Comparison of the two microsporidia that infect honey bees – a review

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ABSTRACT

Two microsporidian species, *Nosema apis* and *Nosema ceranae*, infect honey bees (*Apis mellifera*) worldwide. They are obligate intracellular parasites that multiply in the epithelial lining of the bee's midgut and cause nosemosis. *N. ceranae* infections were primarily found in *Apis cerana* and raised interest in the last decade with the discovery of their presence in the European honey bee (*Apis mellifera*). *Nosema* spp. utilizes hosts' energetic reserves for the purpose of propagation and disrupts the digestive processes of the bee. Nosemosis reduces the lifespan of a single bee and affects the performance of the colony. It also has an economic impact through the reduction in the honey and pollen yield of severely infected colonies or even causes them to collapse. Lack of effective therapy for nosemosis is of special concern and calls for scientific attention. Although *N. ceranae* and *N. apis* are similar in many aspects, there are important differences between them such as clinical signs of infection or the ability to resist low temperatures.

Key words: honey bee, microsporidia, *Nosema ceranae, Nosema apis*

INTRODUCTION

Two microsporidian species have been described to infect European honey bee, *Apis mellifera*, namely *Nosema apis* Zander (1909) and *Nosema ceranae* (Fries et al. 1996). Microsporidia, *Nosema* spp., were previously considered as protozoans but were reclassified to the Fungi cluster based on new molecular and ultrastructural evidence (Adl et al. 2005). Outside the host they are found in the form of very resistant spores that can remain infective for years (Fries 1993). *N. apis* is a long-known and well-studied parasite of the honeybee described in 1909 by a German biologist Zander (cited after Fries 1993).

The natural infection with a new honey bee parasite *N. ceranae* was first reported not more than a decade ago almost simultaneously in Europe (Higes et al. 2006) and Asia (Huang et al. 2007). All infections predating 2005 were previously attributed to *N. apis* but the analyses of stored samples have shown the presence of infection with *N. ceranae* in Europe since at least 1998 (Paxton et al. 2007). The later was primarily the parasite of the economically less important Asian honey bee (*Apis cerana*) (Fries et al. 1996).

Adult workers as well as queens and drones can become infected with *N. apis* or *N. ceranae* (Furgala 1962a, Bailey 1972, Higes et al. 2009). Newly emerged adults are free of infection (Smart and Sheppard 2012). After the bee ingests the infective spores, they invade the midgut epithelial cells and multiply intracellularly, causing nosemosis. As their numbers grow, they begin to exert pressure on the cell wall that eventually ruptures and releases cellular content together with the infective spores (Fries et al. 1992, Higes et al. 2007). Thin-walled primary spores are autoinfective while secondary spores voided with feces infect other individuals (Fries et al. 1992). Because of the damage caused to the epithelial layer, the production of digestive enzymes and the absorption of food is impaired (Liu 1984, Malone and Gatehouse 1998, Matašin et al. 2012). The energy demands of honeybee organism can no longer be met and the lifespan is shortened (Fries 1993, Higes et al. 2007, Mayack and Naug 2010). At the colony level the foragers are lost outside the hive and this can result in reduced pollen collection and honey yield (Anderson and Giacon 1992, Botias et al. 2013) or even death of the colony (Fries 1988, Higes et al. 2008). The aim of this article is to review research that has been made on

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the both microsporidian species known to cause disease in European honey bee, *A. mellifera*, compare their effects on individual bee and colony level and point out the questions that still have to be answered.

NOSEMA SPP.

Taxonomy and morphology

N. apis and *N. ceranae* are the only two microsporidian species known to cause nosemosis in European honey bee (*A. mellifera*). Microsporidia in general are obligate intracellular parasites that use the hollow structure named polar tube for host cell invasion. They belong to the Fungi cluster in Opisthokonta rank based on the results of molecular phylogenetic studies and ultrastructural research (Adl et al. 2005). Microsporidia form oval spores with inner chitin and outer proteinaceous wall, the so-called endospore and exospore. They lack mitochondria. The polar tube or filament is anchored in the anterior end of the spore. It rapidly everts from the spore at the appropriate stimulus and penetrates the wall of the host cell. This is followed by the transport of the sporoplasm through the tube into the host cell. Developmental stages in the cytoplasm can be visualized using electron microscope. Inside the spore, the polar tube is coiled (Vavra and Larsson, 1999). The number of polar filament coils in mature spores is species specific with *N. apis* usually having more than 30 and *N. ceranae* only 20 to 23 coils (Fries 1989, Fries et al. 1996). Besides being slightly larger, *N. apis* spores also appear more uniform in shape and size when observed under the light microscope compared to the spores of *N. ceranae*. Spores of *N. ceranae* measure approximately 4.7 by 2.7 μm and are on average 1 μm shorter than the spores of *N. apis*. When viewed with the scanning electron microscope the surface of the spores is wrinkled, more profoundly in *N. ceranae* (Ptaszynska et al. 2014). Both species are binucleate (Fries et al. 2006).

Geographical distribution

Since their first discovery in Europe in 2005, natural infections of European honey bees with *N. ceranae* are reported worldwide and became more common than *N. apis* infections (Klee et al. 2007).

Besides the European honey bee, *N. ceranae* can also infect the Asian honey bee (*Apis cerana*), the dwarf Asian honey bee (*Apis florea*), the giant Asian honey bee (*Apis dorsata*), *Apis koschevnikovi* and even some bumblebee species of the genus *Bombus* (Plischuk et al., 2009; Chaimanee et al., 2010; Botias et al., 2012). In contrast, natural infection with *N. apis* is found only in the European honey bee, although experimental infection was also confirmed in *A. cerana* (Fries, 1993).

Development of infection

Representatives of *Nosema* spp. are obligate intracellular parasites that need the host cell for their propagation. Only recently a successful grow of *N. ceranae* and *N. apis* in a cell culture of a lepidopteran cell line has been made (Gisder et al., 2011). This finding could provide the means necessary for further studies of their morphology, life cycle and pathogenicity.

The bees usually get infected when cleaning the excreta of diseased bees (Bailey and Ball 1991) but also through trophalaxis (Smith 2012, Huang and Solter 2013) or even when visiting contaminated flowers (Evison et al. 2012). Some spores were randomly found at the mouthparts of infected bees (Huang and Solter 2013). After the spores are ingested, they pass the crop and enter the ventriculus where they germinate. If they successfully penetrate the cell wall of a midgut epithelial cell with their hollow polar filament, they inject the sporoplasm into the host cell and begin to multiply. The epithelial cells become filled with newly formed spores, causing host cells to break and spores are released into the ventricular lumen (Dussaubat et al. 2012, Higes et al. 2013). In a fully developed infection, there are around 40 million spores of *N. apis* present in the midgut (Bailey in Ball 1991) and similar numbers of *N. ceranae* spores. Mature spores pass the alimentary canal and the infected bees release the spores in the environment by defecation. Spores remain viable and capable of infecting a new host for at least 6 months at room temperature, but freezing rapidly causes destruction of *N. ceranae* spores (Fries 2010, Sanchez Collado et al. 2014).

There are reports of *N. ceranae* DNA signal obtained using PCR method (Copley and Jabaji, 2012) or even spore findings in organs and tissues other than the ventriculus (Ptaszynska et al. 2012). But the experiment by Huang and Solter (2013) showed that the positive results could be caused by sampling technique and therefor authors proposed an appropriate alternative sampling to avoid cross contamination. *Nosema* spp. spores were also found in honey (Giersch et al. 2009) and in corbicular pollen collected by foragers (Higes et al. 2008b).

Natural mixed infections of colonies or even single bees with both species of *Nosema* also occur (Paxton et al. 2007, Gisder et al. 2010). Forsgren and Fries (2010) applied an even mixture of *N. apis* and *N. ceranae* spores to worker bees and found no competitive advantage of either within the host. Similar results were obtained in the study by Milbrath and collegues (2015).

Nosemosis caused by *N. apis* shows typical seasonality with low levels of infection in the summer, a low peak in the autumn and a winter rise of infection (Bailey in Ball 1991, Gisder et al. 2010) whereas this pattern appears to be absent in *N. ceranae* infections in the southern (Higes et al. 2010) but not in the northern parts of Europe (Gisder et al. 2010).

DIAGNOSTICS

Finding combs soiled with feces is an apparent sign that the colony might be suffering from *N. apis* infection. On the other hand there is a lack of apparent symptoms connected to

N. ceranae infection besides the poor colony fitness. Samples of live workers are collected for microscopic inspection in both cases. The oldest bees returning from foraging collected at the closed entrance of the hive or bees from side combs and honey super when conditions don't favor flight are appropriate for *Nosema* spores count. Younger bees are often free of infection and do not reflect the actual status of the colony (Doull 1965, Smart and Shepard 2012). At least 60 bees from the colony have to be examined in order to avoid false negative results according to OIE (2008). Most authors consider the proportion of infected bees more informative than the average spore count (Doull 1965; Fries et al. 1984, Higes et al. 2008a). Whole bees, their abdomens or just ventriculi can be squashed depending on the purpose with one ml of water per bee and suspension checked under a light microscope (Cantwell 1970). Key to the study of Nosema disease of honeybees (*A. mellifera*) is the correct identification of *N. apis* and *N. ceranae* spores. With the discovery of *N. ceranae* spores in samples of honeybees (*A. mellifera*) originating from Spain in 2005 (Higes et al. 2006), the use of light microscopy ceased to be a sufficient method for the confirmation of species. Although the spores of *N. ceranae* are slightly smaller than *N. apis* spores occurring in naturally infected bees (Fries et al. 1996). In order to distinguish both *Nosema* species, molecular methods have to be used.

At first, the molecular methods for differentiation of *Nosema* spp. spores were complicated and required a PCR followed by sequencing (e.g. Higes et al. 2006) or the use of restriction analysis of PCR products (e.g. Klee et al. 2007) until a duplex PCR-method was developed (Martin-Hernandez et al. 2007). Single reaction is needed as it simultaneously amplifies 16S ribosomal RNA regions of either of the two microsporidia. More recently a real-time duplex PCR enabled the quantification of *N. apis* or *N. ceranae* in a single reaction (Bourgeois et al. 2010).

PATHOLOGY

Individual bee

All adult representatives of the honeybee colony are susceptible to infection with *N. apis* and *N. ceranae* (Furgala 1962a, Higes et al. 2009, Traver and Fell 2011). However, most of the research regarding *N. ceranae* was done on worker honeybees. The most neglected in terms of research are the effects of *N. ceranae* infection on the honeybee queen - not because of minor importance but possibly due to the more complicated experimental set-up. The queen is indeed less likely to become infected because of her specific role in the colony. She does not clean the comb or feed on her own but is rather fed royal jelly by the attending workers and since the Nosema-infected workers start their tasks outside the hive at an earlier age, they are less likely to pass spores to the queen (Wang and Moeller 1970, Dussaubat et al. 2013, Goblirsch et al. 2013). It was recently noted that younger queens are more susceptible to Nosema infection possibly due to immaturity of immune response (Chaimanee et al. 2014). This finding is of extreme importance for commercial queen producers and stresses the need for the development of the appropriate

technology to ensure good quality and health status of the produced queens. Commonly packed environment of mating nuclei favors the spread of infection (Gregorc et al. 1992). In turn infected queen can represent a source of infection for the re-queened colony (Czekonska 2000).

Although in the short-term *N. ceranae* infected queens have all main physiological functions such as vitellogenin titer, the total antioxidant capacity and the queen mandibular pheromones significantly increased (Alaux et al. 2011), a shorter life span is expected (Higes et al. 2009). Degeneration of ovaries, decreased life span and supersedure is documented in honey bee queens with *N. apis* infection (Hassanein 1951, Furgala 1962a, Loskotova et al. 1980).

In worker bees, *N. ceranae* infection causes imunnosuppression and significant decrease of vitelogenin levels. Vitelogenin normally counteracts with juvenile hormone and prevents premature social maturation from nurse bee to forager (Antunez et al. 2009, Goblirsch et al. 2013).

The mechanisms that caused *N. ceranae* to prevail over *N. apis* are not clear, but it is the species most often determined in infected samples from Mediterranean region (Tlak Gajger et al. 2010, Gregorc 2013). According to some studies infection with *N. ceranae* causes a higher mortality rate than *N. apis* (Martin-Hernandez et al. 2011; Higes et al. 2008) yet others state that the mortality is not significantly higher (Forsgren and Fries, 2010) or is only pronounced in mixed infections (Milbrath et al. 2015). The results of Mayack and Naug (2009) showed that when bees are fed *ad libitum*, the median survival time in *N. ceranae* infected and in uninfected bees is similar. It is likely that the accessibility to unlimited amounts of food masks the level of mortality in experiments utilizing caged bees. Sugar syrup consumption is indeed higher in nosema-infected bees in comparison to the uninfected ones (Martin-Hernandez et al. 2011, Mayack and Naug, 2009). The trehalose level in the haemolymph of free-flying *N. ceranae* infected foragers is lower and reduces at a faster rate if compared to uninfected foragers (Mayack and Naug 2010). As trehalose represents the main source of energy for flight, the lack of it can cause impaired flying abilities of forager bees.

Although there are numerous reports on deleterious effects of *N. apis* on the development and structure of the hypopharyngeal glands of worker bees (Wang and Moeller 1969, Wang and Moeller 1971, Liu 1990), research regarding the status of these glands in *N. ceranae* infected workers is scarce. Underdeveloped glands are probable in such individuals compared to healthy ones since they begin their foraging duties at a younger age (Holt et al. 2013). *N. ceranae* infection diminishes protein content of food glands in infected *A. florea* workers (Suwannapong et al. 2010). Infection early in a workers life most likely results in premature atrophy of hypopharyngeal glands and compromises royal jelly production.

Interactions with other stressors are poorly understood. Exposure of *N. ceranae* infected bees to sublethal doses of pesticides fipronil or thiacloprid results in an increased mortality (Vidau et al. 2011) and bees emerged from brood combs with high level of pesticide residues are more prone to *Nosema* spp. infection (Wu et al. 2012). Certain pathogens such as viruses are found to add to the primary

nosema infection as is the case with Chronic bee paralysis virus (Toplak et al. 2013). Analyses of North American honeybee colonies displaying symptoms of colony collapse disorder often showed the presence of *N. ceranae* spores and Invertebrate iridescent virus (Bromenshenk et al. 2010).

Colony level

N. apis infected colonies have reduced pollen collection ability compared to healthy ones (Anderson and Giacon, 1992). Pollen is essential for normal colony development, for developing bees and the production of royal jelly in the hypopharyngeal glands of nurse bees as it is the main dietary source of protein for honeybees (DeGrandi-Hoffman et al., 2010). If more than 50 % of the bees are *Nosema* infected, the colony is notably affected and may die-off (Pickard and El-Shemy 1989, Higes et al. 2008). In an experiment conducted by Fries and colleges (1984), the honey yield was reduced to half when the average *N. apis* infection within a colony reached about 15 million spores per bee and a little less than 50 % of bees were infected. *N. ceranae* causes a significant increase in the production of ethyl oleate in infected workers, a primer pheromone important in regulation of the division of labor and can disturb the sensitive equilibrium inside a colony (Dussaubat et al. 2010, Dussaubat et al. 2013). *Nosema* spp. infected foragers are less likely to successfully return to the hive because of their impaired orientation skills (Kralj and Fuchs, 2009) and diminished energy stores needed for flight (Mayack and Naug 2009, Mayack and Naug 2010). Both factors potentially lower the transmission of disease inside the colony.

CONTROL

The diagnosis and subsequent treatment of disease in food producing animals is necessary to ensure well-being of the animals, minimize the damage to the productivity and prevent the spread of disease. On the other hand honey bee products have to be safe for the consumer and free of residues of veterinary medicines. Antibiotic substance fumagillin was a long time the therapy of choice for the treatment of nosemosis (Furgala 1962b, Williams et al. 2008). Medicines with fumagillin as active substance are not registered in the European Union. It was found that fumagillin has genotoxic effects (Stanimirović et al. 2007) and also one of the latest studies of fumagillin efficiency in the control of *N. ceranae* showed the level of parasite's reproduction increases significantly at concentrations lower than those prescribed (Huang et al. 2013). Therefore new treatment practices need to be developed. Some of the substances that lowered *N. ceranae* spore levels in preliminary testing include surfactin S2 (Porrini et al. 2010), thymol (Maistrello et al. 2008, Costa et al. 2010) and commercially available product Nozevit (Tlak Gajger et al. 2009). Prophylactic apitechnical measures, such as comb replacement, hygienic handling of the colonies and queen replacement, are effective in lowering the level of infection or reduce spread of disease (Fries 1988, Loskotova et al. 1980). The prevention and control of Nosemosis in queen-breeding apiaries can't be emphasized enough (Gregorc et al. 1992). Freezing of infected beekeeping equipment at -20 °C can diminish *N. ceranae* infectivity as it causes the spores to quickly loose viability. The method is simple, inexpensive and safe. Tests should be performed to establish appropriate duration of freezing. Genome-sequencing of *N. ceranae* (Cornman et al. 2009) was the basis to develop the novel therapeutic strategies. Gene silencing method (RNAi) can successfully reduce the levels of *N. ceranae* spores in the midgut of caged bees, but hasn't been yet tested in field conditions (Paldi et al. 2010).

Another important approach to control Nosemosis is selective breeding of honey bee strains. A recent experiment of selected nosema tolerant honey bees from Denmark showed that they manage to maintain a normal level of apoptotic death rate in infected midgut epithelial cells to prevent spores accumulation in the epithelium (Kurze et al. 2015).

All described measures merely mitigate infection not entirely cure it therefore they should be combined when possible to achieve the best results as is the case with Varroa control.

DISCUSSION

Despite of abundant data on *N. apis* and large efforts to describe in such detail the newly discovered *N. ceranae*, many questions remain open. There is a bulk of knowledge concerning the effects of infection with *Nosema* spp. on survival and certain organs in honeybee workers, mostly under laboratory conditions, but much less in actual field conditions and in combination with other negative factors. A lack of knowledge concerning the outcome of *N. ceranae* infection in honeybee queens is obvious with only four articles covering this topic (Higes et al 2009, Alaux et al. 2011, Traver and Fell 2012, Chaimanee et al. 2014). Negative effects on queen survival and egg-laying similar to those found in *N. apis* infection are plausible. Spread of infection to newly emerged queens in queen-breeding apiaries should be prevented to assure good quality of queens intended for sale. Selective breeding for Nosema resistant bees could also become an important tool in reducing the incidence of Nosema infections in honey bee colonies.

Today we are facing a worldwide decline of the honeybees. The studies that investigated possible causes of colony depopulation could not point out a single factor responsible for it but have rather shown that different detrimental effects work in concert. Less accessible food sources to bees because of intensive monocultural farming, pesticide exposure, new pathogens, overexploitation, the spread of urban areas and climate changes possibly act together with *N. ceranae* infection to cause massive losses of foragers and colony collapse disorder syndrome (Bromenshenk et al. 2010). It is therefore crucial for the beekeeper to minimize all the negative factors possible and should have the ability to do so through availability of controlling agents and advancement in beekeeping technology. The vast negative effects of nosemosis on individual bees and whole colonies call for search of effective therapy without compromising consumer health. Problem solving should be in the focus of further research.

ACKNOWLEDGEMENTS

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Primerjava mikrosporidijev, ki povzročata okužbe pri medonosni čebeli – pregledni članek

IZVLEČEK

Nosema apis in *Nosema ceranae* sta vrsti mikrosporidijev, ki povzročata okužbe pri medonosni čebeli (*Apis mellifera*) širom sveta. Gre za obligatne znotrajcelične parazite, ki se razmnožujejo v črevesnem epitelu čebele in povzročajo nosemavost. Okužbe z *N. ceranae* so prvič odkrili pri *Apis cerana*, zanimanje zanje pa je naraslo v zadnjem desetletju, ko so ugotovili njihovo prisotnost pri medonosni čebeli (*A. mellifera*). Oba parazita iz rodu *Nosema* porabljata energetske zaloge gostitelja za lastno razmnoževanje in škodujeta njegovim prebavnim procesom. Okužba skrajšuje življenjsko dobo čebel in prizadene delovanje celotne čebelje družine. Nosemavost ima negativen učinek na donos medu in cvetnega prahu pri močno okuženih družinah, lahko pa celo privede do njihovega propada. Pomanjkanje učinkovitih metod zdravljenja nosemavosti zahteva razvoj novih. Kljub številnim podobnostim obstajajo med obema povzročiteljema pomembne razlike kot so klinični znaki okužbe in odpornost na nizke temperature.

Ključne besede: medonosna čebela, mikrosporidiji, *Nosema ceranae, Nosema apis*