



Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia)

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Abstract

In this report, an experimental infection of *Apis mellifera* by *Nosema ceranae*, a newly reported microsporidian in this host is described. *Nosema* free honeybees were inoculated with 125,000 *N. ceranae* spores, isolated from heavily infected bees. The parasite species was identified by amplification and sequencing the SSUrRNA gene of the administered spores. Three replicate cages of 20 honeybees each were prepared, along with one control cage ($n = 20$) supplied with sugar syrup only. The infection rate was 100% at the dosage administered. The presence of *Nosema* inside ventricular cells was confirmed in the samples using ultrathin sectioning and transmission electron microscopy. By day 3 p.i. a few cells ($4.4\% \pm 1.2$) were observed to be parasitized, whereas by 6 days p.i. more than half of the counted cells ($66.4\% \pm 6$) showed different parasite stages, this value increasing on day 7 p.i. ($81.5\% \pm 14.8$). Only one control bee died on day 7 p.i. In the infected groups, mortality was not observed until day 6 p.i. ($66.7\% \pm 5.6$). Total mortality on day 7 p.i. was 94.1% in the three infected replicates and by day 8 p.i. no infected bee was alive. After the infection, the parasites invaded both the tip of folds and the basal cells of the epithelium and the autoinfective capacity of the spores seemed to spread the infection rapidly between epithelial cells. On day 3 p.i., mature spores could be seen inside host cell tissue implying that the developmental cycle had been completed. The large number of parasitized cells, even the regenerative ones, the presence of autoinfective spores and the high mortality rate demonstrate that *N. ceranae* is highly pathogenic to *Apis mellifera*. Possible relation with bee depopulation syndrome is discussed by authors. © 2006 Elsevier Inc. All rights reserved.

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1. Introduction

The Class Microsporidia is a large group of obligate intracellular parasites highly widespread in nature and frequently infecting insects. The infection ranges from the most common chronic form to the less frequent acute form of the disease. The genus *Nosema* has more than 150 species, usually infecting invertebrate hosts including at least 12 orders of insects. *Nosema* spp. are commonly found in

Lepidoptera and Hymenoptera, causing some well known diseases such as pebrine disease in silkworms (by *Nosema bombycis*) and dysentery in honeybees (by *Nosema apis*).

Nosema apis infecting *Apis mellifera* was described more than one hundred years ago. Recently a second microsporidian, *Nosema ceranae*, infecting the same host has been reported (Huang et al., 2005; Higes et al., 2006). This parasite species had previously been characterized only in naturally infected *Apis cerana* honeybees (Fries et al., 1996). Like *N. apis* in *Apis mellifera*, *N. ceranae* was shown to develop in the ventricular epithelial cells of *A. cerana*.

In this report, we describe for the first time an experimental infection of *A. mellifera* by *N. ceranae*. Some

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ultrastructural and morphological characteristics with special emphasis on differences compared to *N. apis* infections are described.

2. Material and methods

2.1. Molecular characterization of spores

Spores for the inoculation were isolated from live heavily infected bees of *Apis mellifera iberiensis*, collected in late spring, from a naturally infected hive located in the experimental apiary of Regional Apicultural Center in Marchamalo, Central Spain. After dissecting the intestinal tracts, they were macerated in distilled water using a manual tissue grinder and the suspension was filtered through No. 4 Whatman mesh and the resulting suspension cleaned by centrifugation and resuspension in distilled water. The spore concentration was determined by counting with a haemocytometer chamber and the suspension was freshly prepared before use.

For species identification of the spores used, one aliquot was prepared for molecular characterization. Spore germination was induced in all the samples as follows: the spores were resuspended in 200 μ l of freshly prepared germination buffer (0.5 M sodium chloride, 0.5 M sodium hydrogen carbonate, pH to 6.0 with orthophosphoric acid; De Graaf et al., 1993) and incubated at 37 °C for 15 min allowing spore germination (Rice, 2001). DNA extraction was done as described previously (Higes et al., 2006). PCR amplification and sequencing of the 16S rRNA sequence was carried out four times on all DNA templates. Primers used were MICRO-F (Visvesvara et al., 1995) and MICROCE-R: 5'-TGCTAATGGTTCTCCAACAGC-3'. Two internal primers were also necessary for amplification and sequencing the 16S rRNA gene of *N. ceranae* (INTER-FOR: 5'-TTACAGTAAGAGTGAGACCTATCAGC-3' and INTER-REV: 5'-AAGTTACCTCGCGTTGAGTC-3').

All PCRs were performed using a Mastercycler[®] ep gradient S (Eppendorf) in 50 μ l volumes containing 2.5 mM Mg²⁺, 0.6 μ M each primer, 0.2 mg/ml BSA, 0.1% Triton X-100, 4 μ l of template DNA and Expand High Fidelity PCR System (No. 1732650 Roche Diagnostic, error rate 4.8×10^{-6}) and each with the following profile: an initial PCR activation step of 2 min at 94 °C, followed by 10 cycles of 15 s at 94 °C for denaturation, 30 s at adequate temperature determined for each pair of primers for annealing and extension at 72 °C for 45 s, and 20 cycles consisting of denaturation at 94 °C for 15, 30 s at temperature determined for each pair of primers for annealing and 50 s at 72 °C plus 5 s cycle elongation for each successive cycle and a final extension step at 72 °C for 7 min (Higes et al., 2006).

Amplified PCR products were electrophoresed through 1.5% agarose TAE gel in standard TAE buffer, stained in TAE buffer with 0.01% SYBR[®] Gold (Invitrogen) and visualized using UV illumination.

PCR products were purified with Qiaquick PCR Purification Kit (No. 28104 Qiagen) and sequenced (3730 DNA Analyzer, Applied Biosystem). The sequence data obtained from the ABI sequencer was checked visually using Chromas 1.43 software.

2.2. Experimental infection

Frames of sealed brood obtained from a healthy colony of *A. m. iberiensis* (*Nosema* free confirmed by PCR) sited in an experimental apiary located two kilometres from Regional Apicultural Center, were kept in an incubator at 34 ± 2 °C to provide newly emerged *Nosema* free honeybees. The emergent worker bees were carefully removed, confined to cages in groups of 20, and kept in the incubator for five days. The bees were fed *ad libitum* with a solution of sucrose (50% w/w in water). In addition, 2% Promotor L (Calier Laboratory), a commercial mixture of aminoacids and vitamins, was added as a dietary supplement.

Five days after eclosion, the bees were starved for 2 h and 20 bees per group were each fed with 10 μ l of 50% sucrose solution containing 125,000 spores of *N. ceranae*. To accomplish correct dosing, honeybees were CO₂ anesthetized for ease of handling; then when each bee woke up, it was held with its mouthparts touching a droplet with the spore solution at the tip of a micropipette until it had consumed the entire droplet (Malone et al., 1999). Bees that did not consume the entire droplet were discarded. Three replicate cages of 20 honeybees each were used and in one additionally control cage ($n = 20$) the bees were fed 10 μ l of plain sucrose solution.

Each cage was checked daily and any dead bees removed and counted. Ventriculi of dead bees were individually checked to verify the presence of *Nosema* spores. One bee from each cage was collected 3 h, 3, 6, and 7 days p.i. and their ventriculi processed for microscopic examination. Control bees were sacrificed in day 14 p.i. and analysed to confirm the absence of spores in ventriculus by OIE method.

2.3. Preparation for microscopy

The ventriculus of each collected bee was removed and prefixed in 2% glutaraldehyde—2.5% paraformaldehyde solution for a maximum of one hour, then it was washed three times in phosphate buffer (PBS) pH 7.4 and stored at 4 °C until use. Specimens were post-fixed in 1% osmium tetroxide at room temperature, washed in PBS, dehydrated with an ascending acetone series, and embedded in Epon-Araldite resin.

Semithin (0.5 μ m) sections were cut with a Reichert-Joung ultracut E microtome and stained with a 1% methylene blue in 4% sodium borate water and studied with a photomicroscope Olympus Vanox AHB53. After light microscopic selection of representative tissue areas, the Epon block was trimmed for ultrathin (60 nm) sectioning.

For transmission electron microscopy (TEM) studies, grids were double contrasted with 2% uranyl-acetate in water and lead citrate Reynolds solution for 10 min each, examined and photographed with a Jeol 1010 electron microscope at accelerating voltage of 80–100 kV.

The parasitic ratio was calculated as the proportion between parasitized and non-parasitized cells on representative tissue areas over one hundred cells counted. Counting was done with a computer-assisted image analyzer (Olympus Microimage™ Image Analysis, software version 4.0 for Windows, Media Cybernetics, Silver Spring, MD, USA).

3. Results

3.1. Parasite species determination

The consensus sequence (1171 bp) for *N. ceranae* obtained in this study was deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov>, Accession No. DQ329034) and confirmed the identity of the species used here as *N. ceranae*.

3.2. Infection rates, parasitic ratio, and mortality

Control bees were negative throughout the study and all the experimentally infected bees were positive. In all the spore dosed bees, infection was confirmed inside host cell tissue, except for the 3 h p.i. registration. Spores of microsporidia were also observed in all dead bees in the infected cages.

Table 1 shows the percentage of parasitized cells and the mortality rate. Only a few cells were infected by day 3 p.i. ($4.4\% \pm 1.2$). On day 6 p.i. more than half of the counted cells ($66.4\% \pm 6$) were infected showing different developmental stages of the parasite while $81.5\% \pm 14.8$ cells were visibly parasitized on day 7 p.i.

Only one control bee died on day 7 p.i. and in the infected groups, mortality was not observed until day 6 p.i. ($66.7\% \pm 5.6$). Total mortality on day 7 p.i. was 94.1% in the three infected groups. By day 8 p.i., none infected bee was alive. All the remaining control bees were alive by day 14 p.i. and they were confirmed as uninfected.

3.3. Gross pathology

Control bees looked healthy throughout the experiment. Food was consumed everyday and they were active when

observed daily, especially when light came into the incubator upon inspection. Infected bees also had a similar behaviour until day 5 p.i. when they became visibly less active. From day 6 p.i. onwards, infected bees were immobile and did not react to any stimulus. No signs of diarrhoea were seen throughout the study.

When the ventriculi were isolated, they had a normal appearance in the control bees and infected bees till day 6 p.i. After 6 days p.i. the ventriculi of infected bees appeared whitish and shrunken.

3.4. Light microscopy

Epithelial cell morphology in control bees showed no alterations due to methodology.

By 3 h p.i. mature spores could be observed in the ventricular lumen of infected bees. One of the samples showed irregular basophilic structures close to the epithelial cell membrane.

By day 3 p.i., infections of epithelial cells were detected in all samples from the three replicates. Only a few epithelial cells in each sample contained developmental stages of the parasite, at the tips of the ventricular folds. Infected cells filled with different parasitic stages were scarce and surrounded by non-infected cells. Parasitized cells were enlarged and their cytoplasm contained heterogeneously stained parasitic structures, suggesting different life cycle stages (Fig. 1). Emptied spores were also observed inside infected cells. Non-infected regenerative epithelial cells were seen in nests at the bottom of cup-like depressions of minor ventricular folds.

By day 6 p.i., most epithelial cells were infected with microsporidia in the three dosed replicates. A predominance of basophilic mature spores in the epithelial cells in the tips of the folds was observed. In contrast, cells at the bottom of the folds contained less basophilic but larger stages, indicating more vegetative parasite cells (Fig. 2). Some normal non-infected cells could also be seen close to groups of the infected ones. Mature spores were clearly observed at this stage of the infection as well as empty spores inside the host cell cytoplasm. At the same time, both mature and empty spores could also be seen in the gut lumen.

Seven days after infection, the majority of the epithelial cells showed evidence of degeneration such as presence of vacuoles in the cytoplasm or a brightly stained nucleus. Scarce unaltered cells were also observed, and either at the tips or at the bottom of epithelium folds there were cells

Table 1
Percentage of parasitized cells and mortality rate (mean \pm standard deviation) in infected and control groups

	Infected groups				Control
	3 h	3 d	6 d	7 d	
Parasite ratio (%)	0	4.4 ± 1.2	66.4 ± 6	81.5 ± 14.8	0
Mortality (%)	0	0	66.7 ± 5.6	94.1 ± 0	5.9 (Day 7 p.i.)

Mortality was calculated as a percentage of the number of remaining caged bees at each time point.

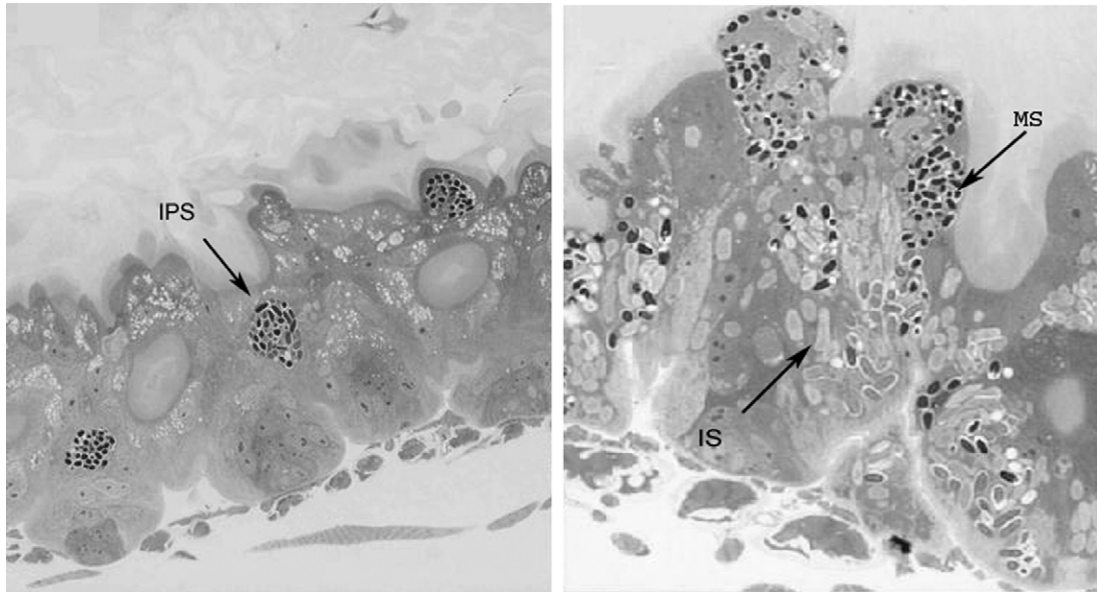


Fig. 1–2. Infection of *Apis mellifera* ventricular (midgut) cells with *Nosema ceranae*. Methylene blue. (Fig. 1) Day 3 p.i. A few epithelial cells at the tips of the folds showing intracellular parasite stages (IPS). 200 \times . (Fig. 2) Day 6 p.i. Heavily infected tissue with tightly packed parasites. Basophilic mature spores (MS) in apical region of epithelial cells. Larger heterogeneously stained parasitic structures at the bottom of the folds, suggesting different life cycle stages 400 \times (IS, immature stages).

contained *N. ceranae* intracellular stages. The distribution of parasitic stages on day 7 p.i. was similar to day 6 p.i., except that cells appeared to be more heavily infected.

3.5. Electron microscopy

All the stages of *N. ceranae* had diplokaryotic nuclei. Three hours p.i. mature and empty spores as well as germinating ones with evertting polar filaments could be distinguished in the lumen. Although some spores appeared empty, infected cells could not be detected at that time. Non-specific electron dense structures were observed next to the apical cells at the tips of folds (Fig. 3) only in infect-

ed bees. These forms were amorphous, surrounded by a single membrane, without vesicles, ER with ribosomes or any other structure.

By day 3 p.i. all the intracellular stages of the life cycle of *N. ceranae* could be observed. In some samples, the amorphous forms seen at 3 h p.i. were also observed. Although empty spores were detected in the lumen, sporoplasms could not be distinguished. Empty spores were also observed inside infected cells (Fig. 4).

All parasitic stages were in direct contact with the host cell cytoplasm and there was no evidence of grouping or enclosure within a vacuolar membrane. Merogonial plasmodia were frequently observed, some of them with four

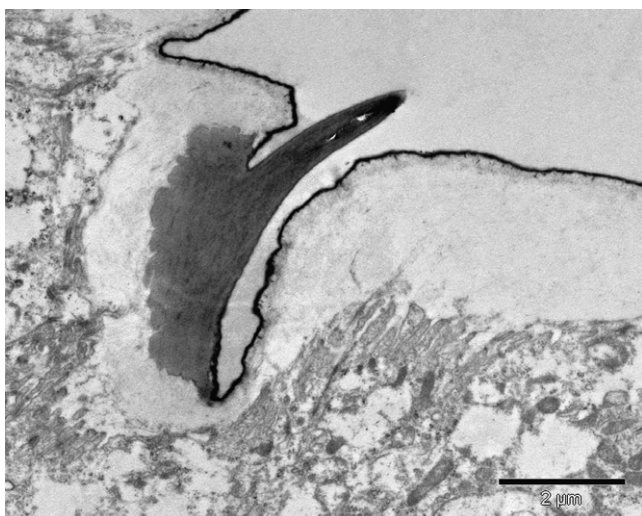


Fig. 3. Hour 3 p.i. Unspecific electron dense structure next to the apical cells at the tip of folds. Bar = 2 μ m.

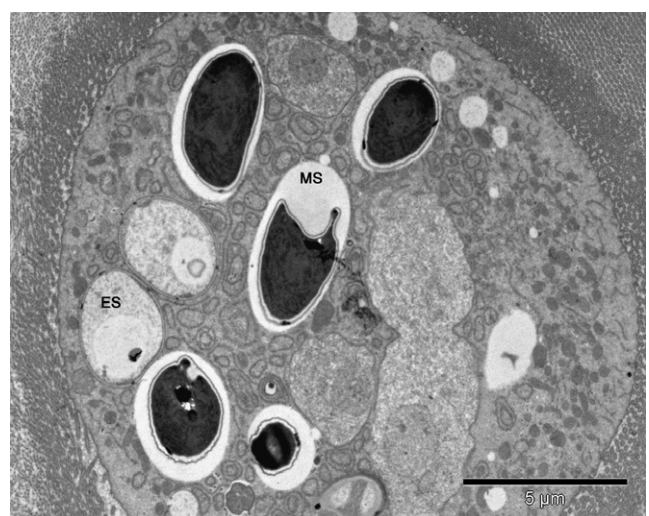


Fig. 4. Day 3 p.i. Infected ventricular cell filled with different parasitic stages: meronts, mature spore (MS) and empty spore (ES). Bar = 5 μ m.

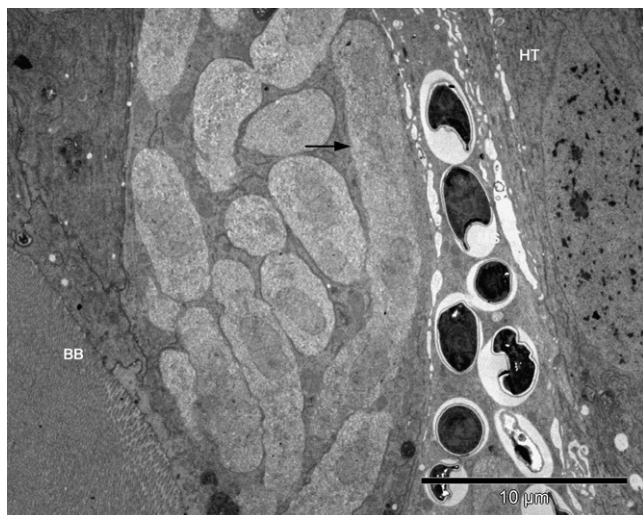


Fig. 5. Day 3 p.i. Ventricular cells infected with *Nosema ceranae*. Merogonial plasmodia with four diplokarya (arrow). BB, brush border; HT, healthy tissue. Bar = 10 μ m.

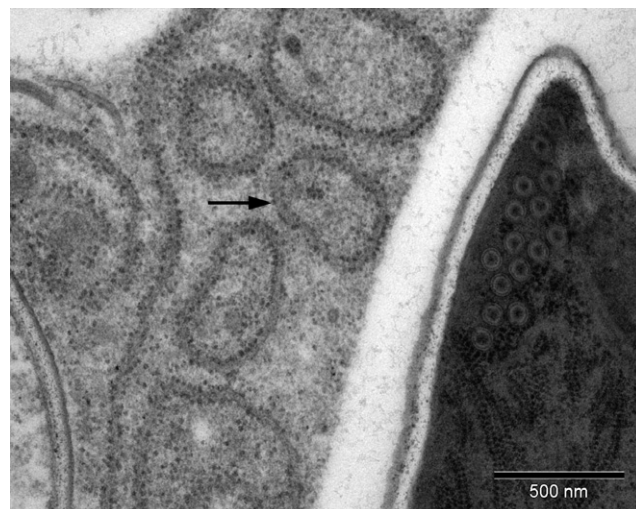


Fig. 7. Day 3 p.i. Host cell ring-shaped rough endoplasmic reticulum (arrow), was usually observed around sporonts. Bar = 500 nm.

diplokarya (Fig. 5); when present they were numerous and rarely associated with the presence of sporogonic stages. Numerous sporonts were recognized with diplokarya of similar size to those observed in daughter meronts. When sporonts were identified, different intracellular parasite stages were found in the same cell, such as mature spores, empty spores or merogonial stages. Infected cells were enlarged and the cytoplasm contained a larger number of mitochondria and free ribosomes. Several mitochondria were close to and surrounded the plasmalemma of meronts (Fig. 6) while RER, usually ring-shaped (Fig. 7), was commonly observed around sporonts. In cells with sporogonial phases, the RER content was greater than when merogonial stages were present.

Similar findings to those observed three days p.i. were seen on days 6 and 7 p.i. but there were a greater number

of infected cells. Most epithelial cells were infected and spores in the bottom of ventricular folds were seen even inside regenerative nests. Cellular nuclei of infected cells had evidence of activity due to the presence of higher quantities of euchromatin (Fig. 8). Numerous lysosomes, most of them secondary, irregularly shaped with heterogeneous electron dense areas, were present in the cytoplasm (Fig. 8).

4. Discussion

This is the first published data on an experimental infection of the European honey bee with the microsporidium *N. ceranae*, although the infectivity to *A. mellifera* was previously mentioned (Fries, 1997). Our results show that the

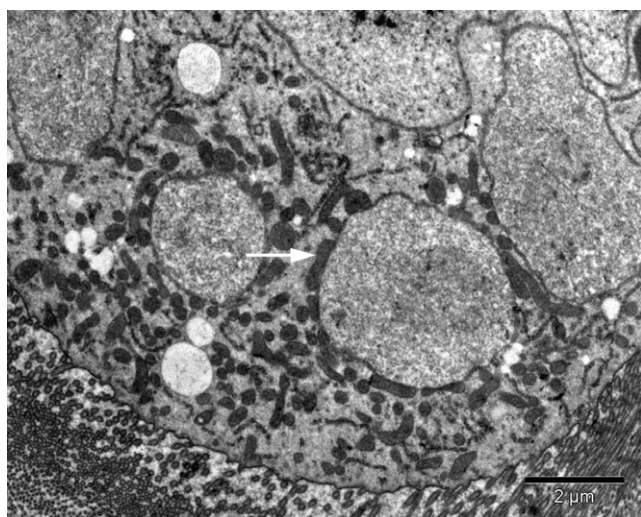


Fig. 6. Day 3 p.i. Meronts surrounded by host cell mitochondria (arrow). Bar = 2 μ m.

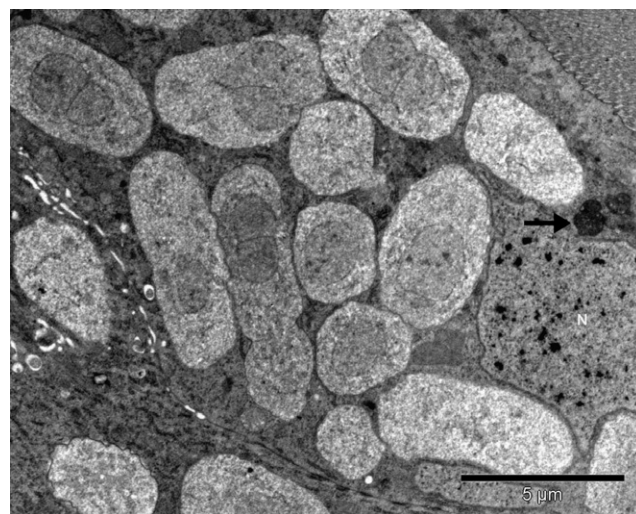


Fig. 8. Day 6 p.i. Some meronts are located in invaginations of the nuclear membrane. Cellular nuclei (N) with hetero and euchromatin are readily identified as regions of granular material of varying electron density. Some secondary lysosomes are present (arrow). Bar = 5 μ m.

parasite developed well in this host and that the intracellular life cycle was completed within 3 days p.i. This study demonstrated the rapid division of the parasite in *A. mellifera* and suggests a high pathological potential.

In the early development of an infection of *N. ceranae* in *A. mellifera*, the presence of merogonial plasmodia, some of them with four diplokarya, and endogenous cytoplasmic development were typical of the genus *Nosema*. The lack of sporophorous vesicles, the presence of diplokarya during all stages of the life cycle and the molecular characterizations are also typical for this genus.

The finding of intracellular emptied spores inside the cells by day 3 p.i. probably indicates that intracellular germination of spores is taking place in *A. mellifera* infected with *N. ceranae* with a resulting horizontal transmission between epithelial cells similar to *N. apis* in the same host (Fries et al., 1992). In infections of *N. ceranae* in *A. cerana*, however, the infection often appears as isolated islands of single infected epithelial cells, interpreted as lack of horizontal transmission between cells, because no empty spores were found inside the cells (Fries et al., 1996). Thus, the host may be an important factor for the transmission of the parasite within the ventricular epithelium. Autoinfective spores that germinate intracellularly in both *in vitro* and *in vivo* experiments have been shown in several species in the genus *Nosema* (Fries et al., 1992; Iwano et al., 1991,1994).

Hypertrophic enlargement of the host cell, including sometimes the nucleus, was frequently observed in parasitized cells. Cell and nuclear hypertrophy are common features of microsporidian infection as a result from the rapid multiplication of the parasite to completely fill the host cell cytoplasm with parasite stages (Becnel and Andreadis, 1999). More than one merogonial cycle may be present in *N. ceranae* when it multiplies in *A. cerana* (Fries et al., 1996). In this host, only meronts with two diplokarya dividing by simple binary fission were observed (Fries et al., 1996) while in *Apis mellifera* several meronts with three or four dividing diplokarya were clearly seen in this study. A similar finding has been described in *Apis mellifera* infected with *N. apis*, where long chains of schizonts, probably an old terminology for merogonial plasmodia, were reported (Gray et al., 1969). These observations could indicate a higher pathological potential of *N. ceranae* in *Apis mellifera* compared to infections in *A. cerana*.

A frame-like structure of mitochondria often formed closely around the plasmalemma of meronts of *N. ceranae* in *A. mellifera*, similar to sporulating *Buxtehucdea scaniae* (Vavra and Larsson, 1999), suggesting a parasite external energy supply. In the early stages of the intracellular development, the microsporidian interferes in the apoptosis of host cell (Aguila et al., 2006), and their pathogenic effect is only seen in later phases of microsporidia multiplication, usually during sporogony. In this study, the host RER closely abuts also the plasmalemma of sporonts and was more commonly observed there in higher quantities compared to merogonial stages. Cells infected with some micro-

sporidia display an increase in cell ribosomes and endoplasmic reticulum suggesting an increase in host cell metabolism probably related to the requirements of the increasing number of parasites. Host cells became filled with developing parasite stages and the presence of secondary lysosomes on day 7 p.i. may be due to the presence of metabolic residues in great amounts.

Nosema apis infection in *Apis mellifera* bees with several different spore doses (the maximum being similar to the one administered in this work) could not establish a discernible relationship between bee longevity and spore dose in two races of European bee (Malone and Stefanovic, 1999). Median survival times of *N. apis* infected and non-infected bees ranged from 18 to 54 days. In our experimental conditions, all bees infected with *N. ceranae* died within 8 days of being exposed to spores. This indicates that *N. ceranae* may be more pathogenic to *A. mellifera* than *N. apis*.

The high regenerative capacity of healthy digestive epithelium commonly compensates for partial cellular destruction. If parasites destroy the basal cells, alterations would be irreversible and may cause the death of the infected bee. In this infection, the parasites multiply well within the basal cells of the epithelium and the autoinfective capacity of the spores seems to rapidly spread the infection. The large number of parasitized cells, even the regenerative ones, the presence of autoinfective spores and the high mortality observed, demonstrate a high pathogenicity of the microsporidium *N. ceranae* to *A. mellifera*.

Such high mortality rates could have important negative effects on bee colonies. It is possible that the bee depopulation syndrome detected over the last few years in several European countries (Higes et al., 2005) may be related to *N. ceranae* infection. This study supports the hypothesis that infected bees must die in few days, with no other associated symptoms, and probably away from the hive while foraging. Depending on the strength of the colony, depopulation should be detected once most foragers have disappeared and the emerging bee population can not compensate the loss of adult bees. However, studies on naturally infected colonies are needed to confirm this hypothesis.

Acknowledgments

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