australia	2013	Malfroy and colleagues (2016)
	C-lineage (EHB)	New Zealand	2010	Frazer and colleagues (2015)

Table 1. *cont.*

Host species	Putative subspecies	Country	Earliest reported sampling year	References
Stingless bees				
Genus *Melipona*				
fasciculata		Brazil	2015	Porrini and colleagues (2017)
quadrifasciata	*anthidioides*	Brazil	2015	Porrini and colleagues (2017)
marginata		Brazil	2015	Porrini and colleagues (2017)
rufiventris		Brazil	2015	Porrini and colleagues (2017)
mandacaia		Brazil	2015	Porrini and colleagues (2017)
Genus *Tetragonisca*				
fiebrigi		Argentina	2014	Porrini and colleagues (2017)
Genus *Scaptotrigona*				
jujuyensis		Argentina	2015	Porrini and colleagues (2017)
Solitary bees				
Genus *Osmia*				
bicornis		Belgium	2012	Ravoet and colleagues (2014)
cornuta		Belgium	2012	Ravoet and colleagues (2014)
Genus *Andrena*				
ventralis		Belgium	2012	Ravoet and colleagues (2014)
Genus *Heriades*				
truncorum		Belgium	2012	Ravoet and colleagues (2014)
Bumble bees				
Genus *Bombus*				
atratus		Argentina	<2008	Plischuk and colleagues (2009)
		Uruguay	2010	Arbulo and colleagues (2015)
		Colombia	2013	Gamboa and colleagues (2015)
morio		Argentina	<2008	Plischuk and colleagues (2009)
bellicosus		Argentina	<2008	Plischuk and colleagues (2009)
		Uruguay	2010	Arbulo and colleagues (2015)
waltoni		China	2008	Li and colleagues (2012)
remotus		China	2008	Li and colleagues (2012)
impetuosus		China	2008	Li and colleagues (2012)
sibiricus		China	2008	Li and colleagues (2012)
brasiliensis		Argentina	2015	Plischuk and Lange (2016)
hortorum		UK	<2013	Graystock and colleagues (2013)
hypnorum		UK	<2013	Graystock and colleagues (2013)
lapidarius		UK	<2013	Graystock and colleagues (2013)
lucorum		UK	<2013	Graystock and colleagues (2013)
pascuorum		UK	<2013	Graystock and colleagues (2013)
pratorum		UK	<2013	Graystock and colleagues (2013)
terrestris		UK	<2013	Graystock and colleagues (2013)
Vespidae				
Polybia scutellaris		Argentina	2010	Porrini and colleagues (2017)

Apis mellifera subspecies or evolutionary lineage are mostly predicted from the known native and introduced distributional ranges. Subspecies marked with an asterisk were mentioned in the reference whereas those marked in bold were identified either morphometrically or molecularly.

where *N. ceranae* is now a common parasite in some areas.

How the spread of *N. ceranae* is affecting the prevalence of *N. apis?*

The increasing worldwide prevalence of *N. ceranae* in the past decade, particularly in Mediterranean countries like Spain, Italy, Israel, Greece or Turkey (Klee *et al*., 2007; Higes *et al*., 2008a; Soroker *et al* 2011; Hatjina *et al*., 2011; Oguz *et al*., 2017), coupled with the absence of *N. apis* in several surveys, has led to the hypothesis that *N. ceranae* might be displacing *N. apis* (Klee *et al*., 2007, Traver and Fell, 2011). The seasonal pattern typical of *N. apis* infection was well known: (i) low levels of infection during the hot summer months; (ii) a short peak in the autumn; (iii) a slow rise in the number of infections during the winter; (iv) and a peak in the spring, with the level of infection rapidly increasing when foraging is limited by humid and cold climatic conditions (Fries, 1993). Accordingly, *N. apis* levels tend to drop off during the summer due to natural controlled mechanisms within the colony itself (Bailey, 1955). Long term studies showed that this pattern was evident for *N. apis*,

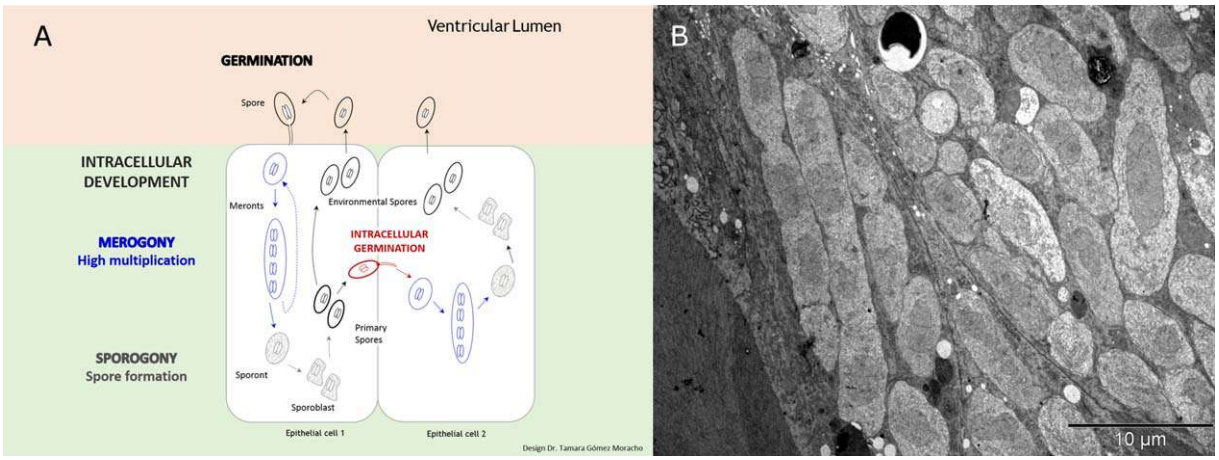

Fig. 1. *Nosema ceranae* and *Nosema apis* life cycle in honey bees. The spores ingested by the bees get to the ventricular lumen. There, spores extrude the polar filament and the sporoplasm is transferred into the epithelial cells. The sporoplasm matures into a Meront and a Merogonic phase starts that comprises binary division of binucleate stages (the number of divisions is still undetermined). Lately, electrondense material is deposited in the outer face of the plasma membrane, which indicates the sporogonial phase. This phase involves the division of the sporonts (*Nosema* spp. have been described as bisporous) and then sporonts and daughter cells mature into spores. The first generation of spores will be primary spores which can re-infect the same cell or infect neighbor cells. The second generation (after secondary meronts) will lead to environmental spores with the spore wall thicker than the primary spores (Huang and Solter, 2013). All parasitic stages develop in direct contact with the host cell cytoplasm and all phases are dyplokariotic.
A. Scheme of the *Nosema* cell-cycle inside the host cell.
B. TEM image taken from a honey bee infected by *N. ceranae.* The ventricular cells can be seen with different parasitic stages.

even though *N. ceranae* was present throughout the year (Higes *et al*., 2008a; Martín-Hernández *et al*., 2012). However, the levels of *N. ceranae* (percentage of bees infected) vary over time with very high levels from the end of summer up to spring and the maximum during winter in Spain (Higes *et al*., 2008a). These profile can be influenced by some undetermined factors since the higher levels were reported in summer in Canada (Copley *et al*., 2012), in March in Serbia (Stevanovic *et al*., 2013) or in spring (reflecting the development over winter) in Germany (Gisder *et al*., 2017). Also, *N. ceranae* can multiply at higher temperatures, displaying a greater biotic potential than *N. apis* (Martín-Hernández *et al*., 2009; Higes *et al*., 2010b; Gisder *et al*., 2017). Indeed, their spores are tolerant to temperatures as high as 60°C and they can survive desiccation (Fenoy *et al*., 2009; Martín-Hernández *et al*., 2009). By contrast, cold has a negative effect on *N. ceranae* whose spores are sensitive to low temperatures and freezing (Fries, 2010; Gisder *et al*., 2010; Sánchez Collado *et al*., 2014).

A first study of the within-host competition effect between *N. apis* and *N. ceranae* did not show any clear competitive advantage for any of them (Forsgren and Fries 2010). However, a later study identified a priority effect when *N. ceranae* was the first infection (Natsopoulou *et al*., 2016). Apparently both environmental variables and interspecies competition are important elements of mathematical models that help explain the differential prevalence of *Nosema* spp. in distinct climatic regions. Although such models can overestimate prevalence, the predictions derived from them are consistent with field data obtained across Europe. Hence, they reveal a transition zone in the relative prevalence of the two species, with *N. ceranae* predominating over *N. apis* in Southern regions (e.g., Spain) and vice versa (e.g., Sweden). Accordingly, the apparent global advantage of *N. ceranae* appears not to be due to differences in spore production or infectivity (as shown by Milbrath *et al*., 2015). The replacement of *N. apis* by *N. ceranae* is unlikely to occur due to a competitive advantage for within-host spore production (Martín-Hernández *et al*., 2012; Gisder *et al*., 2017).

Genetic diversity of *N. ceranae*

Many studies assessing the intraspecific variability in *N. ceranae* rely on the analysis of the ribosomal DNA (*rDNA*) (Huang *et al*., 2008; Sagastume *et al*., 2011; 2014; Suwannapong *et al*., 2011; Roudel *et al*., 2013), which is organized into ribosomal units (Huang *et al*., 2007; Huang *et al*., 2008) present as multiple copies in the genome (Cornman *et al*., 2009). Although intragenomic *rDNA* diversity is usually low as a result of concerted evolution (Eickbush and Eickbush, 2007), *rDNA* markers show extensive sequence heterogeneity in *N. ceranae* (Sagastume *et al*., 2011; 2014) and in other *Nosema* species (Gatehouse and Malone, 1998; 1999; Tay *et al*., 2005; O'Mahony *et al*., 2007). Indeed, the

average nucleotide diversity (π, Nei, 1987) in these regions ranges from 0.14%–0.45% for the small subunit (SSU; Sagastume *et al*., 2011; Roudel *et al*., 2013) to 2.59% for the Intergenic Spacer (IGS; Sagastume *et al*., 2011).

The analysis of single copy genes (Chaimanee *et al*., 2011; Hatjina *et al*., 2011; Roudel *et al*., 2013; Gómez-Moracho *et al*., 2014; 2015a; 2015b; van der Zee *et al*., 2014), which are better suited than multicopy markers for estimating the levels of intraspecific diversity, reveal high diversity within isolate variability in *N. ceranae*, regardless of whether isolates are obtained from a single bee or from homogenized pools of individuals (Hatjina *et al*., 2011; Roudel *et al*., 2013; Gómez-Moracho *et al*., 2014; 2015b; Pelin *et al*., 2015), with an average pairwise diversity at synonymous sites of about 1% (Gómez-Moracho *et al*., 2015a). Most of the variation in *N. ceranae* is contributed by low frequency mutations (Hatjina *et al*., 2011; Roudel *et al*., 2013; van der Zee *et al*., 2014; Gómez-Moracho *et al*., 2014; 2015b; Pelin *et al*., 2015), especially in the parasite populations obtained from *A. mellifera* (Gómez-Moracho *et al*., 2015a), which is largely compatible with the recent expansion of *N. ceranae* in this new host (Roudel *et al*., 2013; Gómez-Moracho *et al*., 2015a; Pelin *et al*., 2015).

The finding of multiple haplotypes within isolates can be explained by (i) the presence of several strains co-infecting honey bee colonies (Hatjina *et al*., 2011; Gómez-Moracho *et al*., 2014; 2015a), (ii) the existence of a diplokaryon with two diploid nuclei (Roudel *et al*., 2013; Pelin *et al*., 2015) or (iii) the combination of both, as these causes are not mutually exclusive. In any case, the occurrence of infections in which many different haplotypes co-exist, is a key factor in maintaining the genetic diversity of *N. ceranae* in its new hosts (Gómez-Moracho *et al*., 2015a).

Another important source of genetic variation is the existence of recombination. Although a clonal mode of reproduction has been proposed for *N. ceranae* on the basis of the detection of high levels of linkage disequilibrium and heterozygosity (Pelin *et al*., 2015), the lack of genetic exchange between nuclei would make their sequences evolve independently and become more divergent over time. This scenario contrasts with the lack of structure observed in the haplotypes obtained from single bees (Roudel *et al*., 2013; Gómez-Moracho *et al*., 2015a), which rather suggests the existence of genetic flow between nuclei. The presence of sex-related loci and genes involved in meiotic recombination (Lee *et al*., 2010) point to the existence of cryptic sexual stages in the life cycle of *N. ceranae*; however, it is still unknown if the recombinant haplotypes (Sagastume *et al*., 2011; Roudel *et al*., 2013; van der Zee *et al*., 2014; Gómez-Moracho *et al*., 2014; 2015a; 2015b) are generated during meiosis or during mitosis, as the outcomes of these processes are difficult to distinguish (Weedall and Hall, 2015). At any rate, the weak, yet significant genetic exchange detected in *N. ceranae* (Gómez-Moracho *et al*., 2015b) has important evolutionary implications, not only because it allows deleterious mutations to be eliminated more efficiently but also because recombination provides a better capacity to adapt to new environments or hosts than a clonal mode of reproduction (Barton, 2010).

How are *N. ceranae* populations structured?

Recent analyses suggest that, despite sharing alleles, there is moderate but significant differentiation among the *N. ceranae* haplotypes found in different *Apis* species (Chaimanee *et al*., 2011; Gómez-Moracho *et al*., 2015a). In *N. ceranae* populations from *A. mellifera* most of the variation occurs within honey bee colonies, which show no genetic differentiation and shared alleles regardless of their geographic origins (Roudel *et al*., 2013; Gómez-Moracho *et al*., 2014; 2015a; van der Zee *et al*., 2014; Pelin *et al*., 2015). In line with these observations, the analysis of the genomes of eight *N. ceranae* isolates from distant locations revealed that more than 98% of the polymorphism detected was shared among at least two of the isolates studied (Pelin *et al*., 2015), confirming that population structuring in *A. mellifera*, if any, is still at an extremely initial stage.

In contrast, when it comes to *N. apis*, a considerable fraction of the genetic variance (between 20% and 34%) corresponds to differences between isolates obtained from distinct *A. mellifera* lineages (Maside *et al*., 2015). Indeed, isolates collected from honey bees of lineage A (which can be found in Africa and the Iberian Peninsula) exhibit different haplotypes from those obtained from lineages C or M (which involve *A. mellifera* subspecies distributed across South Eastern Europe or Western and Northern Europe respectively); the existence of this population structure suggest a far older relationship between *A. mellifera* and *N. apis* than that between the former and *N. ceranae*.

What are the major effects of *N. ceranae* on honey bees?

Since the first report of *N. ceranae* infection in *A. mellifera*, there has been some controversy about the consequences of such infection. However, in recent years most studies have confirmed that *N. ceranae* has a pathogenic effect in this host, expressed at least in a shortening of the workers' lifespan in controlled (cage) experiment (e.g., Mayack and Naug, 2009; Alaux *et al*., 2010; Martín-Hernández *et al*., 2011; Dussaubat *et al*., 2012; Goblirsch *et al*., 2013; Schwarz and Evans, 2013;

Aufauvre *et al*., 2014; Basualdo *et al*., 2014; Roberts and Hughes, 2014; 2015; Williams *et al*., 2014; Doublet *et al*., 2015aHuang *et al*., 2015); only few papers failing to report this effect (Milbrath *et al*., 2013; Retschnig *et al*., 2014; Garrido *et al*., 2016).

The effect of N. ceranae *infection on honey bees' fitness*

The lesions caused by the infection in the bee ventriculi were described in depth some years ago (Higes *et al*., 2007; Garcia-Palencia *et al*., 2010). Here we focus on the studies that enlightened many of the effects that this microsporidia has on the physiology of *A. mellifera*.

Changes in metabolism. The effect of *N. ceranae* infection on the host's gene expression has recently been thoroughly addressed, confirming the effect of this microsporidia in the infected host (Szumowski and Troemel, 2015). Since *N. ceranae* invades the ventriculus (midgut) of honey bees, most studies have focused on this organ. Honey bees consume nectar and pollen as sources of carbon and nitrogen respectively, and both require extensive processing in the gut (Kunieda *et al*., 2006) by enzymes that metabolize carbohydrates and lipids to breakdown the food and to release stored energy and to synthesize the organism's primary energy stores (reviewed by Klowden, 2002). Modifications to carbohydrate metabolism have frequently been reported in *N. ceranae* infected bees suggesting a manipulative activity of the pathogen to ensure the availability of nutrients for its own benefit. In terms of gene expression, this manipulation is apparently reflected in the up-regulation of the α-glucosidase gene and of three genes involved in trehalose transport (the major carbohydrate energy storage molecule in insects; Dussaubat *et al*., 2012) observed, as well as the down-regulation of the trehalase and the glucose-methanol-choline oxidoreductase three encoding genes (Aufauvre *et al*., 2014). These alterations to the expression of genes involved in sugar metabolism were also confirmed in a proteomic study where four proteins involved in energy supply were found to be less abundant in the midgut of *N. ceranae*-infected bees (Vidau *et al*., 2014). Similarly, gas chromatography–mass spectrometry highlighted a decrease in the majority of carbohydrates and amino acids implicated in various biochemical pathways, such as fructose, L-proline, sorbitol and glycerol (Aliferis *et al*., 2012).

The alterations of the carbohydrate metabolism reflect the nutritional and energetic stress experienced by *N. ceranae* infected honey bees (Mayack and Naug, 2010; Aliferis *et al*., 2012; Vidau *et al*., 2014). Interestingly, this has not been observed in *Nosema* tolerant honey bees (Kurze *et al*., 2016). Energetic stress has been described in infected foragers that were hungrier than uninfected bees (Mayack and Naug, 2009), consuming more sugar (Alaux *et al*., 2010; Martín-Hernández *et al*., 2011; Vidau *et al*., 2011). These infected workers appear to be unable to utilize the excess carbohydrates consumed probably because the most of them are used by the pathogen to complete its life-cycle. Moreover, the energetically stressed bees have been reported to experience higher mortality during foraging (Mayack and Naug, 2013). Hence, the mechanisms controlling the mobilization of energy reserves appear to be disturbed and there is poor carbohydrate homeostasis in their haemolymph (Aliferis *et al*., 2012). The stronger sugar demand and higher consumption could be a host response to the infection, directly related to the dependence of microsporidia on host energy. However, the intestinal lesions caused by *N. ceranae* proliferation may decrease the digestive capacity of honey bees and generate signs of starvation, such as impoverishment of hypopharyngeal protein secretions in nurse bees (Vidau *et al*., 2014). It should also be noted that a higher sugar consumption is not always observed in such studies (Aufauvre *et al*., 2012; 2014), suggesting that other unknown factors could influence this parameter. Additionally, Li and colleagues (2018) reported that bees infected by *N. ceranae* show an accelerated lipid loss, suggesting lipids may be used also as a fuel for increased metabolic demands due to the infections.

All these modifications alter the feeding behaviour of infected honey bees and their transition to become foragers (Mayack and Naug, 2009). In fact, the inhibition of fatty acid synthesis and also the starvation can lead bees to begin foraging earlier in life (Schulz *et al*., 1998; Toth, 2005), and energy stressed bees in a colony first altering their activity and then their foraging rate (Mayack and Naug, 2013). Among *N. ceranae* infected bees, the weaker capacity to fly, probably due to the lower trehalose levels, should also be taken into account (Mayack and Naug, 2010). However, it may be that these behavioural alterations are related to the infection itself, since the altered regulation of highly conserved neurohormonal pathways (such as the octopamine pathway) on *N. ceranae* infection was caused by the pathogenesis itself and not indirectly by energetic stress (Mayack *et al*., 2015).

Changes in other vital functions. Other important metabolic pathways for honey bee physiology are also altered by *N. ceranae* infection. For example, oxidative stress has been reported due to the over-expression of genes related to the generation of antioxidant enzymes and increased glutathione-S-transferase activity (Vidau *et al*., 2011; Dussaubat *et al*., 2012), although the appearance of this detoxifying enzyme could be influenced by diet

(Di Pasquale *et al*., 2013). This oxidative response to infection (observed by both transcriptomic and proteomic approaches), and the higher energetic demand, strongly suggests that a negative impact on infected honey bee development may cause a reduction in lifespan (Vidau *et al*., 2014). Additionally, the stress response observed in *N. ceranae* infected bees appears to be derived from the modification of transcriptional profiles in the brain and from changes in the cuticular hydrocarbon profiles (McDonnell *et al*., 2013; Aufauvre *et al*., 2014) that are similar to those produced by the mite *Varroa destructor* (McDonnell *et al*., 2013). The enhanced impact of the infection over time, also seen in response to insecticides (see below), suggests a growing disturbance of the honey bee transcriptome that might reflect the failure of recovery from stress and could explain the higher mortality rates observed (Aufauvre *et al*., 2014).

Conversely, *N. ceranae* infection was reported to prevent the apoptosis of epithelial cells in the bees' ventriculi (Higes *et al*., 2013; Martín-Hernández *et al*., 2017) to avoid the host innate response to the infection. A capacity to inhibit genes involved in cell signalling and in the self-renewal of intestinal cells (Dussaubat *et al*., 2012; Huang *et al*., 2016) and an up-regulation of genes belonging to the IAP family (inhibitors of apoptosis genes; Martín-Hernández *et al*., 2017) has also been reported. As microsporidia can modulate such processes in cell cultures (Del Aguila *et al*., 2006), this may be a mechanism used by the parasite to favour its development. Indeed, there are differences in the transcription of an anti-apoptotic gene (inhibitor of apoptosis protein-2) between *Nosema*-tolerant and *Nosema*-sensitive bees (Kurze *et al*., 2015).

Effects on bee immune response. Microsporidia have been seen to modify the host's immune response. For example, *Nosema bombycis* induces transcriptional changes in 34 out of 70 *Bombyx mori* immune genes, even inducing the down regulation of the serine protease cascade in the melanization pathway, and up-regulating lysozyme and lectins (reviewed in Szumoski and Troemel, 2015). This effect is especially noteworthy given that several studies have addressed how infection by *N. ceranae* might affect immunity at the social (colony) and individual (bee) level. However, while all of these studies reported effects on immunity at the individual level, other controversial results have also been described. In this regard, one of the first studies on *A. mellifera* infected with *N. ceranae* showed a down-regulation of some immune-related genes like *abaecin*, *hymenoptaecin*, *glucose dehydrogenase* (GLD) and *vitellogenin* (*Vg*), suggesting that *N. ceranae* infection suppresses immune defence mechanisms in honey bees (Antúnez *et al*., 2009). Later studies also detected host immunosuppression, reporting a down-regulation of *defensin*, *abaecin*, *apidecin* (Chaimanee *et al*., 2012), *hymenoptaecin* (Chaimanee *et al*., 2012; Aufauvre *et al*., 2014), *serine protease 40*, *catalase* (Aufauvre *et al*., 2014), *basket* (GB16401) and *u-shaped* (GB16457) genes (involved in Drosophila immune responses; Dussaubat *et al*., 2012). However, these changes were associated with the over-expression of other immune related genes, such as *ROS* (reactive oxygen species) and *glutathione peroxidase like 2*. Another study also reported a down-regulation of antimicrobial peptides (Badaoui *et al*., 2017), suppression of Toll and Imd pathways and of the expression of Pattern Recognition Receptors-related genes, this last was persistent and intensified in time (Li *et al*., 2018). By contrast, Schwarz and Evans (2013) reported a complex immune response mounted by bees against *N. ceranae* infection, which is also dynamic over time. In this work, ingestion of *N. ceranae* spores was seen to rapidly enhance Toll and Imd signalling, and to increase the expression of the cellular recognition molecule Dscam and AMP Defensin 2. Moreover, infection caused a diverse and extended effector immune response via *abaecin*, *apidaecin*, *hymenoptaecin*, *defensin 1* and *2*, mainly observed 7 days post-infection. These same genes and some other related to microbial recognition proteins as peptidoglycan recognition proteins and Gram-negative binding proteins were also upregulated in a field assay, both in nurse and foragers infected, mainly these latter (Li *et al*., 2017).

However, in a different study neither haemocyte number nor phenoloxidase (PO)-activity were apparently affected by infection (Alaux *et al*., 2010), although the longevity of *Nosema* infected bees was linked to the latter (Di Pasquale *et al*., 2013). Intermediate results were reported when the immune response was compared between '*Nosema*-resistant selected' and 'unselected' drones, with stronger gene expression in the infected group than in the uninfected controls from day one to five post infection, although the expression of genes from the innate immune system was weaker in the unselected strain (Huang *et al*., 2012). Many factors can influence immune responses, such as the different doses of infection (which vary considerably between studies), the duration of the assays, the tissues in which gene expression was studied (whole bees, abdomen, ventriculi, etc.), or the age of the bees at infection and during the study. In this regard, it is known that the haemocyte number is dramatically lower in early adult life in all the bee castes, and that the dynamics of PO activity is sex and caste specific (Schmid *et al*., 2008). In fact, the immune response of *N. ceranae* infected queens changes as they age, with the expression of *apidaecin*, *eater* and *vitellogenin* varying in queens inoculated at

different ages (Chaimanee *et al*., 2014). Also, the quality and diversity of the pollen supplementing bees' food influences their immunity, as observed through the general activity of Glutathione-S transferase, alkaline phosphatase and PO (Di Pasquale *et al*., 2013). However, little is known about the protective role of immune related peptides after microsporidia infection. Indeed, only the increased expression of *aubergine* has been linked to resistance (or protection) in *Nosema* resistant honey bees (Huang *et al*., 2014a,b).

In terms of other molecules related to the honey bees' immune response, as well as to other physiological functions, some alterations to Vg and Juvenile Hormone (JH) have been also described in *N. ceranae* infected bees. Vg fulfils several functions in workers, such as participating in the synthesis of royal jelly (Amdam *et al*., 2003), promoting immunity, stress resilience and longevity (Amdam *et al*., 2004), and it also proposed to regulates behavioural development along with JH (Robinson and Vargo, 1997; Nelson *et al*., 2007). Adult workers that were infected as larvae with *N. ceranae* spores showed significantly higher Vg titers and lower total haemolymph protein titers than uninfected controls (BenVau and Nieh, 2017). Furthermore, these honey bees infected at the larval stage also had some modification in their sting, which developed a more queen-like sting morphology (BenVau and Nieh, 2017). Moreover, the expression of *Vg* has been found to be higher in bees collected from colonies with low levels of *N. ceranae* infection than in bees from colonies with high levels and it has been suggested to be associated with colony resistance to *N. ceranae* (Antúnez *et al*., 2013).

In addition to modifying the expression of the *Vg* gene, *N. ceranae* infection can also disrupt the physiological regulation of the age-specific behaviour of infected workers by increasing the level of JH (III) in the haemolymph of infected bees (Ares *et al*., 2012), as JH is a promotor of foraging. Indeed, the atypical transcription of Vg and JH is the inverse of what would be expected for healthy, uninfected bees (Goblirsch *et al*., 2013). Similarly, infection of *B. mori* by *N. bombycis* provokes the differential expression of many genes involved in the synthesis and metabolism of JH, which probably leads to the described increase in JH (Ma *et al*., 2013). This alteration in the Vg/JH equilibrium has been proposed to be responsible for the effects on early foraging and the shortened lifespan of *N. ceranae* infected worker bees (Goblirsch *et al*., 2013). Also, *N. ceranae* alters the metabolism of bees increasing EO (ethyl oleate) levels (Dussaubat *et al*., 2010), a primer pheromone which regulates worker behavioural maturation (i.e., inhibits the transition from inside-nest tasks performed by nurse bees) to foraging tasks performed by old bees (Leoncini *et al*., 2004). All these effects fit with the fact that infested bees forage earlier.

Altogether, these alterations reflect the effect of this Microsporidia on immunity at the individual bee and colony level, such that colonies may become more susceptible to other infectious diseases or the effects of pesticides (see below).

Factors related with N. ceranae *infection*

Honey bees live in an environment where they might be exposed to different factors, such as pathogens and pesticides, which may interact with one another.

Interactions between N. ceranae *and other pathogens of honey bees.* The pathogen status of a colony is seasonal-dependent and gaining insights on the interactions between pathogens in the field can become complex. In this regard, several experimental approaches that aimed to study the interaction between *Nosema* and other viruses or parasites contributed to the analysis.

One of the more prevalent viruses of honey bees worldwide is the Deformed wing virus (DWV), named after the main sign of infection observed in adults. Costa and colleagues (2011) reported that varroa-free emerging adults from a DWV-positive colony fed with *N. ceranae* spores showed significantly lower DWV loads in their midgut than their *Nosema*-free counterparts. This difference did not held for other tissues, suggesting that DWV and *N. ceranae* may compete for host cells or specific cell functions in the honey bee midgut. However, in a later field study in Hawaii, no correlation between DWV loads and *N. ceranae* spore counts were observed (Martin *et al*., 2013). A recent survey suggested that the DWV load may negatively impact establishment of *Nosema* spp., as *Nosema*-free honey bees had significantly higher DWV loads than *Nosema*-infected honey bees (Traynor *et al*., 2016). The order of infection seems to be important; prior *N. ceranae* infection inhibited subsequent DWV infection but this was not reciprocal, suggesting asymmetry in the competitive interaction between these pathogens (Doublet *et al*., 2015b). In another experiment, *N. ceranae* fed to emerging bees from a DWV-infected colony appeared to accelerate DWV replication at early stages of viral infection in a dose-dependent manner, but not once DWV titers reached a plateau (Zheng *et al*., 2015). However, the discrepancies among these studies could be attributed to differences in the methodologies applied to determine the viral load, such as the analysis of a specific tissue or the whole bee. Finally, the food could also influence the infection since pollen supply was able to increase the *N. ceranae* impact on DWV replication (Zheng *et al*., 2015) and a negative correlation between *N. ceranae* spore

loads and DWV-B (formerly *Varroa destructor virus-1*) titers was stronger in protein-fed bees than in sugar fed bees, showing that nutrition seems to play an important role also on virus infections in insects (Tritschler *et al*., 2017).

The relationship between *N. ceranae* infection and *V. destructor*, an important vector of DWV, is also unclear. Indeed, although one study found a positive correlation between *Nosema* and varroa in commercial apiaries pretreated against both (Little *et al*., 2016), others did not observe any correlation between these two pathogens but signalled an emerging genotype B of DWV as linked to overwinter worker losses (Natsopoulou *et al*., 2017). In this respect, the infection by *N. ceranae* has been related to a reduced efficacy of varroa treatments (Botías *et al*., 2012b).

Regarding other viruses, in a 6 year survey Traynor and colleagues (2016) observed a strong positive correlation between Lake Sinai virus-2 (LSV-2) and *Nosema* spp. with intensity peaks opposite to varroa-peak infestation and its associated viruses DWV and ABPV (Acute bee paralysis virus). They proposed different hypotheses to explain this positive correlation, including (i) different seasonal life histories of parasites and pathogens, (ii) a double-repression relationship between ABPV/DWV/varroa, which would compete for host resources, with DWV and ABPV outcompeting LSV-2 and (iii) a direct link between LSV-2 and *Nosema* that inhibit the replication of DWV and ABPV. Another study did not found significant association between *N. ceranae* and the DNA virus, the *Apis mellifera* fillamentous virus, AmFV (Hartmann *et al*., 2015).

The Black queen cell virus (BQCV), another very frequent virus infecting bees, induced elevated mortality of adults when fed simultaneously with *N. ceranae*, but this effect was not reflected in a significant increase in the load of one pathogen over the other in the bee midgut (Doublet *et al*., 2015a). However, the detection of pathogen loads was performed 13 days post infection while significant differences in bee mortality started earlier at 9 days post infection.

Experimental simultaneous co-infections of winter honey bee workers with Chronic bee paralysis virus (CBPV) and *N. ceranae* showed differences in CBPV replication but not in honey bee mortality, depending on the inoculation method of the virus, *per oz* or *per cuticula* (Toplak *et al*., 2013). Thus, when adult bees were simultaneously inoculated with CBPV and *N. ceranae per oz*, 50% of the bees had Ct (Cycle threshold) values of CBPV lower than the initial virus inoculum, which contrasted with 71.7% of the bees with lower Ct values than the initial virus inoculum obtained when they were infected with CBPV-only *per oz*. Infection with *N. ceranae per oz* and CBPV *per cuticula* resulted in 71.2% of the bees with Ct values lower than the original virus inoculum, while infection of CBPV alone *per cuticula* resulted in 12% of the bees with Ct values lower than the inoculum. These results suggest a synergistic effect of *N. ceranae* on CBPV replication when the virus is inoculated *per cuticula* and an antagonistic effect when it is *per oz*. The percentage of *N. ceranae* spores in *per oz*- and *per cuti*cula CBPV-infected dead bees were 71.1% and 58. 2% respectively, compared to 54.3% in *N. ceranae*-only infected bees (Toplak *et al*., 2013).

Regarding to other gut pathogens, in the past few years, special attention has been paid to co-infection of *N. ceranae* with *N. apis*. While there is no evidence of host competitive advantage for *N. ceranae* (Forsgren and Fries, 2010), infection intensity and honey bee mortality appear to be significantly greater for *N. ceranae* than for *N. apis* or for their mixed infections (Williams *et al*., 2014). Indeed, while the mortality caused by *N. ceranae* was similar to that of *N. apis*, reduced spore intensity was observed. Moreover, the host competition was evident between the two microsporidia and the order of infection had an important influence (Natsopoulou *et al*., 2015). The first parasite to infect significantly inhibited the growth of the second, although *N. ceranae* provoked stronger inhibition. It was recently reported that mixed infection by *Nosema* species negatively affected honey bee survival more than a single species infection; yet no competitive advantage for *N. ceranae* was observed even when both species coinfected the host simultaneously (Milbrath *et al*., 2015). There is also some controversy to whether *N. ceranae* is more pathogenic than *N. apis*, as this seems to vary greatly among different studies (Huang *et al*., 2015). Nevertheless, the damage to colonies is more closely related to the prevalence under natural conditions than the pathogen's specific effects, which has been shown to be influenced by multiple factors.

Finally, co-infection of *N. ceranae* with the trypanosomatid *Crithidia mellificae* was found to alter the repertoire of systemic antimicrobial peptides as well as dampening the cellular immune response of honey bees (Schwarz and Evans, 2013).

On one hand, the emerging picture from laboratory studies is that the temporal sequence of infection, route of infection, dose of the pathogen and the impact of the infecting agent on host immunity (and metabolic resources of the host) are important factors determining the outcome of the co-infection, since *N. ceranae* appears to have an important effect on the ability of a virus to infect the bee's midgut cells. On the other hand, each of these pathogens, the microsporidia and the virus, can weaken the bee's immune defences facilitating the replication of the co-infecting partner. Consequently, these complex interactions between *N. ceranae* and other

pathogens will require further study to be fully understood.

Interactions between N. ceranae *and pesticides.* Interactions between pesticides and *N. ceranae* can be expected, as both have the potential to disturb similar metabolic functions related to immunity, energetic resources and antioxidant responses (Di Prisco *et al*., 2013). Several studies indicate that a detrimental interaction occurs when honey bees are exposed to both pesticides and *N. ceranae* (Pettis *et al*., 2013). A synergistic effect between *N. ceranae* and neonicotinoids, first observed under laboratory conditions, causes a significantly higher bee mortality along with a reduction in glucose oxidase activity, which is involved in social immunity through the sterilization of the colony and brood food (Alaux *et al*., 2010). This synergism between neonicotinoids and *N. ceranae* in adult bees has been confirmed by others (Vidau *et al*., 2011; Aufauvre *et al*., 2012; Doublet *et al*., 2014) and was also observed in field studies demonstrating an indirect effect of imidacloprid on *N. ceranae* growth, even when honey bees were exposed to levels below those considered harmful (Pettis *et al*., 2012). In contrast with those observations, Gregorc and colleagues (2016) did not detect an effect of a neonicotinoid on *N. ceranae* growth in laboratory conditions but a minor synergistic toxic effect on the honey bee midgut tissue compared to that of both stressors separately. Similar synergism was reported between *N. ceranae* and fipronil (Vidau *et al*., 2011; Aufauvre *et al*., 2012), yet such interaction was later questioned, despite the observation that *N. ceranae*-insecticide combinations significantly enhanced honey bee mortality (Aufauvre *et al*., 2014). Particularly, in new-born queens exposed to both *N. ceranae* spores and a neonicotinoid under laboratory conditions, and introduced later in small mating hives in the field, co-exposure had similar effects to individual exposure to each stressor, rapidly compromising queens' survival and physiology (Dussaubat *et al*., 2016). When using proboscis extension response (PER) only slightly impairment of learning in honey bees infected with *N. ceranae* and no interaction with a neonicotinoid pesticide was observed (Piiroine and Goulson, 2016). Regarding other pesticides, Pettis and colleagues (2013) reported that bees consuming pollen contaminated with fungicides (as chlorothalonil or pyraclostrobin) and acaricides (as 2,4 Dimethylphenyl formamide, an amitraz metabolite, bifenthrin or fluvalinate) have a large increased risk of *N. ceranae* infection. Conversely, Garrido and colleagues (2016) found no interactive effects between sublethal doses of *tau*-fluvalinate or coumaphos and *N. ceranae* on nurse bee mortality and adults, and *N. ceranae* development was not affected by the acaricides.

More research is needed to understand in which environmental context honey bees are more susceptible to both stressors and which interaction effects can become visible and compromise colony survival.

Other interacting factors. Many other factors could influence the development and the course of *N. ceranae* infection. The age of an individual when exposed to a parasite can have a significant effect on its survival, immune-competence and the intensity of infection (Roberts and Hughes, 2014). Foragers have previously been reported to be the most intensely infected individuals in a colony (Higes *et al*., 2008a; Meana *et al*., 2010; Smart and Sheppard, 2012; Li *et al*., 2017). However, older worker bees appear to survive better than younger individuals when challenged with *N. ceranae*, although older bees develop more intense infections and have lower levels of prophenoloxidase, a marker of immunity response, which is negatively correlated with the intensity of infection. This facet has important epidemiological consequences since more strongly infected bees survive longer, facilitating pathogen transmission (Roberts and Hughes, 2014). Similarly, the queen becomes less susceptible to *N. ceranae* infection as she ages, such that the time spent in the mating nuclei is also epidemiologically important (Chaimanee *et al*., 2014). In fact, until recently only adult bees were thought to be susceptible to *N. ceranae* infection, yet larvae and pupae have been shown to develop infection, and this infection confirmed histologically in tissues as early as prepupal stages diminished adult longevity (Eiri *et al*., 2015).

All *A. mellifera* castes (workers, queen and drones) are susceptible to *N. ceranae* infection (Higes *et al*., 2008a), although drones appear to suffer higher mortality than workers and surviving bees have a lower body mass, suggesting sex-specific differences in honey bee susceptibility to *N. ceranae* (Retschnig *et al*., 2014). In fact, drones have been regarded as intracolonial 'super-spreaders', whereby transmission is enhanced when drones rather than workers are the infected individuals. Moreover, the survival of susceptible individuals (workers) maintained with infected drones was generally substantially worse than when they were kept with infected workers (Roberts and Hughes, 2015).

Another factor that must be considered is the dose of infection. It is known that virulence, in the sense of increased mortality rates in infected individuals, increases with the dose of inoculum (Ebert, 1999). Usually, the more parasites infect an individual, the stronger the effects on host fecundity and survival (Anderson and May, 1978; Keymer, 1982; Ebert *et al*., 2000). Higher doses of viral, bacterial and fungal pathogens increase mortality rates and reduce the survival time of infected insects (e.g., van Beek *et al*., 1988; 2000; Hochberg,

1991; Arthurs and Thomas, 2001; Brunner *et al*., 2005). Some of these dosage effects are purely statistical, such that at higher doses the probability of a successful and potentially lethal infection increases, yet this is also the case in terms of the internal dynamics of infection. Consequently, the strong variability in longevity reported in distinct laboratory studies could be influenced by this parameter, as it also varies greatly in these studies.

Diet may also affect tolerance to *N. ceranae*, since nutritional quality and the diversity of pollen nutrition can shape bee health (Porrini *et al*., 2011; Di Pasquale *et al*., 2013; Jack *et al*., 2016; Tritschler *et al*., 2017). Indeed, pollen nutrition improves the survival of healthy and *N. ceranae* infected bees, and pollen quality (reflected in protein content and antioxidant activity) strongly influences the effects of infection on bees (Di Pasquale *et al*., 2013). The source of dietary protein also seems to be important. Infected or uninfected bees fed with a non-natural protein diet as a pollen substitute had lower protein titres in the haemolymph than those fed with bee-bread, and their survival was also worse (Basualdo *et al*., 2014). Also, the mortality of bees infected with *N. ceranae* and fed in the laboratory with only sucrose syrup, supplemented or not with aminoacids and vitamins, was higher than when the bees were fed with pollen (Porrini *et al*., 2011).

Does host variation influence virulence?

The host range of *N. ceranae* is increasingly larger denoting a low specificity and a potentially high capacity of adaption to novel hosts. Despite the high number of host taxa, *N. ceranae* has been restricted to the Apidae. However, this situation changed recently with detection of *N. ceranae* in the Vespidae wasp *Polybia scutellaris*, suggesting that the pathogen is even capable of traversing the family barrier (Porrini *et al*., 2017).

Since first discovery in *A. cerana* (Fries *et al*., 1996), *N. ceranae* was subsequently found in other Asian bee species, including *A. florea*, *A. dorsata* (Chaimanee *et al*., 2010) and *A. koschevnikovi* (Botías *et al*., 2012a). Following the out of Asia host shift (Higes *et al*., 2006; Huang *et al*., 2007), *N. ceranae* has spread worldwide and jumped across numerous social and solitary bee species and genera within the Apidae, including *Bombus*, *Osmia*, *Andrena*, *Melipona*, *Tetragonisca* and *Scaptotrigona* (Table 1). The notable host range and corresponding global distribution, represent a wide variety of climates, from Mediterranean in Spain (Higes *et al*., 2006), temperate in Canada (Williams *et al*., 2008), tropical in Mexico (Guerrero-Molina *et al*., 2016), to hot arid in Saudi Arabia (Ansari *et al*., 2017).

Despite the long list of host taxa that have been shown positive to *N. ceranae* (Table 1), experimental infections have been limited to *A. cerana* (Suwannapong *et al*., 2011), *A. florea* (Suwannapong *et al*., 2010), *Bombus spp*. (Graystock *et al*., 2013) and, to a great extent, to *A. mellifera* (Higes *et al*., 2007; Paxton *et al*., 2007; Mayack and Naug, 2009; Forsgren and Fries 2010; Chaimanee *et al*., 2013; Williams *et al*., 2014; Huang *et al*., 2015; Milbrath *et al*., 2015; Natsopoulou *et al*., 2015), with higher virulence displayed by the two latter species. Whether *N. ceranae* is virulent to all *Apidae* and whether it is equally virulent to every *A. mellifera* subspecies is unclear. What is clear is that the current *N. ceranae* geographical range covers a large portion of the *A. mellifera* diversity with possibly 14 subspecies of the four major evolutionary lineages (C, M, A and the Middle Eastern O; Ruttner, 1988), as putative hosts. It must be noted, however, that most artificial infection studies and population surveys did not identify, or even mentioned, the subspecies or lineage (only three studies provided morphometric or molecular identification; Table 1), and there is a possibility that commercial stock of C-lineage ancestry (either *A. mellifera carnica*, *A. mellifera ligustica* and even buckfast) was the main strain under analysis in many regions.

Infection experiments in *A. mellifera* have produced inconsistent results on *N. ceranae* virulence, with mortality rates of caged bees ranging from 10% in France (Vidau *et al*., 2011) to as high as 100% in Spain (Higes *et al*., 2007) 8–10 days post-infection. Several hypotheses (discussed herein) have been evoked to account for the differences across infection experiments and worldwide *N. ceranae* surveys, among which is the genetic variability of the host (Paxton *et al*., 2007; Martín-Hernández *et al*., 2011; Dussaubat *et al*., 2013a; Branchiccela *et al*., 2017). Predicting from studies focusing on a wide range of host-pathogen systems, which have shown that disease virulence varies with host genotype (de Roode *et al*., 2004, and references therein), it is possible that different subspecies vary in their ability to counter infection. Yet, to our knowledge, there is only one cage artificial infection experiment that compared the susceptibility to *N. ceranae* of molecularly identified honey bees of unspecified subspecies but belonging to lineages C and O (Fontbonne *et al*., 2013). This study suggests that genetic variation among individual bees or colonies within lineage, and not between lineages, is a better predictor of host response to *N. ceranae*. In fact, inter-colony variation in susceptibility to nosemosis has long been recognized and used in Denmark in a breeding programme that selected for low infection rates (Traynor and Traynor, 2008).

In contrast with the cage study of Fontbonne and colleagues (2013), field experiments of natural infection found differential levels of *N. ceranae* between molecularly identified Russian (M-lineage) and Italian bees (C-

lineage) in the USA (Bourgeois *et al*., 2012), and between Africanized (A-lineage) and Italian bees in Uruguay (Mendoza *et al*., 2014), with Italian bees seemingly more susceptible in both cases. Furthermore, the results of Bourgeois and colleagues (2012) provide the first evidence for genetic variation in resistance to *N. ceranae* in the Russian stock.

However, not all studies offer support for bee strain contributing to differential virulence. In another field experiment in the USA, queen genetic origin did not seem to influence *N. ceranae* infection levels (Villa *et al*., 2013). However, the authors did not identify the colonies and, inferring from queen origin (commercial colonies from Northern USA, Canada and Australia), they were most likely of C-lineage ancestry. A similar finding was reported for Spain in a *N. ceranae* survey of colonies identified for mitochondrial DNA (Jara *et al*., 2012). In this study, variation in pathogen prevalence was not linked to A and M-lineage mitotypes.

While it remains to be demonstrated that variation among *A. mellifera* subspecies influences virulence of *N. ceranae*, it has been repeatedly shown that increased genetic diversity within a colony improves its resistance to a diverse array of diseases (Palmer and Oldroyd, 2003; Tarpy, 2003; Tarpy and Seeley, 2006; Seeley and Tarpy, 2007; Desai and Currie, 2015), supporting one of the hypothesis related with the evolution of polyandry (Hamilton, 1987). This hypothesis was recently tested in honey bee colonies headed by queens artificially inseminated with one or 12 drones and proved true for *N. ceranae*, with significantly higher prevalence levels detected in genetically similar colonies as compared to genetically diverse colonies (Desai and Currie, 2015). Interestingly, no significant differences between the two types of colonies were found for *N. apis* (Desai and Currie, 2015), which agrees with a previous study from Woyciechoski and Krol (2001). In conclusion, while all these studies represent first (but not comparable) attempts to address the role of honey bee variability in *N. ceranae* virulence, carefully designed cage and field infection assays with genetically characterized host and pathogen are required for a better understanding of their interaction.

Can *N. ceranae* kill a colony?

Accurate data on nosemosis type C as a main cause of colony mortality is difficult to find in the literature, principally because of the absence of clear clinical signs (Higes *et al*., 2010c). However, there are some reports where nosemosis (without specifying the species) is associated with colony losses. A survey carried out in 2014–2015 in the USA, reported that 5% of the beekeepers attributed to *Nosema* disease a 53.9% of colony mortality (CI 95%: 50.0–57.8) (Seitz *et al*., 2016). Another survey in Europe, developed in 2012–2013 showed 21.77% (CI 95%: 14.14–31.14) winter mortaligy in colonies suffering nosemosis (based on clinical signs for *N. apis*) versus a 12.64% (CI 95%: 6.84–20.78) in colonies without the infection, although significant regional differences in colony losses were observed (Chauzat *et al*., 2016).

The first evidence of a relationship between *N. ceranae* infection and colony loss was recorded in Spain (Higes *et al*., 2006; 2008a; 2009a; Botías *et al*., 2013; Cepero *et al*., 2014; Meana *et al*. 2017). Subsequently, a similar link between this pathogen and honey bee colony weakness/loss was proposed in other countries with comparable climatic conditions such as Greece (Hatjina *et al*., 2011), Israel (Soroker *et al*., 2011), South-East, North and Western-coast of USA, (Villa *et al*., 2013; Bekele *et al*., 2015), Central Chile (Bravo *et al*., 2014), Italy (Lodesani *et al*., 2014; Cavigli *et al*., 2016) and Jordan (Adjlane and Haddad, 2016). On the contrary, it seems that colder climates like Germany, Balkan countries, Switzerland and Northern Greece do not fulfill the specific conditions (climatic and/or beekeeping practices) for *N. ceranae* to compromise colony survival (Gisder *et al*., 2010; Hedtke *et al*., 2011; Stevanovic *et al*., 2011; 2013; Dainat *et al*., 2012; Francis *et al*., 2014). This may well reflect the ability of *N. ceranae* spores to better resist to high temperatures and desiccation than to low temperatures (Fenoy *et al*., 2009; Sánchez Collado *et al*., 2014) and its ability to complete the life cycle more efficiently at high temperatures (Martín-Hernández *et al*., 2009; Higes *et al*., 2010b). Thus, in warmer areas the infection by *N. ceranae* might cause a chronic stress on honey bee colonies will be more intense, ultimately favouring colony death (Higes *et al*., 2008a,b; Maiolino *et al*., 2014) as predicted by recent models (Betti *et al*., 2014; Perry *et al*., 2015, see below).

Is disruption of age polyethism by N. ceranae *linked to colony mortality?*

Considering the studies carried out under field conditions, most researchers agree that *N. ceranae* produces alterations in temporal polyethism. Studies over long periods demonstrated that *N. ceranae* can trigger premature foraging activity and shorten the lifespan of infected worker bees (Dussaubat *et al*., 2013b; Goblirsch *et al*., 2013). Indeed, *N. ceranae* infection appears to accelerate honey bee behavioural development (Higes *et al*., 2008a) and it disrupts the basic underpinnings of temporal polyethism as workers may become less flexible in their response to colony demands, leading to colony decline. It has also been shown that infected bees take longer foraging trips and that they spend less time in the hive between successive trips,

bringing back less sugar from each trip (Naug, 2014; Alaux *et al*., 2014). The changes in foraging activity (Goblirsch *et al*., 2013) have a strong adverse effect on the efficiency of the colony's energetic gain, which has important implications for the individual and colony lifespan, producing a substantial demographic effect on the colony that can lead to a strong decline in population size and ultimately, to colony death. In the same way, Bordier and colleagues (2013), using three hives equipped with optical bee counters, recorded the drifting behaviour of bees parasitized by *N. ceranae* over their lifetime and also their survival. The authors showed that the survival of *N. ceranae*–infected bees was significantly lower than that of control bees, and the survival rate of infected bees decreased faster than the control, especially after 15 days. Also, similarly to Forfert and colleagues (2015), the authors found that *N. ceranae* parasitism did not modify the probability of drifting but *Nosema*-infected drifters performed more but shorter drifts compared to 'healthy' drifters.

As mentioned, *N. ceranae* alters the metabolism of bees increasing EO levels (Dussaubat *et al*., 2010). Consequently, infected bees undertake precocious and more intense flight activity than healthy bees; at the same time, they exert a pheromone pressure on healthy bees that might delay their behavioural maturation, and colonies suffered from higher mortality rates, as observed in a 28-day study with bees infected at birth (Dussaubat *et al*., 2013b). In a 35-day study, *N. ceranae* altered the flight behaviour of infected bees (inducing early foraging activity and longer foraging trips), and a change in EO levels that also resulted in modifications in the colony homeostasis, and a reduction in the survival of *N. ceranae*-infected bees (Alaux *et al*., 2014). Also, an accelerated lipid loss in *N. ceranae*-infected worker bees has been proposed to cause a cascading effect on downstream physiology that may lead to precocious foraging, which is a major factor driving colony collapse (Li *et al*., 2018).

Conversely, short-term studies starting with young honey bees tend to show no effects on behaviour or mortality at the individual or colony level in field conditions. For example, a field study showed no effect of *N. ceranae* on in-hive activity and mortality of worker bees during a 13–14 day observation period (Retschnig *et al*., 2015). Another study using 10 and 17 day-old bees (7 and 14 days post infection) observed no effect on learning and memory tests using PER (Charbonneau *et al*., 2016) or only slightly impaired learning in 16 day-old infected honey bees (8–9 days post infection; Piiroinen and Goulson, 2016).

The altered foraging activity described by most of the works may be due to overexpression of the neuropeptide gene encoding a pheromone synthesized in the brain of the *N. ceranae*-infected bees (McDonnell *et al*., 2013), which suggests that this microsporidia might induce cognitive impairment in bees that affecting their orientation capacity (Higes *et al*., 2008a,b; Kralj and Fuchs, 2010). In this sense, *N. ceranae* was seen to provoke homing defects when harmonic radar technology was employed to characterize its impact on flight and orientation in the field (Wolf *et al*., 2014), expressed as worse flight performance rather than compromised navigation. These alterations potentially compromise the colony by reducing resource input. Conversely, in a study performed in the UK with healthy-looking forager bees with low natural infection levels, no effect was observed regarding duration and distance of flights (Wolf *et al*., 2016), probably due to the different environmental context or genetic background.

Colony chemical communication, based on pheromonal signals, is disrupted not only between workers of infected colonies but also between workers and infected queens. Queens infected with *N. ceranae* showed significantly high levels of two components of the mandibular queen pheromone (QMP) (Alaux *et al*., 2011). Moreover, workers with high spore counts had a significant decrease to queen mandibule pheromone attraction and increased walking and trophallaxis rates (Lecocq *et al*., 2016). All these effects could cause a disruption of chemical communication and they could compromise the colony survival (Dussaubat *et al*., 2016). This would dramatically affect colony resilience, the ability to tolerate the loss of somatic cells (worker bees) as long as the germ line (reproduction) is maintained.

Can chronic N. ceranae *infection break colony resilience?*

A demographic model explored the process of colony failure (Khoury *et al*., 2011). The hypothesis formulated was that colony failure occurs when the death rate of bees in the colony becomes unsustainable and its social dynamics breaks down, producing colony failure. As such, any factor that elevates the death rate of foragers will reduce the strength of social inhibition, resulting in the precocious onset of foraging behaviour in younger bees, as described above. The model suggests that if the high rate of forager death is sustained, nurse bees begin foraging precociously to restore the proportion of foragers in the population, reducing the time each bee contributes to colony growth and brood production. In a second model Khoury and colleagues (2013) suggest that both food availability and the forager bee death rate have a very strong influence on colony growth and development. Low forager death rates and high food availability result in stable populations and in a consequent increase in food reserves. As forager death rates

increase, the food stores reach a finite equilibrium that reflects the balance of food collection and consumption. When the forager death rates exceed a critical threshold as occurs in the presence of *N. ceranae* infection (Higes *et al*., 2008a; Goblirsch *et al*., 2013), the colony fails but residual food may remain. These findings were also reached by applying another model (Russell *et al*., 2013), whereby a colony could fail to display features of colony collapse disorder (CCD). Interestingly, this scenario has been described in several studies of natural *N. ceranae* infection under field conditions (Higes *et al*., 2009a; Botías *et al*., 2012b,; Bravo *et al*., 2014; Cepero *et al*., 2014; Lodesani *et al*., 2014; Simeunovic *et al*., 2014; Bekele *et al*., 2015).

A more complex mathematical model presents different dynamics that are able to disrupt colony health (Betti *et al*., 2014). This model aims to define a possible mechanism linking *N. ceranae* infection to colony collapse through an interplay between the dynamics of infection and those of a normal colony. The model suggests that the key factors in the survival or collapse of a honey bee colony are the rate of transmission of the infection and the disease-induced death rate. An increase in the disease-induced death rate, which can be thought of as an increase in the severity of the disease, may actually help the colony to overcome the disease and survive through the winter, as severely infected bees perish reducing the infected population in the colony. By contrast, an increase in the transmission rate, which means that bees are being infected at an earlier age, has a dramatic deleterious effect. Moreover, it appears that if infection occurs within approximately 20 days of the onset of winter, the colony is more severely affected. In another experiment, the demography of experimental colonies was manipulated to induce precocious foraging and radio tag tracking was used to examine the consequences of precocious foraging on the bees' performance (Perry *et al*., 2015). As indicated above, bees respond to many stressors by foraging earlier in life (e.g., *N. ceranae* infection), yet this is not without significant cost to the individual bees and to the colony as a whole. These precocious foragers (also indicated in Naug, 2014) have a greatly reduced effective foraging life and efficiency compared to normal aged foragers. Such colonies stabilize their populations for a period and brood rearing continues (as described in Higes *et al*., 2008a), yet the population ultimately declines if the stress is maintained chronically as the colony's capacity to buffer its effects becomes exhausted (*N. ceranae* is constantly present in colonies in warm areas). Interestingly, this model produces results that are in agreement with the field observations described previously (Higes *et al*., 2008a).

Can the infection of N. ceranae *in field conditions be easily identified?*

The clinical manifestation of *N. ceranae* infection has become one of the most controversial aspects of beekeeping under field conditions. Years ago, Koch's postulates were followed to show how *N. ceranae* can cause the death of a honey bee colony by inducing chronic stress (Higes *et al*., 2010c), although it is evident that nosemosis type C does not evolve equally around the globe (Higes *et al*., 2013), making difficult to define universal clinical signs. As commonly described in veterinary medicine, a clinical sign is an objective indication of a specific event or a characteristic that can be detected either by examination or by *in vivo*/*in vitro* analysis of the subject. A disease in a group (apiary) often manifests with a spectrum of signs that range from unapparent to subsigns in farm animals. Clinical features of nosemosis type C described in Spanish colonies include a longer breeding period during cold months (even when the winter break should usually occur), a higher proportion of frames containing brood with respect to the number of nurse bees during the warm months, and diminished honey production, infected colonies become clearly weakened and depleted of adult bees, and they collapse in a period of 1.5–2 years (Higes *et al*. 2010a). These subclinical manifestations of nosemosis type C are usually not considered as they are easily confounded with other causal factors. Indeed, in the absence of classical signs such as those of nosemosis type A (Higes *et al*., 2008a), the role of the nosemosis especially caused by *N. ceranae* in colony loss is usually erroneously dismissed.

A common subclinical sign of nosemosis type C is the decline in honey production of about 50% (Bravo *et al*., 2014). Similar results were obtained elsewhere (Botías *et al*., 2013), with a reduction in honey production of between 52% and 67% in *N. ceranae*-infected colonies. Analogous losses in honey production have been observed in colonies headed by three-year-old queens with high *N. ceranae* load as compared to colonies with younger queens (Simeunovic *et al*., 2014). Another interesting side-effect of *N. ceranae* infection is the reduction in the effectiveness of strip-treatment against varroa (Botías *et al*., 2012), which is weakened in colonies with high *N. ceranae* load. The effectiveness of varroa strip treatment depends on bees contacting the strips and their subsequent interaction within the colony. The behavioural and social changes provoked by the presence of *N. ceranae* in colonies could interfere with and weaken this varroa treatment. This effect should be taken into account when assessing acaricide treatments in field conditions, which should be considered as a subclinical consequence of *N. ceranae* parasitism.

Furthermore, *N. ceranae* infection in spring and varroa in summer influence outbreaks of stress-related diseases like chalkbrood (Hedtke *et al*., 2011), a fungal disease of the honey bee brood caused by *Ascosphaera apis* that has a detrimental effect on the colony (Jensen *et al*., 2013). Both parasites affect the adult bee population and *N. ceranae* produces a disruption of the basic foundations of temporal polyethism, as workers and colonies may lose their capacity to respond to the colony's demands (Higes *et al*., 2008a; Goblirsch *et al*., 2013). As a result, there may be too few hive bees to adequately maintain the brood temperature around 34–35°C, thereby increasing the likelihood of chalkbrood outbreaks.

It was suggested that co-infection by an iridovirus and *N. ceranae* was linked with honey bee decline in the USA (Bromenshenk *et al*., 2010), but the presence of iridovirus in both CCD and healthy colonies was not lately confirmed (Tokarz *et al*., 2011). A link was suggested between the presence of *C. mellificae* and *N. ceranae* in summer, and the negative synergy between the two was proposed to be a predictive marker of winter mortality (Ravoet *et al*., 2013), although laboratory infection with both parasites did not differ from the group infected with *N. ceranae* only (Higes *et al*., 2016). The combination of certain pesticides and the effects of *N. ceranae* on bee colonies have also been studied, such as the treatment of sunflowers with the insecticide fipronil and its effect on honey bee colony loss (Bernal *et al*., 2011). Neither the pathogen nor fipronil were detected in residues of adult bee and pollen (corbicular and stored), yet *V. destructor* and *N. ceranae* were prevalent in the apiaries studied. This combination of pathogens was considered to be the determining cause of the high mortality of the colonies in the apiaries surveyed. When exposure to different pesticides was studied, *Nosema* infection was shown to be significantly more severe in the bees from imidaclopride-treated hives (Pettis *et al*., 2012; 2013).

Colonies fed with protein supplement have more *Nosema* (and BQCV) than when they were fed with pollen (from *Brassica rapa*; DeGrandi-Hoffman *et al*., 2015). Also, an increased probability of *Nosema* infection was evident in bees that consumed pollen with a higher fungicide load, an issue that should be taken into account in the future. In this sense, *N. ceranae* and pesticide exposure would appear to contribute to honey bee health decline (Wu *et al*., 2012). Bees reared from brood combs containing more pesticide residues (e.g., chlorpyrifos, endosulfan, fluvalinate, etc.) were more often infected with *N. ceranae* than those reared in brood combs with fewer residues, and at a younger age. These data suggest that the exposure to some pesticides during development in the brood combs increases the susceptibility of bees to *N. ceranae* infection (Pettis *et al*., 2013).

What is the impact of beekeeping practices?

Beekeeping practices may also influence the in-field evolution of nosemosis type C in the host, as well as the evolution of the pathogen. Since the queen's age has an important role on the evolution of *N. ceranae* infection and honey bee colony strength (Botías *et al*., 2012b), replacement of an old queen by a younger one decreases the proportion of *Nosema*-infected forager and house bees. This practice will maintain the overall infection rate at a level compatible with colony viability and productivity (Simeunovic *et al*., 2014). Moreover, this feature should be taken into account in field studies of *N. ceranae* infection and natural or artificial queen renewal must be reported to avoid errors in the interpretation of the results.

It was also recently shown that the therapeutic doses of oxalic acid utilized for varroa control might inhibit the development of *N. ceranae* in laboratory and field conditions, both at the individual and colony levels (Nanetti *et al*., 2015). There is also some evidence that formic acid fumigation may help to suppress *Nosema* (Underwood and Currie, 2009). Effectivity of thymol and resveratrol against *Nosema* were also reported (Costa *et al*., 2010), although thymol and coumaphos were suspected to increase susceptibility to infection by *N. ceranae* (and *C. mellificae*), since both products cause a significant reduction in *Dscam* transcription (Boncristiani *et al*., 2012), an important element in the honey bee immune response to these parasites (Schwarz and Evans, 2013). The fact that the use of chemicals to treat varroa infestation is not uniform in different regions could explain the conflicting data on the importance of *N. ceranae* on honey bee health. The action of some therapeutic products on bee physiology can also affect *N. ceranae* infection and disease development. The effect of other beekeeping practices, that highly differ between countries, on *N. ceranae* infection should also be borne in mind, since such effects remain unknown (Higes *et al*., 2013).

Over the years of confronting nosemosis, much effort has been invested in search of effective cure against it. So far, bicyclohexylammonium fumagillin, an antibiotic isolated from the fungus *Aspergillus fumigatus*, is one of the few drugs known to be active against microsporidia (McCowen *et al*., 1951), suppressing their reproduction and multiplication at recommended concentrations (Higes *et al*., 2011; Huang *et al*., 2013). Fumagillin is extensively used to control *Nosema* disease in apiculture for over 60 years. Its mode of action involves binding to the active site of MetAP-2 (Methionine

aminopeptidase 2) enzyme, thus inhibiting its activity. Fumagillin activity is unspecific to *Nosema*, affecting mammalian as well as honey bee MetAP-2. van den Heever and colleagues (2014) further suggested that fumagillin toxicity to bees may explain some reports of bee mortality. In the commercial formulation, fumagillin is present as a salt in an equimolar quantity with dicyclohexylamine (DCH). The toxicity of both components to humans caused by residues remaining in hive products is suspected (van den Heever *et al*., 2014). DCH contamination in the hive products is also of concern due to its stability and lipophilicity. Thus, Fumagillin-B® is currently not licensed in most countries of the European Union due to the side effects risk of its commercial formulation, like genotoxic and tumorigenic properties and stability in honey (van den Heever *et al*., 2016b). Anyhow, Fumagillin-B® or Fumidil B® is the only registered chemical treatment available to combat *Nosema* disease in apiculture. To reduce the residues problem in the honey, fumagillin treatment is prohibited in US and Israel during the foraging season. It is commonly prophylactically applied to the hives in late fall and early spring in most of the US and Canada (Huang *et al*., 2013; Williams *et al*., 2011) or in November-December in Israel. The impact of this treatment on colony survival is not yet clear (Soroker, unpublished). In Uruguay, different winter fumagillin treatments were able to provide temporal decrease in *Nosema* spores but did not affect colony survival, irrespective of dose or application strategy (Mendoza *et al*., 2017). It has also been reported that *N. ceranae* seems to reproduce even better at lower concentrations of fumagillin, which also affects the bees' physiology, such that its use may augment the prevalence of *N. ceranae* (Huang *et al*., 2013).

Several semisynthetic and synthetic fumagillin analogues were shown to possess biological activity against *N. ceranae* under laboratory conditions but none were as effective as Fumagillin-B® (van den Heever *et al*., 2016). The most popular alternative treatments against nosemosis in Europe are Api Herb, Nozevit+, Vita Feed Gold, Protofil, Hive Alive and Nosestat. Other treatments, such as acetylsalicylic acid with extract of *Artemisia absinthium* L. and extracts of *Aster scaber and Artemisia dubia*, are currently under study (Kim *et al*., 2016; Michalczyk *et al*., 2016). Alternative products have been tested under laboratory conditions with some success (Porrini *et al*., 2010; Bravo *et al*., 2017), although they are not commercially available.

The effects of probiotics and prebiotics on *N. ceranae* infection have also been analysed. *Lactobacillus rhamnosus* (a commercial probiotic) and inulin (a prebiotic) showed no beneficial effect on the survival rates of honey bees infected with *N. ceranae* (Ptaszyńska *et al*., 2016). Similarly, a mixture of different species belonging to *Lactobacillus*, *Bifidobacteria*, *Pediococci* and *Lactococci* genera showed no advantageous effect on the infection (Endler, 2014). Another study including nutraceutical, prebiotic and probiotics showed acacia gum as the most effective prebiotic, although with a high mortality as side effect, and the probiotic Protexin Concentrate© single-strain (ProtexinC1) as able to reduce the spores, increasing the bee survival (Borges, 2015).

Finally, a promising assay has shown that the administration of autoclaved spores to larvae was able to reduce the infection levels of adults by 57% without significantly altering larval or adult longevity (Endler, 2014).

What's in the future?

In spite that *N. ceranae* infection is widespread in both healthy and declining honey bee colonies, recent research has shed light on its contribution to honey bee losses. However, research is needed to clarify some basic questions: (i) what is the role of larval stage infections in the epidemiology of nosemosis type C and (ii) what causes the different clinical signs between *N. apis* and *N. ceranae* when tissue lesions look the same?, (iii) what are the interactions with other nosogenous biotic and abiotic agents?, as well as (iv) what is the impact of the newly discovered *N. neumanii* and its distribution and prevalence?. There is also a need to develop better measures to reduce the impact of these diseases in worldwide beekeeping, especially focused in the selection by host tolerance mechanisms at the individual and social levels.

Another challenge is to achieve a better comprehension of the host-parasite dynamic in specific environmental contexts like temperate regions, this would allow to evaluate the contribution of factors like climate, other honey bee pathogens such as viruses and varroa, exposition to agro-chemicals and food resources, and in-hive treatments against other hive pests and pathogens that may negatively interact with *N. ceranae* and endanger colony survival.

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Conflict of Interest

Authors declare no conflict of interest.

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