

NOSEMOSIS OF HONEY BEES

SUMMARY

To date, two microsporidian parasites have been described from honey bees: *Nosema apis* (Zander) and *Nosema ceranae* (Fries). *Nosema apis* is a parasite of the European honey bee (*Apis mellifera*) and *Nosema ceranae* of the Asian honey bee (*Apis cerana*) (11) and the European honey bees). The latter has recently been detected in several geographically separated populations of European honey bees in Europe (12), South and North America (14) and Asia (13). The pathological consequences of *Nosema ceranae* in *Apis mellifera* are not well known. In the following chapter, only *Nosema apis* is described. Both types are presumably very similar. *Nosema apis* is a parasite that invades the epithelial cells of the ventriculus of the adult honey bee. Infections are acquired by the uptake of spores during feeding or grooming. The disease occurs throughout the world, but treatment of bees can help to prevent the spread of infection to unaffected bee colonies.

The parasite invades the posterior region of the ventriculus, giving rise to large numbers of spores within a short period of time. The parasite is ubiquitous. *Nosema* levels generally increase when bees are confined, such as in the autumn and winter in colder climates when the amount of brood is decreasing and perhaps in the early spring when there is an increase in the brood. The disease is transmitted among bees via the ingestion of contaminated comb material and water, and by trophallaxis; honey stores and crushed infected bees may also play a role in disease transmission. Spores are expelled with the faeces where they may retain their viability for more than 1 year. Spores may also remain infective after immersion in honey and in the cadavers of infected bees; however they may lose viability after 3 days when submerged in honey at hive temperature. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood. However, it seems likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for *N. apis* to be successfully transmitted to the next generation of bees. The spores are inactivated by acetic acid or by heating to 60°C for 15 minutes. To be effective, these treatments, which inactivate spores on hive surfaces and combs, can be combined with feeding colonies with the antibiotic fumagillin to suppress infections in live bees. The EU prohibits the use of antibiotic fumigation (EU 3/01/081).

Identification of the agent: In some acute cases, brown faecal marks are seen on the comb and the front of the hive, with sick or dead bees in the vicinity of the hive. However, the majority of colonies show no obvious signs of infection, even when the disease is sufficient to cause significant losses in honey production and pollination efficiency. During winter, there may be an increase in bee mortality. In affected bees, the ventriculus, which is normally brown, can be white and very fragile. Microscopic examinations ($\times 400$ magnification) of homogenates of the abdominal contents of affected bees will reveal the oval spores of *Nosema apis*, which are approximately $5\text{--}7 \times 3\text{--}4 \mu\text{m}$ with a dark edge (*Nosema ceranae* is slightly smaller). Their internal contents can be distinguished after staining with Giemsa's stain. *Nosema apis* spores have a distinctive appearance, with a thick unstained wall and a blue-stained featureless interior. The nuclei within the spores are not visible. This method can help to distinguish *N. apis* from other microbes found in bees.

The appearance of *Nosema apis* spores can be confused with yeast cells, fungal spores, fat and calciferous bodies or cysts of *Malpighamoeba mellificae*. The latter are similar in size to *Nosema* spores, being $6\text{--}7 \mu\text{m}$ in diameter, but are completely spherical instead of oval.

Positive identifications can be made only by observation of typical spores in the ventriculus or faeces. Very mild infections may not be demonstrable. The extent of infection is determined by counting the spores on a microscope grid and calculating the average number of spores per area and estimating from that the number of spores per bee.

Serological tests: *There are no applicable serological tests.*

Requirements for vaccines and diagnostic biologicals: *There are no biological products available.*

A. INTRODUCTION

The microsporidium *Nosema apis* (Zander) is a protozoan parasite exclusive to the epithelial cells of the ventriculus of adult bees *and* the disease occurs throughout the world (16). Infection occurs by the ingestion of spores in the feed (5, 19), via trophallaxis (19) or perhaps after grooming of the body hairs (6, 10, 19).

The polar tube of the spore is everted and penetrates the peritrophic matrix of the intestine, particularly in the posterior region of the ventriculus. The sporoplasm passes down the tube and enters the cytoplasm of the epithelial cells, where it reproduces. Autoinfections can occur at the same time as new infections. After a short interval, spores develop in large quantities. The parasite is ubiquitous and multiplies at a specific rate throughout the year. *Nosema* levels generally increase when bees are confined, such as in the autumn and winter in colder climates when the amount of brood is decreasing and perhaps in the early spring when there is an increase in the brood (19, 20). In winter, spores are rarely to be found, or are only found in heavily infected bees.

Any inherent natural defence by a bee colony against a heavy infection with the parasite depends on the colony size as well as on the prevailing weather conditions during the early part of the autumn of the previous year (18). If these conditions are unfavourable, the overall life expectancy of the colony is reduced. This may lead to the premature death of bees during winter or early spring. In a typical case of a colony being depleted because of a *Nosema* infection, the queen can be observed surrounded by a few bees, confusedly attending to brood that is already sealed.

In faecal droppings, spores may retain their viability for more than 1 year (3). Spores may also remain viable for up to 4 months after immersion in honey (21) and for up to 4.5 years in the cadavers of infected bees (18). The spores may lose viability after only 3 days when submerged in honey at hive temperature (17). It is likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for *N. apis* to be successfully transmitted to the next generation of bees. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood and it seems that temperature may have a marked effect on the rates at which spores lose viability, regardless of their medium (17).

Spores may be killed by heating hive equipment or tools to a temperature of at least 60°C for 15 minutes. Combs may be sterilised by heating to 49°C for 24 hours (8). Fumes from a solution of at least 60% acetic acid will inactivate any spores within a few hours, depending on the concentration; higher concentrations are even more effective and will kill spores within a few minutes (2, 9). Such procedures come under the jurisdiction of national control authorities with protocols that vary from country to country. Disinfection can be carried out, for example, by putting acetic acid solution into bowls or on to sponges that can soak up the liquid. Following disinfection after an outbreak, all combs should be well ventilated for at least 14 days prior to use. Suppression of *Nosema* disease can also be achieved by feeding an antibiotic, fumagillin, in sugar syrup to the colony (8). This is forbidden in many countries and in the EU.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

In acute forms of infection, especially in early spring, brown faecal marks may be noted on the comb and the front of the hive (4). At the entrance to the hive, sick and dead bees may be seen, although other causes, such as pesticide poisoning and diseases of adult honey bees (such as acarapodosis) should be eliminated first if this is the case. The detection of these infectious diseases requires microscopic examination. During winter, *Nosema apis*-infected colonies may become severely depleted of bees or die out altogether. The majority of *Nosema apis*-infected colonies will appear normal, with no obvious signs of disease even when the disease is sufficient to cause significant losses in honey production and pollination efficiency (1, 11). A proper diagnosis can be made only by microscopic examination of adult bee abdomen or ventriculus. To diagnose a *Nosema apis* infection, the posterior pair of abdominal segments is removed with a forceps to reveal the ventriculus, complete with the malpighian tubules, the small intestine and rectum. The ventriculus is normally brown but, following a *Nosema* infection, it can become white and fragile. However, this appearance is given by other causes of intestinal disturbance, for example feeding on indigestible food stores, such as syrup containing actively growing yeast. For a reliable diagnosis, a number of bees in a sample should be examined.

a) Microscopy

It is necessary to attempt to distinguish between a *Nosema apis* infection and an infection caused by *Malpighamoeba mellificae* (19). There is quite often an indication of dysentery in a *Nosema apis* infection. In an *M. mellificae* infection, there may be diarrhoea, often of a sulphur-yellow colour and with a distinct odour. Characteristics of *M. mellificae* cysts are described later. Secondary mixed infections may occur (17). A simple, nonquantitative method for detecting *Nosema apis* infection is as follows: sampled bees should be obtained from the hive entrance in order to avoid sampling individuals under the age of 8 days, which would lead to 'false negatives' because no spores from the protozoan in question would be determined. At least 60 bees should be collected in order to detect 5% of diseased bees with 95% confidence (10). Before sending to the laboratory, the bees should be fixed in 4% formal, 70% ethyl alcohol or frozen in a standard freezer in order to prevent them from decomposing and to improve their reception and organisation in the laboratory. The abdomens of the bees to be examined are separated and ground up in 2–3 ml of water. Three drops of the suspension are placed on a slide under a cover-slip and examined microscopically at $\times 400$ magnification, under bright-field or phase-contrast optics. This is a slight simplification of Cantwell's original method (7). The spores are about 5–7 μm long and 3–4 μm wide (*Nosema ceranae* is slightly smaller than *Nosema apis*). They are completely oval with a dark edge. Their contents, consisting of nucleus, sporoplasm and polar tube, cannot be seen. Dyes are usually not necessary.

Nosema spores must be differentiated from yeast cells, fungal spores, fat and calciferous bodies, and from *M. mellificae* cysts, which are spherical and approximately 6–7 μm in diameter.

When air-dried, ethanol-fixed smears of infected tissue are stained with Giemsa's stain (10% in 0.02 M phosphate buffer) for 45 minutes. *Nosema apis* spores will have a distinctive appearance, with thick unstained walls and an indistinct blue interior, without visible nuclei. Insect cells, fungal spores and other protozoa stained in this way will generally have thinner walls, blue/purple cytoplasm and magenta-coloured nuclei.

In order to obtain accurate, reliable and meaningful quantification of levels of *Nosema* infections in honey bees, a standardised procedure must be used. A suitable protocol is as follows:

A sample of older worker honey bees is taken, from which the abdomens of ten individuals are macerated in 5 ml of water using a mortar and pestle. When tissue pieces have become quite fine, the suspension is filtered through two layers of muslin (thin loosely woven cotton fabric) in a funnel leading to a graduated centrifuge tube. A second 5 ml of water is used to rinse the pestle, swirl around the inside of the mortar and pour through the subsample in the funnel. Water levels are equalised in the tubes and the suspensions are centrifuged for 6 minutes at 800 g . The supernatants are decanted and the tubes are refilled to the 10 ml level. Using disposable pipettes and a rubber bulb, the pellets are resuspended by repeated uptake and forcible ejection through the pipette tips. When the solution appears to be homogenous, a sample is taken to fill the calibrated volume under the cover-slip of a haemocytometer (blood cell counting chamber). After a few minutes the spores will have settled to the bottom of the chamber. *Nosema* spores appear transparent but with a very distinct dark edge and are 5–7 μm long and 3–4 μm wide. They are best seen using a magnification of $\times 400$ and bright-field or phase-contrast optics. The number of spores in each square is counted. Where a spore lies over the edge of a square, count only those spores that straddle the left and upper edges of the square, not the right and bottom edges. One *Nosema apis* spore, observed in the haemocytometer's entire central square millimetre grid ($25 \times 16 = 400$ small squares), is equal to an average of 10,000 spores per bee. If no spores are seen, the result should be designated 'not detected', but that does not mean that the bees are not infected. Regulatory agencies will decide on the level of infection useful for their purposes.

A laboratory method for the simultaneous detection of *Nosema* spores and *M. mellificae* cysts consists of the individual examination of the colonies using 30–60 bees per colony. A suspension of the abdomens of dead bees is prepared by grinding with 5–10 ml water; the volume of water depending on the number and condition of the bees. The suspension must be filtered to remove debris that would interfere with the examination, first through a 100 μm and then a 40 μm filter. Parts of the malpighian tubules pass through the 100 μm filter, but are collected on the 40 μm filter. They are placed on a slide or bacterial counting chamber and examined at $\times 400$ magnification. Only a few tubules are filled with cysts after an *M. mellificae* infection. The normal structure of malpighian tubules is not visible in this case. Only cysts inside the malpighian tubules can be taken as a positive result, because *M. mellificae* cysts are often confused with fungal spores and yeast cells.

b) Culture

There are no cultural methods for growing these organisms.

c) Polymerase chain reaction (PCR)

Different methods have been developed to distinguish *N. apis* from *N. ceranae*. A multiplex PCR is described below with which both pathogen types can be clearly identified at the same time (15).

o **Sample preparation for PCR**

The abdomens of 10–20 adult honey bees from each sample are macerated in 10 ml distilled water (PCR grade) and the suspension is then filtered and centrifuged at 800 **g** for 6 minutes. For DNA extraction, spore germination is induced with 200 µl freshly prepared germination buffer (0.5 M sodium chloride, 0.5 M sodium hydrogen carbonate, pH to 6.0 with orthophosphoric acid), and the mixture is incubated at 37°C for 15 minutes. The DNA extraction can be easily carried out using routine procedures or commercial kits, such as High Pure PCR Template Preparation Kit (No. 1796828 Roche Diagnostic).

o **Multiplex PCR**

With this technique both microsporidians (*N. apis* and *N. ceranae*) can be distinguished in just one PCR because of the use of specific primers with no interference. PCR reactions are performed in 50-µl volumes containing 5 µl of template DNA, 25 µl of High Fidelity PCR Master Mixture (catalogue no. 12140314001; Roche Diagnostic), 0.4 µM of each primer, 0.4 mM of each deoxynucleoside triphosphate, 3 mM Cl₂Mg, 0.2 mg/ml bovine serum albumin, 0.1% Triton X-100, and 5 µl of *N. apis* or *N. ceranae* DNA template. The parameters for amplification are: an initial PCR activation step of 2 minutes at 94°C, followed by 10 cycles of 15 seconds at 94°C, 30 seconds at 61.8°C, and 45 seconds at 72°C, and 20 cycles of 15 seconds at 94°C, 30 seconds at 61.8°C, and 50 seconds at 72°C plus a 5-second elongation cycle for each successive cycle and a final extension step at 72°C for 7 minutes. Negative controls (from DNA extraction) are included in all PCR experiments.

The molecular weights of PCR products are determined by electrophoresis in a 2% agarose TAE (Tris-acetate-ethylene diamine tetra-acetic acid) gel in standard TAE buffer, stained with ethidium bromide, and visualised using UV illumination.

Primers selected for detection of *N. ceranae* and *N. apis* in multiplex PCR:

Primer	Sequence ^a	PCR product size (bp)	Specificity
218MITOC FOR	5'- <u>CGGCGACGATGTGATATGAAA</u> -ATATTAA-3'	218–219 ^b	<i>N. ceranae</i>
218MITOC REV	5'- <u>CCCGGTCATTCTCAAACAAAA</u> -AACCG-3'		
321APIS FOR	5'- <u>GGGGGCATGTCTTTGACGTACTATGTA</u> -3'	321	<i>N. apis</i>
321APIS REV	5'- <u>GGGGGGCGTTTAAAATGTGAAACA</u> ACTATG-3'		

^aCG tails added to primers are underlined.

^bThere is a 1-bp difference in the *N. ceranae* amplicon size depending on the sequences for *N. ceranae* available in GenBank (<http://www.ncbi.nlm.nih.gov>).

2. Serological tests

There are no serological tests available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No biological products are available.

REFERENCES

- ANDERSON D.L. & GIACON H. (1992). Reduced pollen collection by honey bee (Hymenoptera: Apidae) colonies infected with *Nosema apis* and sacbrood virus. *J. Econ. Entomol.*, **85**, 47–51.
- BAILEY L. (1957). Comb fumigation for *Nosema* disease. *Am. Bee J.*, **97**, 24–26.
- BAILEY L. (1962). Bee diseases. *In*: Report of the Rothamsted Experimental Station for 1961, Harpenden, UK, 160–161
- BAILEY L. (1967). *Nosema apis* and dysentery of the honey bee. *J. Apic. Res.*, **6**, 121–125.

5. BAILEY L. (1981). Honey Bee Pathology. Academic Press, London, UK.
6. BULLA (1977). *In: Comparative Pathobiology*. Vol. 1: Biology of *Microsporidia* (1976); Vol. 2: Systematics of the *Microsporidia*, Lee A. & Cheng T.C., eds. Plenum Press, New York, USA, and London, UK.
7. CANTWELL G.E. (1970). Standard methods for counting nosema spores. *Am. Bee J.*, **110**, 222–223.
8. CANTWELL G.E. & SHIMANUKI H. (1970). The use of heat to control *Nosema* and increase production for the commercial beekeeper. *Am. Bee J.*, **110**, 263.
9. DE RUITER A. & VAN DER STEEN J. (1989). Disinfection of combs by means of acetic acid (96%) against *Nosema*. *Apidologie*, **20**, 503–506.
10. FRIES I. (1988). Contribution to the study of *Nosema* disease (*Nosema apis* Z.) in honey bee (*Apis mellifera* L.) colonies. Rapport 166, Sveriges Landbruksuniversitet, Institutionen för husdjurens utfodring och vård, Uppsala, Sweden.
11. FRIES I., FENG F., DA SILVA A., SLEMENDA S.B. & PIENIAZEK N.J. (1996). *Nosema ceranae* n.sp. (Microspora, Nosematidae), morphological and molecular characterization of a Microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). *Eur. J. Protistol.*, **32**, 356–365.
12. HIGES M., MARTÍN R. & MEANA A. (2006). *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. *J. Invertebr. Pathol.* **92**, 93–95.
13. HUANG W.F., JIANG J.H., CHEN Y.W. & WANG C.H. (2005). Complete rRNA sequence of *Nosema ceranae* from honey bee (*Apis mellifera*). <https://gra103.aca.ntu.edu.tw/gdoc/F90632004a.pdf> (Date: 2005-11-25).
14. KLEE J., BESANA A.M., GENERSCH E., GISDER S., NANETTI A., TAM D.Q., CHINH T.X., PUERTA F., RUZ J.M., KRYGER P., MESSAGE D., HATJINA F., KORPELA S., FRIES I., PAXTON R.J. (2007). Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *J. Invertebr. Pathol.*, **96**, 1–10.
15. MARTIN-HERNANDEZ R., MEANA A., PRIETO L., SALVADOR A.M., GARRIDO-BAILON E. & HIGES M. (2007). Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Appl. Environ. Microbiol.*, **73**, 6331–6338.
16. MATHESON A. (1996). World bee health update 1996. *Bee World*, **77**, 45–51.
17. MORGENTHALER D. (1939). Die ansteckende Frühjahrsschwindsucht (*Nosema-Amoeben*-Infection) der Bienen. Erweiterter Sonderdruck aus der Schweizerischen Bienenzeitung Heft 2, 3 und 4.
18. STECHE W. (1985). Revision of ZANDER & BOTTCHER. Nosematose. *In: Krankheiten der Biene*, Handbuch der Bienenkunde.
19. WEBSTER T.C. (1993). *Nosema apis* spore transmission among honey bees. *Am. Bee J.*, **133**, 869–870.
20. WEISER J. (1961). Die Mikrosporidien als Parasiten der Insekten. Verlag Paul Parey, Hamburg and Berlin, Germany.
21. WHITE G.F. (1919). *Nosema* Disease. United States Department of Agriculture Bull., No. 780, 54 pp.

*
* *

NB: There are OIE Reference Laboratories for Bee diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).