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Detection of *Nosema* spp. in worker bees, pollen and bee bread during the honey flow season

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Abstract

Nosema apis and *Nosema ceranae* are responsible for nosemosis in the honey bee (*Apis mellifera*). The aim of the study was to identify *Nosema* spp. during the honey flow season in bee colonies, for co-infection or no infection with *Nosema apis/Nosema ceranae*. Hive bees, forager bees, pollen grains brought by them, and bee bread were analysed. In the infected group, 12 of 30 samples of hive bees were infected with *Nosema ceranae*, 7 were co-infected, 3 were infected with *Nosema apis*. In samples of forager bees, 21 of 30 were co-infected, 8 were infected with *Nosema ceranae.* The analysis of pollen demonstrated that only 2 of 30 samples were infected with *Nosema ceranae,* 3 of 30 samples of bee bread were infected with *Nosema ceranae,* 3 were co-infected and 1 of 30 were infected with *Nosema apis*. In the uninfected group, 13 of 30 samples of hive bees were co-infected, 2 were infected with *Nosema ceranae*. In the forager bees group, 12 of 30 samples were co-infected, 2 were infected with *Nosema ceranae.* Only 1 pollen sample was infected with *Nosema ceranae* and 1 was co-infected*,* 2 of 30 samples of bee bread were co-infected, 2 were infected with *Nosema ceranae.* The study demonstrated that during the honey flow season, infection by microsporidian species occurs mainly in forager bees. Pollen and bee bread may be a source of spores mainly at the beginning of the honey flow season.

Apiology, coinfections, PCR

Nosema apis (*N. apis*) and *Nosema ceranae* (*N. ceranae*) are highly-specialised, parasitic unicellular eukaryotes from the division of Microsporidia (Adl et al. 2005). In the honey bee (*Apis mellifera*) they are responsible for nosemosis, considered one of the most common adult honey bee diseases in the world. Currently, most honey bees in Europe are infected with *N. ceranae*, which has also been reported from other regions of the world (Higes et al. 2006; Williams et al. 2008; Michalczyk et al. 2011; Martin-Hernandez et al. 2012). Bees become infected with the parasite microspores by ingesting water or pollen (Webster et al. 2004; Chen et al. 2008). Weather conditions during the honey flow season play an important role in the development of nosemosis. For *N. apis*, seasonality of the disease has been observed, while the prevalence of infections with *N. ceranae* remains on a similar level during the whole honey flow season (Higes et al. 2006, 2007; Paxton et al. 2007; Chen et al. 2009; Bourgeois et al. 2010; Forsgren and Fries 2010). The literature provides very limited data on the sources of bee infection with *Nosema* spp. spores or the routes of transmission. Pollen and bee bread have been indicated as potential sources of infection (Higes et al. 2008a). Disease in the apiary and bee colony is usually transmitted via combs infected with spores, water, and between insects by means of trophallaxis (OIE 2013).

The aim of our study was to identify *Nosema* spp. present during the honey flow season in infected (co-infected with *N. apis/N. ceranae*) and uninfected honey bee colonies. The analysis included 2 castes of worker bees: bees working on combs sampled directly from the central part of the nest, and foragers bringing pollen to the hive, as well as pollen grains collected by foragers and bee bread stored in newly built combs.

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Materials and Methods

Material for the study was sampled in March 2014 from a 50-hive apiary (53°47'N, 20°30'E). A haemocytometric method (Neubauer chamber) was used to detect *Nosema* spp. spores in each honey bee colony by analysing 60 worker bees randomly sampled from winter hive debris. Multiplex polymerase chain reaction (PCR) demonstrated that 90% of positive samples were co-infected with *N. apis/N. ceranae.* Therefore, further tests were carried out on 5 honey bee colonies (group 1) without nosemosis symptoms, infected with *Nosema* spp. (more than 1.2×10^6 spores/worker bee), and with a co-infection confirmed by PCR (*N. apis/N. ceranae*) and 5 bee colonies (group 2 – control) free from microsporidians. Every month from April to September 60 hive bees (HB) were sampled randomly directly from honey combs in the central part of the nest, and 60 forager bees returning to the hive with pollen (FB) were sampled from the beehive entrance using a special tool. Multiplex PCR was used to separately analyse worker bees and pollen brought by them (Sokół and Michalczyk 2012). Pollen grains from forager bees were collected using sterile tweezers. A single sample contained 20 randomly collected pollen grains. In addition, about 5 g samples of bee bread stored in newly built marked combs were collected. Fifteen mg samples of bee bread were used for analysis. Deoxyribonucleic acid (DNA) was isolated from bees, pollen and bee bread using the same technique.

Genomic DNA was isolated using the Genomic Mini A&A Biotechnology kit (Gdynia, Poland) for DNA extraction by relying on genomic DNA's ability to bind to silica in the presence of high concentrations of chaotropic salts. The isolation process was carried out in accordance with the manufacturer's guidelines, and purified DNA was stored for further analyses in test tubes at a temperature of -20 °C. The multiplex PCR assay involved the amplification of small subunit ribosomal ribonucleic acid (rRNA) sequences (16S rRNA) of *Nosema apis* and *Nosema ceranae*. The following primers were used: for *N. ceranae* - MITOC FOR (5'-CGGCGACGATGTGATATGA-AAATATTAA-3') and MITOC REV (5'-CCCGGTCATTCTCAAACAAAAAACCG-3') amplifying a 218 bp product for *N. apis* and 321 bp for *N. ceranae* (OIE 2013). Multiplex PCR analysis was carried out using the HotStarTaq *Plus* Polymerase (Qiagen, Germany) and the HotStarTaq *Plus* Master Mix Kit (Qiagen, Germany). The reaction mix of 20 µl comprised around 120 ng isolated DNA, 10 µl HotStarTaq *Plus* Master Mix 2 times, 2 µl CoralLoad Concentrate × 10 and 0.1 µl of each primer, supplemented with RNase-Free Water to 20 µl. Every reaction involved three controls: two positive controls with the DNA of *Nosema apis* and *Nosema ceranae* (Centro *Nosema* Apicola Regional, Dirección Genera de la Producción Agropecuaria, Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha, Spain) and one negative control where DNA was replaced with water. The reaction was carried out in the Eppendorf Mastercycler thermocycler. The PCR commenced with initial denaturation at 95 °C for 5 min. The reaction mixture was then cycled × 35 consisting of the following steps: denaturation at 94 °C for 45 s, primer annealing at 55 °C for 45 s and extension at 72 °C for 1 min. The last reaction was followed by final chain synthesis at 72 °C for 10 min. The products of the multiplex PCR reaction were separated by electrophoresis in 2% agarose gel containing 0.5 µg/ml ethidium bromide for visualizing the resulting DNA fragments of *Nosema* spp. The size of the obtained products was evaluated by comparison with the GeneRuler™ 100 bp 36 Ladder Plus (Fermentas, USA) molecular size marker. Electrophoresis results were archived using the GelDoc (Bio-Rad, USA) gel documentation system.

Results

The analysis carried out in April in honey bee colonies from group 1 (co-infection with *N. apis/N. ceranae* in winter hive debris) revealed that 2 HB samples were co-infected (*N. apis/N. ceranae*), two were infected with *N. ceranae*, and one was free from spores. In the same group, four FB samples were co-infected with *N. apis/N. ceranae* and one with *N. ceranae.* In May, all HB samples were infected with *N. ceranae*, and FB samples were co-infected (*N. apis/N. ceranae*). In June, three HB samples were free from spores, and two were infected with *N. apis*. In May, four FB samples were co-infected, and one was infected with *N. ceranae.* In July, two HB samples were co-infected (*N. apis/N. ceranae*), two were infected with *N. ceranae*, and one was free from spores, while four FB samples were infected with *N. ceranae* and one was free from spores. In August, three HB samples were free from spores, one was infected with *N. ceranae*, and one was co-infected (*N. apis/N. ceranae),* while three FB samples were co-infected (*N. apis/N. ceranae),* and two were infected with *N. ceranae.* In September, two HB samples were co-infected (*N. apis/N. ceranae),* two were infected with *N. ceranae*, and one was infected with *N. apis*, while all FB samples were co-infected (*N. apis/N. ceranae*) (Table 1).

The analysis of pollen collected from FB workers from group 1 across the study period revealed the presence of *N. ceranae* only in one sample taken in May and in one sample taken in June.

Table 1. Detection of *Nosema* spp. in worker bees, pollen and bee bread in uninfected and infected colonies depending on the time of examination in individual colonies and months.

Month of examination/ number of colonies	Type of *Nosema* spp.							
	Worker bees				Pollen (pollen grains)		Bee bread	
	Hive bees HB		Forager bees FB					
	Group							
	1	2	1	2	1	2	1	2
April								
n = 5	C	-	C	-	-	-	AC	-
	-	-	AC	-	-	-	AC	-
	C	-	AC	-	-	-	AC	AC
	AC	-	AC	-	-	-	-	-
	AC	-	AC	-	-	-	-	AC
May								
n = 5	C	AC	AC	-	-	-	-	-
	C	-	AC	-	C	-	-	-
	C	AC	AC	-	-	-	-	-
	C	AC	AC	AC	-	-	C	-
	C	AC	AC	AC	-	-	-	-
June								
n = 5	A	-	AC	-	-	AC	-	C
	-	AC	AC	AC	-	-	-	-
	-	-	C	C	-	-	C	-
	A	-	AC	-	-	-	C	-
	-	AC	AC	AC	-	-	-	-
July								
n = 5	-	-	C	AC	-	-	-	-
	AC	C	C	-	-	-	-	-
	AC	-	C	AC	-	C	-	-
	C	-	-	-	C	-	-	-
	C	C	C	-	-	-	-	-
August								
n = 5	-	AC	C	AC	-	-	-	C
	-	-	AC	AC	-	-	-	-
	AC	-	AC	-	-	-	-	-
	-	AC	C	C	-	-	-	-
	C	-	AC	-	-	-	-	-
September								
n = 5	C	AC	AC	AC	-	-	A	-
	AC	AC	AC	AC	-	-	-	-
	AC	AC	AC	AC	-	-	-	-
	A	AC	AC	-	-	-	-	-
	C	AC	AC	AC	-	-	-	-

A – infection with *N. apis*, C – infection with *N. ceranae*, AC – co-infection with *N. apis* and *N. ceranae*, - – no infection

In April, three samples of bee bread were co-infected (*N. apis/N. ceranae*). One sample taken in May and two samples taken in June were infected with *N. ceranae*. In July and August bee bread samples were free from spores, and one sample taken in September was infected with *N. apis*.

In group 2 (control, winter hive debris free from spores), all HB and FB samples taken in April were free from infection. In May, four HB samples were co-infected (*N. apis/N. ceranae)* and one was free from spores. Two FB samples were co-infected (*N. apis/N. ceranae)* and three were free from spores. In June, 2 HB samples were co-infected (*N. apis/N. ceranae)* and three were free from spores, Two FB samples were co-infected (*N. apis/N. ceranae*), two were free from spores and one was infected with *N. ceranae*. In July, two HB samples were infected with *N. ceranae*, three were free from spores, while two FB samples were co-infected (*N. apis/N. ceranae*) and three samples were free from spores. In August, three HB samples were free from spores, two were co-infected (*N. apis/N. ceranae),* while two FB samples were co-infected (*N. apis/N. ceranae*), two were free from spores, and one was infected with *N. ceranae.* In September, all HB samples were co-infected (*N. apis/N. ceranae),* while four FB samples were co-infected (*N. apis/N. ceranae*), and one was free from spores.

In group 2, only two samples of pollen taken from FB workers across the whole honey flow season were infected with *Nosema* spp., one sample collected in June was co-infected (*N. apis/N. ceranae*), and one collected in July was infected with *N. ceranae.* The analysis of bee bread collected during the whole study period demonstrated infection with spores in four samples, co-infection (*N. apis/N. ceranae*) in two samples taken in April, and infection with *N. ceranae* in two samples taken in June and August (Table 1).

Discussion

To date, *Nosema* spp. microsporidians have been detected in honey bee colonies at different levels of their structure and in different periods of the honey flow season (Higes et al. 2008b). Our study was carried out in normally developing bee colonies free from pathogens other than nosema, including *Varroa destructor*. Despite the detection of *Nosema* spp. spores in individual months, no symptoms of nosemosis, e.g. diarrhoea or bee death, were observed in the analysed bee colonies. Nosema spores were more frequently detected in forager bees (FB) than in hive bees (HB). Other researchers also reported a high level of infection with spores in forager bees (Higes et al. 2008a; Meana et al. 2010; Botias et al. 2012; Smart and Sheppard 2012).

Higes et al. (2008a) explained the presence of *Nosema* spp. spores in pollen and bee bread by self-infection of foragers during pollen collection. Pollen may contain *Nosema* spp. spores, and foragers collecting it *ad libitum* may contribute to the spread of nosemosis (Higes et al. 2008a; Pettis et al. 2012; Human et al. 2013; Williams et al. 2013). Pollen stored in comb cells in the form of bee bread, infected during foraging, may be a potential source of infection for new generations of bees (Martin-Hernandez et al. 2007; Higes et al. 2008a; Bourgeois et al. 2010). Our study demonstrated the presence of *Nosema* spp. spores in 11 samples of bee bread and 4 samples of pollen. We demonstrated that pollen, but more frequently bee bread, may be a source of spores, particularly at the beginning of the honey flow season, when the demand for food to feed larvae is high.

Currently, molecular techniques are used to detect mild infections with microsporidians and to identify *Nosema* spp. by analysing a single insect or its body parts, e.g. salivary glands (Higes et al. 2008b; Human et al. 2013). The severity of infection depends on the microsporidian species. Our study demonstrated that mixed infection (co-infection) with *N. apis/N. ceranae* (45 samples) and infection with *N. ceranae* (25 samples), and with *N. apis* (5 samples) were the most frequent in the analysed bee colonies during the honey

flow season. Recent studies indicated that infections with *N. ceranae* are more common in *A. mellifera* than infections with *N. apis*. Honey bees infected with this microsporidian species die promptly, usually outside the hive, without showing previous clinical symptoms (Higes et al. 2007; Paxton et al. 2007; Chen et al. 2009; Forsgren and Fries 2010). The presence of *Nosema* spores in worker bees from colonies may also be associated with subclinical symptoms of the disease, or result from recovery after infection in at least some worker bees. Collected food (honey, pollen, bee bread) or comb cells contaminated with spores by sick worker bees during comb cleaning may be a source of infection second to trophallaxis (Martin-Hernandez et al. 2007; Bourgeois et al. 2010). It can be assumed that *Nosema* spp. spores are permanently present in a honey bee colony, and microsporidians, depending on the environmental conditions and immune status of individual insects, cause either subclinical or fully-symptomatic infections in colonies. However, the mechanism underlying the infection of honey bees with different *Nosema* species has not been fully explained. On one hand, bees can be easily infected by ingesting food containing spores, but the progression of infection depends on the age of the insects. It is also important whether or not sick (infected) worker bees infect healthy individuals or larvae by trophallaxis, and at what time of the honey flow season this infection takes place. Undoubtedly, the progression of the disease is determined by the level of food intake in a single insect and the strength of a bee colony, i.e. its demand for food, or suggested contamination of worker bees with pesticides (Nozal et al. 2008; Alaux et al. 2010). Energetic stress may depopulate a honey bee colony, and impaired immunity can lead to fully-symptomatic nosemosis or promote the onset of other diseases (Campbell et al. 2010; Hedtke et al. 2011; Botias et al. 2013). It seems that pollen and probably water are more important epizootic sources of infection with *Nosema* spores, and the spread of the disease in a honey bee colony is promoted by trophallaxis and the hygiene-related behaviour of bees. However, infection can be transmitted from other castes of bees, including queens and drones (Czekońska 2000; Alaux *N. ceranae* et al. 2010; Traver and Fell 2011).

Pollen or bee bread are a potential source of infection *Nosema* spp. like workers bringing different products to the hive in the water.

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