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PERMANENT PREVALENCE OF *NOSEMA CERANAE* IN HONEY BEES *(APIS MELLIFERA)* IN HUNGARY

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Nosema ceranae is present in honey bee (*Apis mellifera* L.) colonies worldwide. Studies on the comparative virulence of *N. ceranae* and *N. apis* showed significant differences in individual mortality, and the prevalence of *N. ceranae* seems to be predominant in both the continental and the Mediterranean climate regions. This study attempted to monitor the geographical and seasonal distribution of these two *Nosema* species in Hungary, using a simple laboratory method. The distribution of *N. ceranae* and *N. apis* infection rates along all seasons was homogeneous ($P = 0.57$). In co-infected samples, the intensity of *N. ceranae* infection was always significantly higher than that of *N. apis* infection ($P < 0.001$). The infection rate of infected bees in exterior samples was higher than in interior samples in each season; however, the differences were not statistically significant. The species *N. ceranae* had been present in Hungary already in 2004. Statistical analysis of data shows that the infection level is best represented by sampling exterior bees to establish the proportion of infected bees rather than by determining the mean spore count.

Key words: Honey bee, nosemosis, *Nosema ceranae*, spore count, multiplex PCR

Nosema disease in honey bees is caused by two types of microsporidian parasites, *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Higes et al., 2006), and it is a significant economic issue worldwide (Botías et al., 2013; Goblirsch et al., 2013; Higes et al., 2013). *Nosema ceranae* had been described as a parasite of the Asian bee *(Apis ceranae)* in the late 1990s (Fries, 1997); however, one

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decade later it was detected in *A. mellifera* (Higes et al., 2006) and soon thereafter it was reported as the predominant microsporidium infecting honey bees in many countries (Martín-Hernández et al., 2007, 2011; Botías et al., 2012). Colonies affected by *N. apis* generally display latent infection during summer, a small peak in autumn, and a slow increase of infection during winter (Bailey, 1955). *Nosema ceranae* can be detected in samples throughout the year, even in the summer months, as there is no evidence of seasonality under certain climatic conditions (Martín-Hernández et al., 2007). The presence of *N. ceranae* was first reported in Hungary by Tapaszti et al. (2009) in 2007 in 37 out of 38 *Nosema*-infected bee samples collected from Hungarian apiaries using PCR-RFLP methods. All of these 37 samples contained only *N. ceranae*, which indicated the dominance of *N. ceranae* in Hungarian apiaries.

Using the hypothesis that *N. ceranae* dominance over *N. apis* may prevail in all four seasons in Hungary, a monitoring research was designed to investigate the distribution of the two different *Nosema* spp. The prevalence of *N. ceranae* in Western Europe was observed in historical samples of honey bees (Chauzat et al., 2007) and honey (Botías et al., 2012) as far back as 2000, which may indicate that *N. ceranae* had also been present in Hungary at that time. Correct identification of the two *Nosema* species is the key to the study and control of nosema disease (nosemosis) of honey bees. Fingler et al. (1982) found a correlation between the percentage of infected bees and the average spore count of the sample as a whole. The mean spore count per bee has traditionally been used to determine the extent of colony infection (Furgala and Hyser, 1969) and was later developed to indicate the presence of *N. apis* infection. The mean spore count of *N. ceranae* was found not to be directly related to the infection level of the whole colony under field conditions (Higes et al., 2008; Meana et al., 2010). The spores produced by the two *Nosema* species are quite similar and can rarely be distinguished using traditional light microscopy. Genetic typing has to be used to characterise infections or co-infections by these two pathogens (Fries, 1997).

The first purpose of present study was to detect the presence of *N. ceranae* in Hungarian honey bee colonies prior to the 2007 samples collected by Tapaszti et al. (2009). The second purpose was to propose investigation of the distribution of the two different *Nosema* spp. throughout the year and determine the existence and levels of infections in interior and exterior bees. Another objective was to determine the spore count and the proportion of infected bees to quantify the degree of *Nosema* spp. infections for a detailed statistical analysis. A significant part of the work was carried out at the Spanish Laboratory of Bee Pathology (CAR = Centro Apícola Regional) in Marchamalo, Spain, but the final objective of the project was to adapt the methods of molecular genetic identification of *Nosema* species in our laboratory (RET = Regional University Center of Excellence in Environmental Industry) at Szent István University in Gödöllő, Hungary.

Materials and methods

Sampling

Nationwide sampling campaigns of live adult honey bees (*Apis mellifera carnica* P.) were organised during the spring (April), summer (July) and autumn (October) of 2010 across Hungary. To have a representative assessment of the scope of *N. apis* and *N. ceranae* prevalence, 44 apiaries were involved in the sampling campaigns representing all main geographical regions of the country (Fig. 2). Three colonies were randomly sampled in each apiary. Two types of samples were collected from each colony. Interior house bees were collected from the side frames that do not contain uncapped brood. To collect the exterior returning forager bees, entrances were closed for a 20-min period. The forager bees were collected after they had settled in front of the blocked entrances. Sampling of a minimum of 60 individual bees was performed before 9 a.m. or after 3 p.m. to avoid collecting young bees during their exploratory flight.

Historical sampling

Stored samples from the Research Centre for Farm Animal Gene Conservation were taken, where a total of 26 samples of adult worker bees were stored from the year 2004. These samples had been collected from weak colonies and contained 10 to 20 adult bees per sample. The samples represented 11 out of 20 counties in Hungary.

Sample processing at the Centro Apícola Regional (CAR), Marchamalo, Spain

Spring and summer samples from the year 2010 were processed in the laboratory at the CAR in Spain. A total of 30 adult bees from each sample were macerated with 5 ml molecular biology grade H_2O (MilliQ system) for 2 min at high speed in a Stomacher 80 Biomaster (Seward, West Sussex, UK) using strainer bags (BA6040/STR, Seward). The filtered macerates were recovered in 15-ml tubes and centrifuged for 6 min at 800 × *g*. The supernatant was discarded and the final pellet resuspended in 1 ml of H_2O. Each sample was visually checked by phase contrast microscope to verify the presence of *Nosema* sp. spores (OIE, 2008). To determine the presence of *Nosema* species, PCR analysis of the resuspended pellets was also performed using the methods previously described (Martín-Hernández et al., 2007; Botías et al., 2012). The sensitivity level of this technique is 2.5 spores of *N. ceranae* or 25 spores of *N. apis* in 150 µl of bee macerate (CAR Laboratory, unpublished data).

Sample processing at the RET (Szent István University, Gödöllő, Hungary)

The samples from October 2010 together with the Hungarian historical samples were processed in the laboratory of RET in Hungary. Sample processing at the CAR was carried out using 96-well microtitre plates. A total of 30 adult bees from each sample were macerated manually with 5 ml molecular biology grade water (H_2O) in individual LPDE zip lock bags (M6080B, Labsystem) for the PCR until the material became homogeneous. This process took at least 1 min per bag. The macerates were filtered through a disposable plastic net and recovered in 15-ml tubes for centrifugation at 800 × *g* for 6 min. The supernatant was discarded and the pellet was resuspended in 1 ml of H_2O. Each sample was than visually checked under a light microscope to verify the presence of *Nosema* sp. spores (OIE, 2008). The 1-ml suspensions were centrifuged for 15 min at maximum rpm at 4 °C in 1.5-ml Eppendorf tubes, then the supernatant was removed. Three hundred µl of CTAB buffer (Cornman et al., 2009) and 200 mg of glass beads (425–600 mm, Sigma-Aldrich) were added. The tubes were taped to the shaker part of a vortex mixer (ZX3m, Velp Scientific) and vortexed with maximum rpm for 5 min. Using this method we were able to process 10 tubes at one time. After the addition of 1000 mg proteinase K, the suspensions were incubated overnight (12 h) at 56 °C with continuous shaking (200 rpm). DNA was extracted using four cycles of centrifugation at 13,000 rpm for 15 min at 4 °C with the supernatants (300 µl) transferred to new tubes between cycles: first cycle with equal volumes of suspension and phenol (1:1), second cycle with equal volumes of supernatant, phenol and chloroform (1:1:1), third cycle with equal volumes of supernatant and chloroform, and fourth cycle with 1:2.5 volumes of supernatant and cold (–20 °C) ethanol. After 2 min the pelleted DNA was collected by centrifugation (at 13,000 rpm for 15 min at 4 °C). The ethanol was discarded, the pellet was dried and 30 µl of H_2O was added. The extracted DNA was stored at −20 °C until used. Quantity, quality and purity of DNA were analysed spectrophotometrically with Implen Nanophotometer (Implen GmbH, Germany) and the concentration was adjusted to 20 ng/µl by water. The extracted DNA was analysed by PCR to determine the presence of *Nosema* sp. using the specific 218MITOC F/R and 321APIS F/R primers as described previously (Martín-Hernández et al., 2007). As positive control, COI-F and COI-R primers were used (Botías et al., 2012). The PCR reaction mix components were as follow, in 25 µl containing 1 × AmpliTaq Gold Polymerase buffer (Applied Biosystems), 1.5 mmol of MgCl, 2 µMol of dNTP 2.5 BSA (200 mg/ml), 3.3 µl Triton X-100, 1 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 0.9 µM of each pair of 218MITOC and 321APIS primers, 0.2 µM of COI-F/R primers, and 9 µl of DNA template. The thermocycler program was the same as described by Botías et al. (2012). Each PCR product was analysed by agarose electrophoresis (SeaKem LE-Lonsa) (Fig. 1). In order to detect possible contamination and to assess the reliability of sample processes between the two laboratories, the same

negative and positive controls were processed in parallel during the extraction and PCR phases of analysis. Also, the leftovers of a total of 100 samples from the year 2010 (the original macerates and the leftovers of adult bees) processed at the CAR from spring were reprocessed at the RET.

Fig. 1. Amplicons of different lengths, specific for the detection of *Nosema ceranae*, *Nosema apis* and *Apis mellifera* from multiplex PCR using the three pairs of primers (218MITOC F/R, 321APIS F/R, COI-F and COI-R). Lane 1 shows positive control sample from *A. mellifera* infected with *N. ceranae*, lane 2 shows positive control sample from *A. mellifera* infected with *N. apis*, lane 3 shows positive control sample from *A. mellifera* infected with both *Nosema* species, lane 4 shows positive control sample from *A. mellifera* without infection of the two types, lane 5 shows negative control sample without bee DNA, lanes 6 to 12 show the actual samples from the collection in the year 2010, and lane M is a 100 bp DNA ladder

Spore counts and proportions of infected bees

Mean spore counts were calculated using the previously macerated samples collected in autumn (October) of 2010 using the method of Cantwell (1970). A separate count to determine the proportion of infected bees per hive was calculated using the leftovers (n = 30) of each autumn 2010 sample. The individual bees were macerated with 1 ml H_2O and checked for spore presence under a light microscope. To determine a negative result for *Nosema*, multiple visualisations were performed on each sample from triple view focusing on different parts of the samples. A sample was declared to be not infected if no spores were detected in any of the three microscopic views.

Statistical analysis

Z-test was conducted to compare the independent probabilities. To test the homogeneity of distributions, Chi-square test was used. Linear quantile regression using MS Excel was used to calculate the connection between the mean spore count and the rate of infected bees and to calculate the extent of infection.

Results

A less expensive DNA isolation method and multiplex PCR analysis were used to detect and identify the two *Nosema* species in Hungarian honey bee colonies. Genetic typing identified *Nosema* sp. even in those samples in which light microscopy did not confirm infection.

Microscopic versus PCR identification

Results of the microscopic evaluation of samples from 2010 were supported by the findings of PCR analysis. Data obtained from phase contrast microscopy are comparable to those found by light microscopy. Table 1 shows slightly higher infection rate using PCR than with either form of microscopy: 89% positivity rate with microscopy versus 95% with PCR on spring samples, 97% positivity with microscopy versus 98% PCR on summer samples, 95% positivity with microscopy versus 97% PCR on autumn samples, respectively. However, according to the Z-test, there were no significant differences between the infection rates detected by microscopy and PCR in any season ($P > 0.05$).

Table 1

Comparison of the sensitivity of methods for *Nosema* sp. infection

Season	Method	Positive samples (%)
Spring	Microscopy	89
	PCR	95
Summer	Microscopy	97
	PCR	98
Autumn	Microscopy	95
	PCR	97

Seasonal and geographical distribution of Nosema *sp. during the study*

Using the data from 2010, the number of infected hives checked from season to season showed no significant difference (Z-test: $P > 0.05$). As shown in Table 2, the percentage of hives infected with *Nosema* sp. ranged between 95% and 98%. The distribution of *N. ceranae* and *N. apis* infection rates along the seasons was homogeneous (Chi-square test: $P = 0.57$). The prevalence of *N. ceranae* was always significantly higher than that of *N. apis* and of co-infection (Z-test: $P < 0.001$). The rate of *N. ceranae* ranged from 95% to 98% in the infected samples (Fig. 2).

Exterior and interior samples

Regression analysis shows significant correlation between the mean spore count of the sample as a whole percentage of infected bees, and the percentage of infected bees per sample ($R_{int} = 0.65$ with $n = 130$; $R_{ext} = 0.43$ with $n = 138$; $P < 0.001$ in both cases). The infection rate of the infected bees in the exterior samples (Table 3) was higher than in the interior samples in each season; however, no significant differences were detected (Z-test: $P_{spring} = 0.14$; $P_{summer} = 0.08$; $P_{autumn} = 0.28$).

Table 2

Percentage of infected hives checked from different seasons

Season	Rate of the infected hives (%)	Infection type		
		Nosema ceranae (%)	*Nosema apis (%)*	Co-infection (%)
Spring	95	95	1	4
Summer	98	97	0	3
Autumn	97	98	1	1

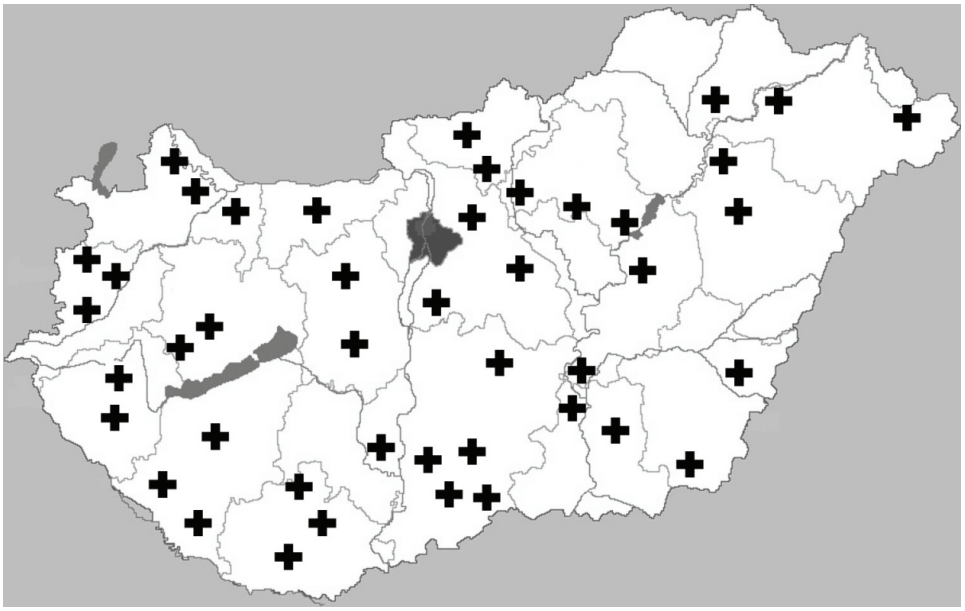

Fig. 2. The distribution of Hungarian sampling locations where *Nosema ceranae* infection of the samples was confirmed

Table 3

The rate of infected bees in exterior and interior samples in different seasons

Season	Sample type	
	Exterior (%)	Interior (%)
Spring	90	84
Summer	98	95
Autumn	94	90

Quantile regression of the rate of infected bees depending on the mean spore count

First we defined the amount of mean spore count as low (+), moderate (++) or high (+++), and calculated the quantiles of the mean proportion of infected bees (5%, 10%, 50%, 90% and 95%, respectively) (Table 4). A linear model was fitted to each quantile.

Table 4

Definition of spore count levels

Mean spore count (thousands)			
Exterior	Interior	Mean spore count rate	
0–24.99	0–9.99	+	Low
25–49.99	10–24.99	++	Moderate
50–160	25–100	+++	High

According to our qualification system, a colony is at a high risk (HR) if more than 50% of the population is infected (Higes et al., 2008, 2013; Botías et al., 2012). A moderate risk (MR) exists when less than 50% but more than 30% of the population is infected. Infection rates below 30% mean a low risk (LR) of infection (Table 5). By risk we mean the appearance of colony weakening and reduction in population on colony level.

Table 5

Definition of the rate of risk depending on the rate of infection

Rate of infection (%)	Extent of risk
< 30	Low risk
30 –50	Moderate risk
50 <	High risk

Figure 3 shows that the probability of low risk (less than 30% of the interior population infected) is more than 0.9, and in this case the mean spore count is below 10,000. If the spore count is between 10,000 and 25,000, the population has a low risk of infection with a probability of about 0.5. If the spore count is above 25,000, the population has a moderate or high risk with a probability of more than 0.9.

Figure 4 illustrates that the exterior population can have a high risk (more than 50% of the population infected) with a probability of about 0.5 even in those cases where the mean spore count is below 25,000. If the spore count is between 25,000 and 50,000, the population has high or moderate risk of infection

with a probability of about 0.9. If the spore count is above 50,000, the population has high or moderate risk of infection with a probability of more than 0.95.

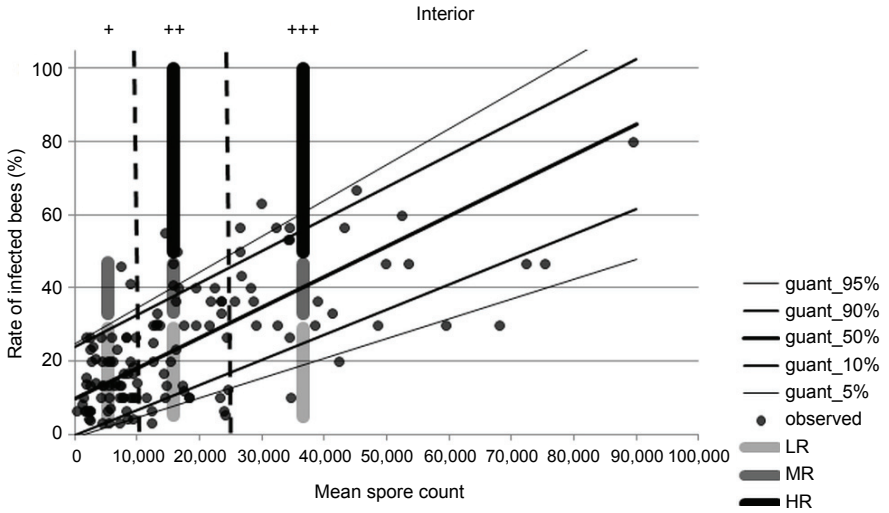

Fig. 3. Quantile regression of the rate of infected interior bees depending on the mean spore count with the rate of spore amount [low (+), moderate (++) and high (+++)] and with the extent of infection risk [high risk (HR), moderate risk (MR) or low risk (LR)]

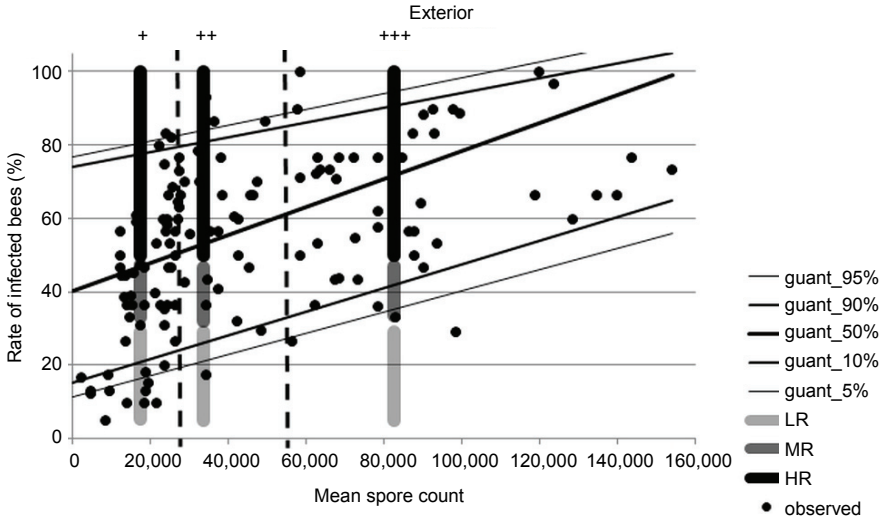

Fig. 4. Quantile regression of the rate of infected exterior bees depending on the mean spore count with the rate of spore amount [low (+), moderate (++) and high (+++)] and with the extent of infection risk [high risk (HR), moderate risk (MR) or low risk (LR)]

Historical samples

All of the 26 available samples were visually verified to contain *Nosema* sp. spores using light microscopy. However, PCR methods confirmed 6 samples (23%) only as being positive for the presence of *Nosema* sp. Out of these, 5 samples were co-infected and only one was confirmed to contain only *N. ceranae*.

Discussion

The results of previous and recent studies confirm that *Nosema ceranae* has been present in Hungary at least since 2004. The low rate of positive confirmation by PCR compared to the microscopic methods is assumed to be attributable to the storage conditions of historical samples. These samples were thawed and frozen multiple times over the years.

As an extremely low rate of infection by *N. apis* was found in each season, the theory of cyclic infection rate of *N. apis* as stated by Bailey (1955) could be revised. In contrast, *N. ceranae* is persistent. Tapaszti et al. (2009) showed that *N. ceranae* was dominant in the summer season, and data from the current study prove that it is dominant throughout the year.

Knowing that *N. ceranae* persists throughout the year, the use of treatment designed for *N. apis* may not always be appropriate. This is supported by the terminology used based on the type of infection, as Nosemosis C is a disease different from Nosemosis A. Previously, nosemosis was being treated when the classical disease signs appeared in early spring and late autumn. Unexpected colony weakness and losses were observed in the summer months by many beekeepers in Hungary. Since *N. ceranae* was not expected to be present, no treatment was applied against it. Forsgren and Fries (2010) have reported that there may not be significant differences in virulence between the two *Nosema* species; however, treatment against a single type may have allowed the spread of *N. ceranae*. This was confirmed in previous works by Martín-Hernández et al. (2011), Higes et al. (2013), Van der Zee et al. (2014) and Williams et al. (2014).

The results of this study also confirm the recommendation of Meana et al. (2010) that the mean proportion of infected bees may be a more reliable indicator to establish colony health. This is especially important because *N. ceranae* is a persistent issue throughout the year. Proactive monitoring for *Nosema* sp. by collecting exterior bees gives beekeepers a tool to better control nosemosis.

Antúnez et al. (2009) reported that the titre of vitellogenin in bees decreased after infection with *Nosema* sp., especially with *N. ceranae*. Nelson (2007) observed that vitellogenin titre in bees paces the onset of foraging behaviour, where the lower the titre of vitellogenin, the earlier bees mature for foraging. It may be concluded that infection with *Nosema* sp. lowers the titre of vitellogenin in bees, thus making the infected bees mature for foraging. If this con-

clusion is right, it can explain why the infection rate of exterior bees is higher than that of interior bees.

Regular vortexing and centrifugation of the phenol-chloroform mixture were substituted for commercial tissue lysers and cell disrupters and biosprint for DNA extraction. The capacity was still low for analysing the numbers of samples required, but it was suitable for daily routine tests in a laboratory having modest conditions.

Study on nosemosis in Hungarian apiculture should adapt methods for collecting data on the correlation of the percentage of parasitism found and the collapse of bee colonies. Studies on *N. ceranae* causing mortality of bee colonies in the field or loss of honey production should be conducted (Botías et al., 2013).

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