

Short Communication

## Detection of infective *Nosema ceranae* (Microsporidia) spores in corbicular pollen of forager honeybees

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### Abstract

*Nosema ceranae* is a Microsporidia recently described as a parasite in *Apis mellifera* honeybees in Europe. Due to the short time since its description, no epidemiological data are available.

In this study, spore detection in both pollen baskets and pollen collected from commercial traps is described (PCM, TEM and PCR methods). Spore infectivity is shown after artificial infection of *Nosema*-free adult bees.

The epidemiological consequences of the presence of *Nosema* spores in corbicular pollen require more study and must be considered in beekeeping practices.

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Pollen is the only dietary source of protein, lipids, vitamins and minerals for the nutrition of developing larvae and adult bees. While overwintering, colonies rely on pollen reserves stored during the previous foraging season to provide nutrients, especially for brood rearing, beginning in early spring (Seeley, 1985). Forager bees gather loads of pollen from flowers and pack it on the outer surface of their hind legs, in the pollen basket. This corbicular pollen is deposited in a cell, usually just above or beside the broodnest.

*Nosema ceranae* is a Microsporidia recently described as a parasite in *Apis mellifera* honeybees in Europe (Higes et al., 2006) and Asia (Huang et al., 2007). *Nosema ceranae* has been described as highly pathogenic for this new host (Higes et al., 2007), but no epidemiological data are available due to the short time since its description. No data of

any kind on spore sources for bees and ways of infection transmission are available.

In this study, spore detection in both pollen baskets and pollen collected from commercial traps is described. Spore infectivity after artificial infection of *Nosema*-free adult bees is also shown.

Forager bee samples were obtained from weak colonies belonging to a commercial beekeeper and from a naturally infected *Nosema* colony located in our experimental apiary. Thirty forager honeybees were taken from the previously sealed hive entrance. Corbicular pollen was present in more than 95% of the bees and was removed from the honeybees' corbicular by using sterile tweezers. This pollen was kept in frozen storage until its analysis. Another sample of commercial pollen obtained by using a trap was analysed at the same time.

The presence of microsporidia was first confirmed in bee and pollen samples. Samples were macerated in ddH<sub>2</sub>O and analysed in phase-contrast microscopy (PCM) with 400×

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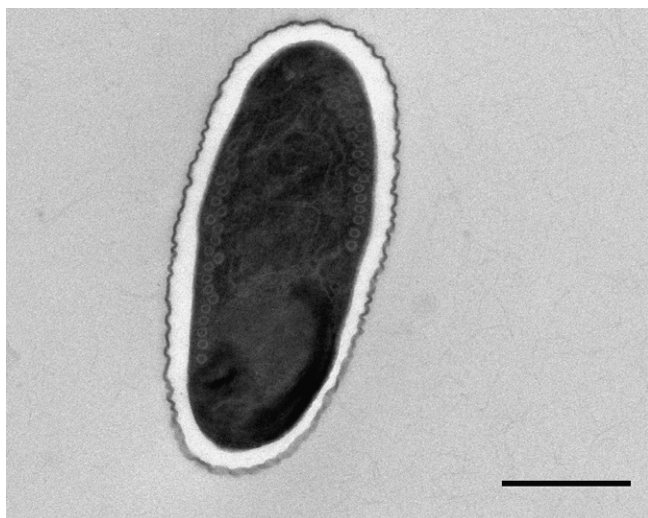


Fig. 1. Mature spore detected in corbicular pollen. Bar = 1  $\mu$ m.

magnification in corbicular pollen balls, bees and a 1 g of trap pollen. Spores were identified by a previously described morphology (Fries et al., 1996) while ultrastructural analyses of spores were done in recently obtained corbicular pollen that were isolated from a hive located in our experimental apiary. Pollen balls were prefixed in 2% glutaraldehyde–2.5% paraformaldehyde and processed by Transmission Electron Microscopy (TEM) analyses (Higes et al., 2007). *Nosema* species was determined by PCR as previously described (Higes et al., 2006).

*Nosema* spores were identified in all the samples by either PCM or TEM (Fig. 1), and the species identified was *N. ceranae* (PCR).

To confirm corbicular spores infectivity, newly emerged *Nosema*-free honeybees were infected in a similar way as previously described (Higes et al., 2007). An aliquot of previously diluted pollen samples (in ddH<sub>2</sub>O) was centrifuged (800g for 6 min.), resuspended three times again, eliminating supernatant, and then counted to prepare infection doses.

Three replicates of 20 newly emerged workers were starved for 5 h. Afterwards, two of them were collectively dosed with 100,000 spores in 1 ml of sucrose (50% w/w in water), and 2% of Promotor L (Calier lab.) was added as a dietary supplement for 24 h. The third cage acted as uninfected control. Once the total infection dose was consumed, bees were fed *ad libitum* with the same control food. Bees were checked and the food changed daily. Afterwards, infection was determined in one bee per group after 3, 7 and 21 days p.i.

Infected bees were positive to PCM spore detection both after 3 days p.i. and 7 days p.i. Quantification of spores showed a 10-fold increase in the number of spores from day 3 to day 7. Around a million spores per infected bee were present a week after the infection with the corbicular pollen spores. After 20 days p.i. all the infected bees became visibly less active, with sluggish movements, or immobile, not reacting to external stimulus. The next day

all of them died. No similar signs were observed in control groups, with all bees alive except one by day 21 p.i. No spores were detected in any of them, including the dead bee. Spore quantifications of dead infected bees showed a mean of 21.5 million per bee in group 1 and 22.1 million spores per bee in group 2. Spore identification by PCR confirmed that they were *N. ceranae*.

This is the first report about the detection of *N. ceranae* infective spores in corbicular pollen. Pollen stored in comb cells of the hive has been reported as a bee pathogen reservoir (Mehr et al., 1976; Moffet et al., 1978; Gilliam et al., 1988; Chen et al., 2006). Viable spores of *Ascospaera apis* have been shown to survive in “bee bread” for at least 12 months (Hale and Menapace, 1980). Gilliam et al. (1989) have shown that the pollen stored in comb cells of the hive differs biochemically from the corbicular pollen. The conversion of corbicular pollen to bee bread has often been postulated to be the result of microbial action, principally a lactic acid fermentation caused by bacteria, yeast and moulds, but its effect on *Nosema* spores is yet to be evaluated. *Nosema* spores’ viability for months in bee bread could be an important epidemiological factor in the transmission of the pathogen to the next bee generation, once the source of infective spores through corbicular pollen has been demonstrated.

Infectivity of spores was demonstrated in all infected bees. Sudden death happened by day 21 p.i. in all infected bees, which was probably due to the collective dose. In a previous study (Higes et al., 2007), sudden death also appeared on the 8th day p.i. in bees that had received an individual dose of 125,000 spores. Clinical symptoms were observed in both cases only a day before.

The presence of *Nosema* spores in corbicular pollen must be due to self-contamination during the process of pollen collection. As the forager bee moistens the surface of its body with its protruding tongue and brushes the pollen that it has collected, the spores inside its body become mixed with the pollen in some way. Spores can either come directly from intestinal content after regurgitation or is present in the saliva, but may be worth investigated to explain how spores end up there. The possibility of collecting the spores directly from contaminated flowers cannot be ignored, but this seems highly improbable.

The epidemiological consequences of the presence of *Nosema* spores in pollen can be a source of nosemosis for the colony, but requires more study and must be considered in beekeeping practices.

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