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Review article

Nosema ceranae in Europe: an emergent type C nosemosis*

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Abstract – In this review, relevant data is presented on an emerging disease of the 21th century in European countries, caused by *Nosema ceranae*. Within a few years after it was detected in Spain in 2005, the rest of European countries that had technical capacity to differentiate *Nosema apis* from *N. ceranae* reported its presence. In a similar way as the initial detection of *Varroa* in Europe, active scientific work is raising many questions due to the absence of clinical symptoms in infected colonies and a long incubation period of the pathogen. *N. ceranae* presents a different epidemiological pattern and pathology compared to *N. apis*. The disease caused by *N. ceranae* is now named nosemosis type C (COLOSS workshop, 2009) and is characterized by the ability to detect the disease-causing agent throughout the year. The continuous death of highly infected bees, mostly foragers, has a clear effect on colony population and productivity. Although there has been a huge effort in the last years to increase knowledge about this disease, significant research is still needed on epidemiology, pathology, prophyllaxis and treatment.

***Nosema ceranae* / *Nosema apis* / *Apis mellifera* / nosemosis / Europe**

1. INTRODUCTION

The detection of *Nosema ceranae* (Microsporidia: Nosematidae) in *Apis mellifera* Linnaeus was reported in 2005 in both Europe and Asia (Higes et al., 2005, 2006; Huang et al., 2007). Originally described in *Apis cerana* (Fries et al., 1996), the spread of *N. cerana* from *A. cerana* to *A. mellifera* probably occurred within the last decade (Klee et al., 2007), although this must still be confirmed (Fries, 2009). To date, two microsporidian species infect honey bees worldwide: *Nosema apis* Zander and *N. ceranae*. *N. apis* was shown to infect *A. mellifera* more than one hundred years ago and was one of the first microsporidia to be described. Indeed, the disease caused by this microsporidia in honey bees was even recognized before the etiological agent was discovered. The bright oval corpuscles found in the digestive tract indicated the fungal origin of the disease (Donhov, 1857; Higgins, 1858; data from Neveu-Lemaire, 1938). In Germany, the corpuscles were finally attributed as the cause of the disease, and were classified as *N. apis* by Zander in 1909. There have been several studies concerning the biology and epidemiology of nosemosis (see particularly Fantham and Porter, 1912; Maassen, 1912, 1914; and Trappmann, 1920: data from Neveu-Lemaire, 1938), and there is a huge body of work related to nosemosis due to *N. apis*, although it was detected molecularly only recently (Webster et al., 2004).

In contrast, studies on *N. ceranae* were limited to the discovery of the parasite in *A. cerana* (Fries et al., 1996). Until 2005, it was assumed that when microsporidian spores were present in honey bees they were always *N. apis*. However, in 2005, a sequence was obtained from a microsporidian isolate in a study

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of diseased *A. mellifera* workers thought to be infected by *N. apis* (Huang et al., 2007). In the same year but in a different continent, *N. ceranae* was isolated in European worker honey bees while trying to standardize the molecular detection of *N. apis* from samples collected from colonies suffering unexpected population loss and/or lower honey production (Martín-Hernández et al., 2005; Higes et al., 2006).

Traditionally, the term "nosemosis" is considered to be the infection of ventricular cells of adult honey bees by *N. apis*, the etiological agent (OIE, 2004). This disease was characterized, in acute forms, by trembling of honey bee workers, bees with a dilated abdomens, brown faecal marks on combs and the front of the hives, sick or dead bees in the vicinity of the hives, and a decrease in brood production and in the size of bee colony particularly in spring (Bailey, 1955; Cornejo and Rosi, 1975; Faucon, 2005; OIE, 2008).

However the emergent disease caused by *N. ceranae,* now known to be present in all five continents (Martín-Hernández et al., 2007; Klee et al., 2007; Giersch et al., 2009; Higes et al., 2009a), presents a different epidemiological pattern, symptomology and pathology. Indeed, it is now considered a major health problem in both individual honey bees (Paxton et al., 2007; Martín-Hernández et al., 2009a; Antúnez et al., 2009) and in whole colonies (Higes et al., 2008a, 2009b). Prior to the identification of *N. ceranae* in honey bees, a "dry nosemosis" was observed with different symptoms from the acute form described for *N. apis* (Faucon, 2005). The different symptoms presented by these *Nosema* species in honey bees highlights the need to consider two different clinical patterns: nosemosis type A caused by *N. apis* and nosemosis type C caused by *N. ceranae* as agreed in a recent International Meeting (COLOSS, 2009).

Most studies on *N. ceranae* in Europe have been reported at meetings of a European working group, EU COST Action (FA0803) that initially met in Wageningen in March 2007. This group, now known as COLOSS (Prevention of Honey Bee Colony Losses; www.coloss.org, organized by Dr. Neumann) currently has more than 150 members from 40 countries, including non-EU countries Our aim is to summarize the findings of all the studies on *N. ceranae*, highlighting the pathology caused in honey bees and the epidemiological factors related to this emergent disease in Europe.

2. COLONIZATION AND SPREAD OF *NOSEMA CERANAE* IN EUROPE

In the last few years, infection of honey bees (*A. mellifera*) by *N. ceranae* has been reported in a number of European countries, including Spain, France, Germany, Switzerland, Denmark, Finland, Greece, Hungary, Holland, United Kingdom, Italy, Serbia, Poland, Slovenia, Bosnia I Herzegovina and Sweden (Higes et al., 2006; Fries et al., 2006; Martín-Hernández et al., 2007; Klee et al., 2007; Chauzat et al., 2007; Topolska and Kasprzak, 2007; Paxton et al., 2007; Tapaszti et al., 2009; Kryeger, 2009; Santrac, 2009), as well as in other continents (Huang et al., 2007; Cox-Foster et al., 2007; Giersch et al., 2009; Higes et al., 2009a). The exact date of the arrival of *N. ceranae* into Europe has not been determined. The concurrent detection of the parasite in Europe and Asia may not have been related to its jump to *A. mellifera* but rather to the development of new highly sensitive and specific molecular tools. *N. apis* and *N. ceranae* spores are not easily distinguished from each other so molecular tools to identify each species have played a decisive role in studies on them (Weiss and Vossbrinck, 1999, in Klee et al., 2007).

The colonization of *A. mellifera* by *N. ceranae* first implied that the parasite reached new geographical areas. Retrospective analysis of data and samples suggest that *N. ceranae* has been in Europe for the past decade (Higes et al., 2006; Martín-Hernández et al., 2007; Klee et al., 2007; Paxton et al., 2007). This is not surprising since the dynamics of population growth in a new habitat usually follow a characteristically sigmoid curve (Bush et al., 2001). Currently, the near-exponential increase in population size of *N. ceranae* is associated with a high prevalence due to the stable population growth in almost all countries

where *A. mellifera* is reared. A similar situation was described for the mite *Varroa destructor* (formerly *Varroa jacobsoni*: Anderson and Trueman, 2000), that spread around the world after its first detection in Asian honey bees in 1904. Although the historical samples are still limited, the data obtained clearly indicate that *N. ceranae* is an exotic pathogen of *A. mellifera* in Europe.

Understanding the origins and spread of *N. ceranae* through colonies of *A. mellifera* and other Hymenoptera worldwide will require new molecular genetic markers to plot the phylogeography of this pathogen (Paxton et al., 2007). The microsporidian rRNA gene has been used in phylogenetic analyses (Rice, 2001; Keeling and Fast, 2002; Williams et al., 2008; Chen et al., 2009b), although some genetic variations exist in the 16S rRNA gene of related microsporidia (Tay et al., 2005). Cornman et al. (2009) found forty-six contigs containing sequences that matched *N. ceranae* ribosomal sequence and indicated that *N. ceranae* ribosomal loci contain abundant polymorphisms and/or error-prone sequences. Indeed, rRNA are present in multiple copies and some variations may exist between copies in *N. ceranae* rRNA genes, precluding clear genotyping of different isolates in Europe (Henriques, 2009). Therefore, epidemiological or phylogenetic relationships between isolates of *N. ceranae* based on repeated sequences such as rDNA must be clearly established. The existence of multiple rDNA copies in the genome of *Nosema* suggests that part of the genetic variation detected may represent between-copy, within-spore diversity (O'Mahony et al., 2007). This also indicates that new polymorphic markers will be needed to define an isolate that can serve to differentiate genotypes or strains (COLOSS workshop, 2009).

The genome sequence of *N. ceranae* based on pyrosequencing data was recently published (Cornman et al., 2009) including initial gene models and genomic comparisons with other members of this highly derived fungal lineage. It was shown that the genome of *N. ceranae* is extremely reduced and strongly AT-biased (74% A+T). The genome sequencing should be of interest for further studies concerning honey bee-*Nosema* interactions, the identification of new epidemiological markers and new targets for therapeutics.

According to the epidemiological characteristics of *N. ceranae*, infection by this microsporidian presents a different pattern to that described for *N. apis* in temperate areas of Europe. Specifically, colonies affected by *N. apis* generally display low levels of infection during summer, a small peak in autumn and the usually slow increased in infection during winter (Bailey, 1955). The main difference between both agents is that *N. ceranae* can be detected in samples throughout the year (collected in each season or month; Martín-Hernández et al., 2007). The lack of seasonality was suspected when analysing the monthly prevalence of *Nosema* spore detection between 1999 and 2005 in 5776 samples across Spain (Martín-Hernández et al., 2007). Accordingly, the seasonal pattern described for *N. apis* was conserved during the initial years (1999–2002), although it was gradually lost in the later period (2003–2005). This lack of seasonality was linked to an increase in colony death reported by beekeepers, as well as the absence of classical signs of *Nosema* in colonies. A similar lack of seasonality has also been suggested in other studies (Tapaszti et al., 2009).

It has been proposed that *N. ceranae* is more prevalent in warmer climates compared to *N. apis* (Fries, 2009). It appears that *N. ceranae* is better adapted to complete its endogenous cycle with a higher biotic index at different temperatures reflecting the epidemiological differences between both microsporidian species in field conditions and at the colony level (Martín-Hernández et al., 2007, 2009a). There also are differences in the resistance of the spores of both microsporidia. For example *N. ceranae* spores are more resistant to desiccation and have a higher thermo-tolerance at 60 ^{o}C for 6 hours (Fenoy et al., 2009) compared to *N. apis* spores that die after 15 min at 60 ˚C (Cantwell and Shimanuki, 1970). In contrast, *N. apis* has a higher rate of infectivity when frozen compared to *N. ceranae* (Fries and Forsgren, 2009, in Fries, 2009), although the loss of viability of *N. ceranae* spores was not so dramatic when they were maintained

in standard medium (RPMI+10%DMSO), in which more than 80% of spores were viable after 3 weeks of freezing (Fenoy et al., 2009). Thus, it seems that the method of spore preservation is an important factor that must be considered in all experimental protocols. Nevertheless, assays under experimental conditions cannot necessarily be extrapolated to natural conditions and consequently, long-term assays on spore viability should be performed under different climatic conditions in Europe, and in different structures, media and solutions to obtain conclusive data.

In most European honey bee samples parasitized by *Nosema* spp. analyzed to date (Chauzat et al., 2007; Martín-Hernández et al., 2007; Klee et al., 2007; Tapazsti et al., 2009; van der Zee, 2008; Granato et al., 2009; Korpela, 2009), a higher rate of *N. ceranae* detection led to the apparent displacement of *N. apis*. However the lack of seasonality, the higher prevalence detected in most studies, a wider thermal reproductive range and higher resistance to spore desiccation, led to a increased chance of detecting *N. ceranae* over *N. apis*, which could have biased the impression that *N. apis* has been displaced. Such a conclusion cannot be made from the short amount of time (less than 5 years) that studies have been carried out in Europe on *N. ceranae.*

It is generally accepted that the earth's temperature is progressively increasing and the consequences of this effect on the endogenous and external life cycles of parasites is a matter of concern (Brooks and Hoberg, 2007). As described for *Aethina tumida* (Le Conte and Navajas, 2008), increasing temperatures due to climatic change will promote the range expansion of honey bee pathogens or pests. Changes in climate may affect the distribution, seasonality and severity of infectious diseases such as nosemosis in honey bees (De la Rocque et al., 2008), and the plasticity of species to adapt to new triggers will increase the probability of their spread but also to consolidate the invasion of new ecosystems under different environmental conditions. In this sense, more research will be needed to establish the epidemiological field characteristics of nosemosis type C in different European countries.

3. BIOLOGY AND TRANSMISSION OF *NOSEMA CERANAE* IN *APIS MELLIFERA*

Microsporidia are a large and diverse group of ubiquitous eukaryotes, exhibiting a very wide range of hosts, including nearly all the invertebrate phyla (especially insects) and all five classes of vertebrates (Canning and Lom, 1986; Mathis, 2000; Franzen and Muller, 2001; Weiss, 2003). They are obligate intracellular single-celled, spore-forming parasites that belong to the Microspora phylum, and there are more than 1300 formally described species in 160 genera, almost half of which have an insect host (Wittner and Weiss, 1999; Becnel and Andreadis, 1999; Keeling, 2009). It is one of the most outstanding groups of organisms in many aspects, recently reclassified from the protozoa to the fungi, within the Opisthokonta (Adl et al., 2005).

From the point of view of both parasitism and evolutionary biology, microsporidia represent a fascinating and important model to understand issues such as host cell manipulation, host-parasite molecular interactions and extreme adaptation to an intracellular environment (Williams, 2009). Honey bee infection takes place after ingestion of mature microsporidia spores, probably during cleaning activities or trophallaxis. The spores enter the bee through the food canal and germinate in the midgut where the epithelial cells become infected after spore germination (polar tube extrusion and host cell invasion). Some features of the endogenous life cycle of *N. ceranae* infection in *Apis mellifera iberiensis* have been described previously (Meana et al., 2007; Higes et al., 2007, 2008b).

The Nosema microsporidia is diplokaryotic and it is in direct contact with the host cell cytoplasm at all parasitic stages, completing its life cycle in less than 3 days. The highly resistant mature spores of *N. ceranae* (Fig. 1) are small, ovocylindrical, and can vary from 3.3 to 5.5 μm in length, and from 2.3 to 3 μm in width (Fries et al., 1996; Chen et al., 2009a). Fresh spores can be easily distinguished in the faeces or intestinal contents of bees because they are extremely refractive when viewed by phase contrast microscopy.

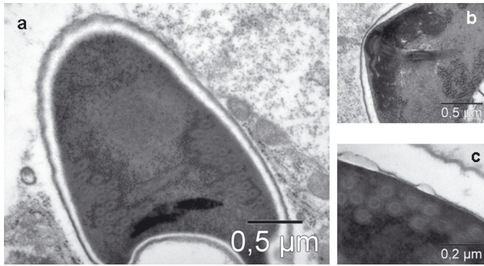

Figure 1. Transmission electron images of *Nosema ceranae* spores. Longitudinal section of a spore (a). Anterior portion of a spore showing the mushroom-shaped anchoring disk connected to the manubroid polar filament. The polar filament and anchoring disk are surrounded by a limiting membrane. The thin endospore area above the anchoring disk is clearly seen (b). Detail of polar filament coils (c).

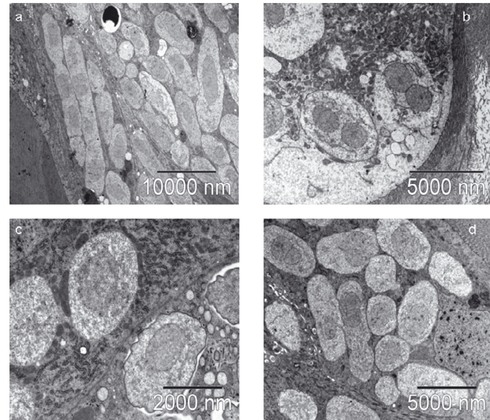

Figure 2. Honeybee ventricular cells infected by different stages of *Nosema ceranae*. Merogonial plasmodia were frequently observed, some of them with four diplokarya (a). Several mitochondria were close to and surrounded the plasmalemma of meronts (b) while RER, usually ring-shaped, is commonly observed around sporonts (c). Parasitized epithelial cells showing the nucleus apically displaced with some immature and mature stages of *N. ceranae* in invaginations of the nuclear membrane (d).

Electron microscopy reveals a typically binucleate spore (Fries et al., 1996; Higes et al., 2007), the outer coat of which has an electron-dense region called the exospore that measures about 14–17 nm in *N. ceranae* and that surrounds an inner electron-lucent region called endospore (Fries et al., 1996) (Fig. 1). The anterior end is filled with the anchoring disk and the start of the polar tube (Fig. 1). Lamellar and tubular polaroplast membranes dominate the spore content and a diplokaryon is surrounded by the polar tube. This structure is arranged in the posterior and mid-part of the spore, with 18–23 coils (Fries et al., 1996; Higes et al., 2007, 2008c; Chen et al., 2009a), and it follows the periphery of the spore, enclosing its contents or the sporoplasm. At the posterior end, a vacuole contains floccular material or a dense body composed of tubular material that may appear glomerular. Under appropriate conditions, the spore is activated in the honey bee midgut environment and triggered to evert its polar tube (polar tube extrusion also called spore germination), which becomes a hollow tube. The spore becomes primed or activated for infection (host cell invasion) by shifts in pH and cation/anion concentration on entering the digestive system, or by approximation to a potential host (reviewed by Keohane and Weiss, 1999; Williams, 2009). The signalling pathways that lead to germination have not been elucidated, although there is an indication that calcium/calmodulin binding at the spores' surface may commence a signalling cascade that causes spore activation (Weidner and Byrd, 1982).

When the everted polar tube pierces a honey bees' intestinal cell, the sporoplasm is injected into it to initiate its dissemination. Once inoculated the proliferative period commences, in which *N. ceranae* maintains direct contact between the plasmalemma and the cytoplasm of the host cell in the absence of an interfacial envelope and a parasitophorous or sporophorous vesicle. Once injected, the binucleate sporoplasm undergoes the vegetative stages of merogony. The cytoplasm surrounding the nuclei, sometimes with four diplokarya (Fig. 2), elongates into a ribbon-like structure, which divides by forming indentations of the plasmalemma between the diplokaryons. The number of proliferative cycles may vary and is yet to be established.

Infected cells are enlarged and the cytoplasm contains many mitochondria and free ribosomes. Several mitochondria can be seen

close to and around the parasite plasmalemma (Fig. 2), which probably facilitates ATP uptake from the host cell (reviewed by Williams, 2009). In this phase, epithelial cell nuclei are displaced to an apical position and they change morphologically. Numerous invaginations and parasitic proliferative cells can be observed close to the epithelial cell nuclear membrane (Fig. 2).

The sporogonic phase involves the formation of sporonts, sporoblast and spores. Sporoblasts are the cells derived from the final division of the sporont and they give rise to spores. They form the extrusion apparatus that will produce the polar filament, its anchoring disk complex, the polaroplast membranes and tubules, and the posterior vacuole. In cells with sporogonial phases, the RER content is higher than when proliferative stages are present, and it closely abuts the parasite plasmalemma during development.

The increase in cytoplasmic density is a result of the rapid production of RER and the enlargement of ribosomes, and the internal development of spore structures. Less than three days after infection, mature spores can be observed as emptied spores inside the infected cells (Higes et al., 2007). This phenomenon is indicative of intracellular germination in *A. mellifera* infected with *N. ceranae* and the ensuing horizontal transmission between epithelial cells, as observed in *N. apis*. To date, no empty spores have been observed in *Apis cerana*, indicating that intracellular germination may not occur (Fries et al., 1996) that there is a weaker capacity for dissemination inside the host. Indeed, the histopathological lesions in the Asian bee host usually involve a single infected cell full of parasites but are surrounded by healthy uninfected epithelial cells.

Faecal marks in hive structures are usually reported in *N. apis* infected colonies and it is commonly accepted that the infective spores of this microsporidia can be transmitted between bees via ingestion (Bailey, 1954; Cornejo and Rossi, 1975). However, those faecal marks have not been observed in colonies infected solely with *N. ceranae* (Higes et al., 2008a, 2009b) and hence, the mechanisms of transmission might be different. Contamination of material with infective spores cannot be ruled out since this seems to be how infections are established in some isolated islands (Van der Zee, 2009). Indeed, infected bees can contaminate beekeeping material when they are crushed during manipulation of the hives (Malone et al., 2001).

The queen honey bee is susceptible to most of the diseases that attack her offspring, and *N. ceranae* infection is no exception. In experimental conditions, *N. ceranae* can be transmitted horizontally from infected worker honey bees to queens by feeding (Higes et al., 2009c). Thus, trophallaxis may be an important means of transmission from bee to bee, or from colony to colony, due to drifting. However, the behavioural changes described in caged honey bees may modify this mode of transmission since infected bees are less inclined to share the sucrose solution with other bees (Naug and Gibbs, 2009).

Pollen stored in combs within the hive is a reservoir of bee pathogens (Mehr et al., 1976; Moffet et al., 1978; Gilliam et al., 1988; Chen et al., 2006), as is the case for *N. ceranae* infection (Higes et al., 2008c). Forager bees gather pollen from flowers and pack it into the pollen basket on the outer surface of their hind legs. This corbicular pollen is deposited in a cell, usually just above or beside the broodnest. The presence of infective *N. ceranae* spores in corbicular pollen must be due to self-contamination during the process of pollen collection (regurgitation, saliva etc.), although the mechanism involved remains to be defined. However, the possibility of collecting spores directly from contaminated flowers cannot be ignored, although this seems to be highly improbable (Higes et al., 2008c). The conversion of corbicular pollen to bee bread is postulated to be the result of microbial action, principally a lactic acid fermentation caused by bacteria, yeast and moulds (Gilliam et al., 1989). However, the effect of this process on *Nosema* spore viability has yet to be evaluated. Honey (Giersch et al., 2009) and royal jelly (Cox-Foster et al., 2007) have been reported as sources of spores of *N. ceranae* although the viability of spores in this matrix is still unclear.

The presence of *N. ceranae* spores in the regurgitated pellets of the bee-eater bird, *Merops apiaster*, may potentially have

epidemiological repercussions in the dissemination of infective *N. ceranae* spores across Europe (Higes et al., 2008b), particularly since honey bees and bumble bees constitute the main part of this birds' diet (Fry, 1983; Martínez, 1984; Cramp, 1985). Bee-eaters prey on thousands of foraging honey bees (Galeotti and Inglisa, 2001), which is the bee population that contains the highest spore burden (Higes et al., 2008a). Adult bee-eaters spend almost half their time flying looking for food, and the spores ingested with the infected bees could be dispersed over large distances. Apiaries are usually stop-over sites in the migration corridors of *M. apiaster* (Yosef et al., 2006), most of which are strategically located and used year after year. Local dispersal of spores can also occur due to their wide feeding area, around 5 km from the gregarious colony nests (Reviewed by Cramp, 1985). Recent data on the breeding of *M. apiaster* in more northernly areas than those previously recorded (Volet and Burkhardt, 2006) might be related to climate change, and could have important consequences if birds are considered to be an epidemiological source of pathogen dispersal.

The world trade of honey bee products and beekeeping material may also play an important role in the dispersal of infective spores of *N. ceranae* from apiary to apiary over different geographical areas (Klee et al., 2007). In this way, commerce of queens and their worker bee escorts may be a source of infection in some areas (Giersch et al., 2009). All these means of spreading and transmitting infective *N. ceranae* spores should be taken into account to explain the presence of *N. ceranae* in *A. mellifera* colonies in remote geographical sites theoretically isolated from any source of infective spores (Colin et al., 2009).

Additionally, *N. ceranae* infects a wide high range of other *Apis* species (e.g. *Apis koschevnikovi*; Botías et al., 2009b) and some species of bumble bees (Plischuk et al., 2009). Although this has been only confirmed in Asia and South America respectively, the possibility of other host species should be studied in Europe as well.

4. EFFECTS ON BEES

The first reported experimental infection of *A. mellifera* by *N. ceranae* (Higes et al., 2007) clearly showed that this parasite was highly pathogenic to its new host, and it induced significantly higher bee mortality compared to *N. apis* (Paxton et al., 2007). Subsequently, differences in the rate of mortality have been reported (Chauzat et al., 2009; Hartmann et al., 2009). Thus, several undetermined factors might influence this rate of mortality, including spore storage, purification and viability, age of healthy uninfected newborn bees, host subspecies, parasitic strain and even accurate spore identification. The variability highlights the importance of standardizing the protocol for infection experiments, as encouraged during COLOSS conferences (Neumann, 2009).

Infected caged worker bees present a clear degeneration of epithelial ventricular cells during the first week after infection, such as the presence of vacuoles in the cytoplasm, lysosomes (most of them secondary), the disruption of cell membranes and nuclear condensation (pyknosis: Higes et al., 2007). The most parasitized epithelial cells have an apically displaced nucleus with some immature and mature *N. ceranae* stages in invaginations of the nuclear membrane. The infected epithelial cells exhibit extensive lysis, evident by the vacuoles present and the ribosome and lysosome aggregates. Heavily infected cells may either be dead or dying, and will eventually lead to the early death of bees due to starvation as described for *N. apis* (Liu, 1984).

Similar pathological lesions to those seen in caged workers bees also have been detected. The lesions result in the death of honey bee queens (Higes et al., 2009c), and can be observed in dying foragers bees collected in the field, and in foragers and nurse bees collected in a naturally infected colony (Higes et al., 2008a, 2009b). These bees had infected cells all along the ventriculus epithelium with similar alterations to those described experimentally (Higes et al., 2007). Hence, the accessibility of fresh pollen in field conditions from Spain does not prevent the pathological repercussions of *N. ceranae* in infected honey bees,

as described for *N. apis* infection (Mattila and Otis, 2006; Avilez and Araneda, 2007).

No spores have been found in histological sections of the crop epithelium, proventriculus, Malpighian tubules, small intestine or rectum of worker bees, or in the ovary and fat body of the queens. Hence, *N. ceranae* seems to be a tissue specific parasite in both infected queens and worker bees (Higes et al., 2009c). However, PCR detection suggests that other tissues may be infected by *N. ceranae* (Chen et al., 2009a), although these tissues were not histologically analyzed. To confirm the infection of different tissues by *N. ceranae*, TEM studies may provide more reliable information on its pathology compared to molecular detection.

As mentioned previously, there is a different effect of temperature on the endogenous life cycle of *N. apis* and *N. ceranae* (Martín-Hernández et al., 2009a). Indeed, early stages of infection by *N. ceranae* are associated with more immature parasitic stages (70%) than mature spores at 33 °C. This differs from *N. apis* infected bees, in which an equal proportion of mature and immature stages (50%) were found at the same point after infection at the same temperature. This phenomenon may explain why the pathological consequences of infection by each of these parasites are different, even when similar spore counts are detected.

Another important effect of *N. ceranae* infection is related to the response of the bee's immune system. Seven days after experimental infection of workers bees the expression of abaecin, hymenoptaecin, glucose dehydrogenase (GLD) and vitellogenin (Vg) genes decrease significantly (Antúnez et al., 2009). Hence, *N. ceranae* would appear to partially suppress the humoral and cellular defence mechanisms in bees, which does not occur after *N. apis* infection. The effect on Vg may produce a specific behaviour in infected colonies since this peptide helps to integrate social organization through its pleiotropic effects on the division of labour and on foraging specialization (Amdam and Omholt, 2003; Nelson et al., 2007). In addition, resistance to oxidative stress in honey bees has been linked to the expression of Vg (Seehuus et al., 2006; Corona et al., 2007; Nelson et al., 2007). The decrease in Vg expression after *N. ceranae* infection is consistent with the reduced lifespan of infected bees (Nelson et al., 2007; Remolina et al., 2007). *N. ceranae* also has an effect on the behaviour of infected foragers through energetic stress, as demonstrated by increasing their hunger and compromising their survival. Together, all these effects probably alter feeding behaviour and the transition to becoming foragers (Mayack and Naug, 2009).

Finally, although the pathological features of *N. ceranae* infection closely resemble those of *N. apis* (Martín-Hernández et al., 2009a), some clinical symptoms usually associated with *N. apis* infection are not observed, such as crawling bees, and dysentery evident through the presence of fecal spots in the hive structures (Higes et al., 2008a). The mechanisms underlying these differences in infected bees remain unclear since both microsporidia infect the same tissue and cause similar lesions. Hence, it is important to analyze in more depth the functional consequences of infection by both *Nosema* spp.

5. EFFECTS ON THE COLONY

Koch's Postulates have been shown to hold for colonies infected with *N. cerana* (Higes et al., 2008a; COLOSS workshop, 2009), as previously confirmed in individual bees (Higes et al., 2007). The pathogen was extracted from diseased colonies and transmitted to healthy colonies, inducing disease and colony death, as well as the capacity to recover *N. ceranae* from these newly infected colonies. Multiplication of the parasite occurs throughout the year with no pauses in its life-cycle. Moreover, no differences in the pathological alterations to infected bees were observed in different seasons, confirming previous observations from pathological samples (Martín-Hernández et al., 2007). A dense, closely connected social group such as a honey bee colony can be considered as a complex living system of organisms that functions as a whole. Thus it is essential to comprehend the difference between bee and colony diseases. This social group is a particularly attractive host for a pathogen

due to the excellent opportunities for transmission through the host's homogeneous microenvironment (Naug and Gibbs, 2009). Indeed, a particular pathogen can be lethal to individual bees but the colony may be able to compensate for individual losses because the social organization within the colony also provides an instrument to potentially resist the rapid spread of the pathogen (Naug and Camazine, 2002; Naug and Smith, 2007). In this sense, the queen is essential to maintain the colony population at the correct levels (Higes et al., 2008a).

The result of parasitism of individual bees by *N. ceranae* has a clear effect at the colony level due to the continuous death of highly infected bees (Fig. 3). *N. ceranae* spores are frequently detected in bees from dead or weakened colonies where there is an evident loss of adult bees and reduced colony vigour (Higes et al., 2008a, 2009b). The weakening of the colony is probably due to the fact that the heavily infected honey bees (foragers preferably) do not return to the hive, as it is presumed that adult honey bees die far from the colony (Higes et al., 2008a, 2009b; Kralj and Fuchs, 2009). On the other hand, an important reduction in honey production has been reported in infected but asymptomatic colonies when compared to colonies that were given treatments to control the microsporidium (Botías et al., 2009a). *Nosema* controlled colonies (Fumagillin treated) produced fivefold more honey than uncontrolled colonies (untreated) and had more brood cells and adult bees.

One consequence of the high mortality rate of foragers is that even uninfected bees may forage earlier. The age at which worker honey bees begin foraging varies under different colony conditions and the age at which foraging starts seems to be delayed in the presence of foragers (Huang and Robinson, 1996). Accordingly, bees will begin foraging at an earlier age when the number of foragers is depleted (Huang and Robinson, 1996; Amdam and Omholt, 2003), due to *N. ceranae* for example. In addition, workers infected or affected by other harmful factors begin to forage and perform other risky tasks at an earlier age compared to their healthy nest mates (Tofilski, 2009). Vg and the juvenile hormone (JH) may fulfil an important role in this phenomenon, acting as mutual repressors in the nurse-forager transition (Amdam and Omholt, 2003; Guidugli et al., 2005). Nurse bees have low levels of JH but

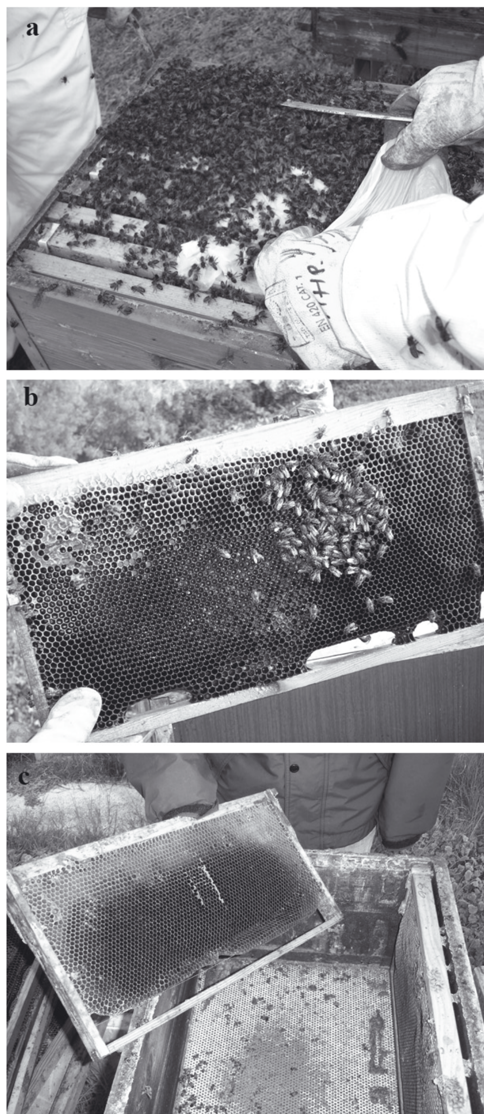

Figure 3. Naturally infected colony by *N. ceranae* in May 2005 (asymptomatic phase) (a) and the same colony one year and a half later (December 2006) showing clear depopulation symptoms (b). This colony died some days later with just a handful of bees and the queen (c).

high levels of Vg, while forager bees display an inverse pattern (Robinson et al., 1991). It has been suggested that an increase in JH titers or suppression of Vg influences the hive bee-forager transition (Whitfield et al., 2006; Nelson et al., 2007) and early foraging behaviour, provoking a reduced lifespan (Nelson et al., 2007). Likewise, an earlier foraging age has been described in *N. apis* infected colonies (Hassanein, 1953; Woyciechowski and Morón, 2009; Lin et al., 2009), although the suppression of Vg expression was only observed in *N. ceranae* infected bees and not following *N. apis* infection (Antúnez et al., 2009). This decrease in Vg probably affects the JH expression and the alterations to the Vg-JH axis may influence the earlier foraging work of infected bees, although this hypothesis remains to be confirmed (M. Spivak, pers. commun.).

The presence of parasites and pathogens, such as *Varroa destructor* mite and *Nosema* spp., influences the flight behaviour of forager bees so that they may not return to the colony (Kralj and Fuchs, 2006, 2009). This effect was interpreted as an adaptative behaviour of the bees to expel the pathogens from the colony, a process called "suicidal pathogen removal". However, this population must be replaced by nurse bees, which would presumably start flying some days earlier than usual due to the hormonal alterations described and the changes in social behavior. Finally the depopulation of colonies becomes evident when the queen cannot compensate for the loss of forager and infected bees, and death becomes imminent (Fig. 3) (Higes et al., 2008a).

Although it is clear that nosemosis due to *N. ceranae* has different epidemiological patterns in Europe (COLOSS workshop, 2009), this disease has a long asymptomatic incubation period at the colony level, which could explain the absence of evident symptoms prior to colony death. The mean spore count in infected bees fluctuates greatly in interior bees from the start to the end of the disease, and it is not a reliable measure of the colony's health when bees are infected with *N. ceranae.* Indeed, almost 20 years ago it was established that *Nosema* infection cannot be established at the colony level on the basis of bees sampled from the broodnest (El-Shemy and Pickard, 1989). The proportion of forager bees infected with *N. ceranae* is the most useful indicator of the extent of the disease in the colony, while the mean forager spore count is possibly an alternative parameter (Higes et al., 2008a).

While *N. ceranae* infected colonies have a long incubation period that usually appears to be asymptomatic, some clinical features of infection have been described in Spain that are usually passed over by beekeepers (Higes et al., 2008a). These include a longer breeding period during cold months (even when the winter break should usually occur), a higher proportion of frames containing brood relative to nurse bees during the warm months, and diminished honey production. Finally, infected colonies become clearly weakened and depleted of adult bees, and they collapse in a period of 1.5–2 years. Two different mechanisms of collapse have been observed that could reflect the moment at which the colonies die. When collapse occurs during the cold months, more than 50% of the dead bees found inside the hive were infected. Indeed, the mean spore count in these bees was always higher than 10 million and the queens (when found) were infected. However, when collapse occurred later in early spring, the proportion of infected bees and the mean spore counts were lower. Moreover, under these circumstances the queens were not usually infected. It is likely that the differences between these two situations lie in the numbers of old and young bees in each season. In early spring, the proportion of newborn uninfected bees will reduce the infection parameters, thereby delaying the infection of the queen (Higes et al., 2009c).

N. ceranae was recently proposed to be a key factor in colony loss in professional apiaries in Spain (Higes et al., 2009d). However, there are different views on the consequences of *N. ceranae* infection in European colonies. While a relationship has been observed between honey bee colony loss and *N. ceranae* infections across Europe, this is not always the case. In Poland, the Netherlands, Austria and France (Topolska et al., 2008; Gajda and Topolska, 2009; Van der Zee et al., 2008; Derakhshifar et al., 2009; Borneck, pers. commun.), the presence of *N. ceranae* has been

related to colony death, including the recently reported massive colony loss in North Europe (Korpela, 2009). However, in other studies infection by *N. ceranae* does not seem to have any effect on colonies (Charriére, 2009; Gómez, 2008). It is also important to carry out parallel studies with comparable parameters on the consequences over long periods of time under different climatic conditions and beekeeping management regimes, since it is evident that such parameters might influence the effects of infection at the colony level.

6. DIAGNOSIS AND PROPHYLAXIS

The control of infectious diseases in food producing animals is an essential aspect of veterinary medicine, and it includes the diagnosis of the disease as well as measures to treat the sick and prevent the spread of disease. The control of honey bee diseases is necessary to maintain their role as food producers (honey, pollen, etc.), and as pollinators of crops and wild vegetation.

As previously mentioned, forager bees are the more reliable sample for *N. ceranae* detection. They should be collected at the hive entrance (avoiding collection of young bees during their orientation flights) or the oldest bees can be collected individually from frames by an experienced person.

Since the sensitivity of the diagnosis is highly influenced by the number of bees analyzed (Martín-Hernández et al., 2009b; Gajda, 2009) it is important to collect the highest number possible of forager bees and to record the number (or weight) of bees analyzed.

Detection of *N. ceranae* relies on microscopic analysis (OIE, 2008) (Fig. 4), molecular methods or transmission electron microscopy (for instance the size of the spores or number of polar filament coils). Several PCR protocols have been described including PCR-RFLP (Klee et al., 2007; Tapaszti et al., 2009), PCR with specific primers (e.g. Chen et al., 2008), real-time PCR (Cox-Foster et al., 2007; Chen et al., 2009b; Bourgeois et al., 2010), or multiplex PCR (Martin-Hernandez et al., 2007). The latter is recommended by the OIE (OIE, 2008). Detection should not be considered as a diagnosis of the disease since from a pathological point of view, diagnosis relies on defined clinical signs that are not easily recognized in the disease caused by *N. ceranae*. It is also necessary to consider type C nosemosis in different geographical areas, and clinical parameters should be defined to accurately establish the prognosis of the disease, such as the proportion of infected foragers to nurse bees.

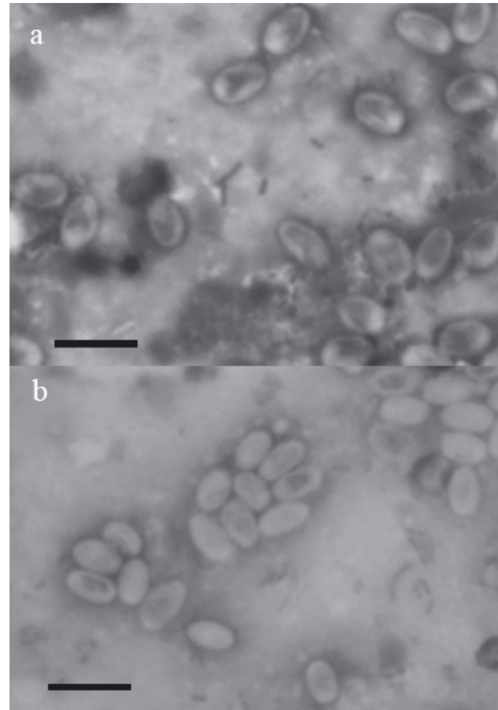

Figure 4. Spores of *Nosema apis* (a) and *Nosema ceranae* (b) stained with methylene blue (light microscopy ×1000). The spores of *N. ceranae* are smaller than spores of *N. apis.* Bars = 5 µm.

The antibiotic fumagillin is effective against both *Nosema* species and it is effective for six months (Higes et al., 2008a, 2009b). No fumagillin residues are found in honey collected in spring or summer from colonies treated with different doses of fumagillin during autumn and winter (Nozal et al., 2008). Fumagillin is derived from *Aspergillus fumigatus* (Bailey, 1953; Didier, 1997) and it is one of the few drugs known to be active against microsporidia (MacCowen et al., 1951). However, due to the absence of an

established maximum residue limit in honey, it is currently forbidden throughout the European Union. Accordingly, it is important to find alternative treatments. Infected caged worker bees fed with thymol and resveratrol candy showed significantly lower infection rates, and resveratrol also increased the lifespan of the bees (Maistrello et al., 2008). These products could be useful alternatives to control *Nosema* disease, although more studies under field conditions are still necessary. Other alternatives like Protofil (Chioveanu et al., 2004), Vita Feed Gold® (Costa et al., 2009), Api-Herb (Nanetti, 2009; Giacomelli et al., 2009), Nonosz® (Bekesi et al., 2009), mainly based on herbal extracts, have potential in controlling *N. ceranae*. Alternatively, general beekeeping management of disease prevention, such as replacement of combs and queens and hygienic handling of colonies, seem to be useful in the control of nosemosis (Korpela, 2009; Kryeger, 2009).

7. CONCLUSIONS

N. ceranae has caused an imperceptible pandemia in the last decade or so. Hence, it can still be considered an emergent disease, causing a major health problem in both individual honey bees and in whole colonies, probably due to the impact of the recent colonization of the European honey bee.

There is little information about the epidemiological factors and clinical symptoms of this disease in different areas in Europe and other parts of the world, which have distinct beekeeping management and climatic conditions. Studies on type C nosemosis by *N. ceranae* have been limited in their duration to a few years, and longer studies will undoubtedly clarify the role of this microsporidia on colony health, as well as increasing our general understanding of the many unknown factors related to this new disease. It is possible that nosemosis type C is at the same point that *Varroa destructor* disease was during the first half of the 20th century, when it was published: *"... bee pathologists pointed out that varroosis was not a disease because one varroa mite on a bee does no harm, and that heavy losses of colonies might well have been caused by the poisons which had been used or by other, especially viral diseases.."*, and *"... this acari was only of interest to a few serious entomologist..."* (Mobus and de Bruyn, 1993). However, currently *Varroa* is considered a major honey bee pest (OIE, 2008).

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Nosema ceranae en Europe : émergence d'une nosémose de type C

Nosema ceranae / _Nosema apis_ / _Apis mellifera_ / nosémose / Europe

Zusammenfassung – _Nosema ceranae_ in Europa: eine neu auftretende Nosemose Typ C. Im Jahr 2005 wurde sowohl aus Asien als auch aus Europa über Nachweise von *Nosema ceranae* in der

Honigbiene berichtet. Bis dahin wurde angenommen, dass die Nosemose der Bienen durch eine Infektion der Ventrikelzellen von erwachsenen Bienen mit *Nosema apis* verursacht wird. Allerdings weicht die durch *N. ceranae* verursachte, neu auftretende Krankheit in ihrer Epidemiologie, sowie ihrer Symptomatik und Pathologie ab. Daher ist es erforderlich, zwei verschiedene klinische Verläufe zu differenzieren: Nosemose Typ A, verursacht durch *N. apis*, und Nosemose Typ C, verursacht durch *N. ceranae*.
Die Infektion der Bienen geschieht durch das Aufnehmen von reifen Sporen. Der Lebenszyklus von *N. ceranae* wird in weniger als 3 Tagen komplett durchlaufen; die intrazelluläre Keimung der Sporen wurde beobachtet. *N. ceranae* kann während des ganzen Jahres in Bienen nachgewiesen werden, die Übertragungsmechanismen sind jedoch noch nicht vollständig aufgeklärt. Sowohl Königinnen als auch Arbeiterinnen können sich mit *N. ceranae* infizieren. Pollen aus Pollenhöschen, von Bienenfressern ausgewürgte Pellets, sowie Imkereigeräte wurden als Reservoir von infektiösen Sporen beschrieben.
Experimentelle Infektionen von *A. mellifera* mit *N. ceranae* zeigten eine höhere Pathogenität in diesem Wirt, die im Vergleich zu *N. apis* eine höhere Sterblichkeit zur Folge hatte. Jedoch wurden von anderen Autoren Unterschiede in den Sterblichkeitsraten beschrieben, möglicherweise beeinflussen bisher noch unbekannte Faktoren die Ergebnisse. Infizierte Arbeiterinnen wiesen eine deutliche Degeneration der Epithelzellen des Ventrikulums auf, wobei in histologischen Schnitten aus anderen Geweben keine Anzeichen einer Infektion mit Sporen gefunden wurden. *N. ceranae* kann die humoralen und zellulären Abwehrmechanismen der Bienen teilweise unterdrücken, was bei Infektionen mit *N. apis* nicht der Fall ist.
Es wurde nachgewisen, dass die Postulate von Koch sowohl für Völker als auch für Einzelbienen gelten. Die Parasitierung einzelner Bienen durch *N. ceranae* hat einen deutlichen Effekt auf der Volksebene, was zu einem kontinuierlichen Totenfall hochinfizierter Bienen führt. Die lange und symptomfreie Inkubationszeit auf der Volksebene kann die Abwesenheit von sichtbaren Symptomen vor dem Zusammenbruch des Volkes erklären. Der Erreger wurde auch als Schlüsselfaktor für die Völkerverluste in Berufsimkereien in Spanien diskutiert. Es gibt jedoch einander widersprechende Berichte über die Folgen einer Infektion von Völkern mit *N. ceranae* aus verschiedenen Teilen Europas.
Der Nachweis von *N. ceranae* erfordert den Einsatz von molekularbiologischen Methoden. Das Antibiotikum Fumagillin wirkt gegen beide Arten von *Nosema*, obwohl sein Einsatz in der Europäischen Union verboten ist. Einige neue potenzielle Bekämpfungsmittel sind Thymol und Resveratrol, sowie ApiHerb oder Nonosz®. Eine gute imkerliche Praxis ist entscheidend für die Vermeidung und Kontrolle dieser Krankheit.
Es fehlt noch an Wissen über die epidemiologischen Faktoren und klinischen Symptome in verschiedenen Regionen Europas und anderer Teile der Welt, wo unterschiedliche klimatische Bedingungen herrschen und verschiedene Formen der Imkerei ausgeübt werden. Zukünftige Studien über die Nosemose Typ C werden zweifellos ihre Rolle bei Völkerzusammenbrüchen aufklären und werden den Wissensstand über viele noch unbekannte Faktoren im Zusammenhang mit dieser neuen Krankheit verbessern.

***Nosema ceranae* / *Nosema apis* / *Apis mellifera* / Nosemose / Europa**

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