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Original article

Twenty-five-year study of *Nosema* spp. in honey bees (*Apis mellifera*) in Serbia

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ABSTRACT

A total of 7386 samples of adult honey bees from different areas of Serbia (fifteen regions and 79 municipalities) were selected for light microscopy analysis for *Nosema* species during 1992–2017. A selection of honey bee samples from colonies positive for microsporidian spores during 2009–2011, 2015 and 2017 were then subjected to molecular diagnosis by multiplex PCR using specific primers for a region of the 16S rRNA gene of *Nosema* species. The prevalence of microsporidian spore-positive bee colonies ranged between 14.4% in 2013 and 65.4% in 1992. PCR results show that *Nosema ceranae* is not the only *Nosema* species to infect honey bees in Serbia. Mixed *N. apis*/*N. ceranae* infections were detected in the two honey bee samples examined by mPCR during 2017. The beekeeping management of disease prevention, such as replacement of combs and queens and hygienic handling of colonies are useful in the prevention of *Nosema* infection.

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1. Introduction

Nosema ceranae (N. ceranae) and *Nosema apis (N. apis),* two microsporidian species, can infect adult honey bees (Forsgren and Fries, 2010). Spores *N. apis* are larger than spores *N. ceranae* and at the ends rounded, symmetrical while spores *N. ceranae* at the ends end sharply (Fries et al., 1996, 2006). Spores of *N. apis* are approximately 6 × 3 μm and the number of coils of the polar filament inside spores are >30 (Zander and Bottcher, 1984; Fries, 1989; Liu, 1984). According to Fries et al., (1996) spores of *N. ceranae* are approximately 4.4 × 2.2 μm and the number of coils of the polar filament inside spores are 18–21. Natural infections where hosts are infected by a single parasite are rare (Read and Taylor, 2001). Within the bee host, interactions between parasites can influence disease severity, and mixed infections with *N. apis* and *N. ceranae* are common (Paxton et al., 2007; Fries and Forsgren, 2008; Chen et al., 2008; Milbrath et al., 2015; Huang et al., 2007). One species can significantly influence transmission rates of the other or have no measurable effect on transmission (Pilarska et al., 2006).

Both microsporidia are widespread worldwide today and each microsporidia crossinfects the other host. (Ansari et al., 2017; Sinpoo et al., 2018). Martín-Hernández et al. (2007); Fries and Forsgren (2008) state that *N. ceranae* infections appear to dominate in warmer climates compared to more temperate regions in Europe. The climate could be an important factor explaining differences in parasite species distribution and impact (Pacini et al., 2016; Ansari et al., 2017). Martín-Hernández et al. (2009) compared the increase in spore numbers in bee abdomens at different times post-infection and found *N. ceranae* numbers increased over a wider temperature range compared to *N. apis* (Milbrath et al., 2015).

Higes et al., (2006) found 11 out of 12 samples from 2005 from Spanish apiaries positive for microsporidia to contain *N. ceranae*, based on homology to the original 16S ssrRNA GenBank entry for *N. ceranae*.

Nosemosis in European honey bees was attributed to *N. ceranae* and *N. apis* (Higes et al., 2006; Gisder et al., 2010; Huang, 2011; Colin et al., 2009; Odnosum, 2017). In Sweden, 83% of colonies

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had *N. apis* and mixed *N. apis/N. ceranae* infections were detected in 17% colonies (Fries and Forsgren, 2008). In the 70.4% of colonies in Scotland, had mixed *N. apis/N. ceranae* infections (Bollan et al., 2013). Also, in Argentina and Australia the presence of both types of microsporidia has been proven (Giersch et al., 2009). In Greek, Italy, the eastern Azerbaijan and Saudi Arabia, only spores of *N. ceranae* were detected (Bacandritsos et al., 2010; Papini et al., 2017; Razmaraii et al., 2013; Ansari et al., 2017). *N. ceranae* is highly pathogenic when experimentally inoculated into European honey bees (Higes et al., 2007) and is associated with reduced honey production and increased winter mortality, it has been shown in Spain (Higes et al., 2006). Serbia has a long tradition of beekeeping (Ivanović et al., 2015; Matović et al., 2018). In Serbia and neighbouring countries (Croatia, Bosnia and Herzegovina, Montenegro, the North Macedonia), *N. ceranae* dominates in microsporidia infections in honey bees (Stevanovic et al., 2011). Stevanovic et al. (2011) indicated that *N. ceranae* has been present in Serbian bees (collected between 2000 and 2005) since at least the year 2000.

Because *N. ceranae* has been diagnosed in Serbia (Stevanovic et al., 2011) and because of the high prevalence of *Nosema* infections, we have suspected its presence in mixed infections with *N. apis*. The aim of this study was to investigate and determine the prevalence of *N. ceranae* and *N. apis* in different locations in Serbia (encompassing almost 70% of the total territory), because is still, using microscopic examination and multiplex PCR (mPCR).

2. Materials and methods

2.1. Sample collection

A total of 7386 samples from fifteen regions (79 municipalities) in Serbia were examined for *Nosema* infection between 1992 and 2017. Selected apiaries (a total of 250) were situated at locations distributed in both flatland and mountainous zones, i.e. from different climatic conditions (Fig. 1). Beside dead honey bees collected during winter loss, adult live bees and feces were sampled (in June and/or October). The first sampling was conducted in March 1992, and the last in June 2017. Each sample honey bees consisted of 60 adult bees (minimum). The live bees were collected from the hive entrance after closing it for 30 min (Meana et al., 2010; Botías et al., 2012). During 2009–2017, after light microscopy, collected samples were frozen at −20 °C and stored until molecular analysis Figure 1.

2.2. Spore detection

The abdomens of 60 live young or corpses of honey bees were macerated in 5 ml of distilled water (Sigma-Aldrich, Germany). Microscopic analyses of homogenates collected fecal samples were also performed. Pellets were analyzed by light microscopy (x400 magnification) to verify the presence of spores, according to OIE Manual (1992–2017). This methodology was employed for determination of the presence of *Nosema* spores in all the samples used in this study during 1992–2017. Part of the macerated bee suspensions collected during 2009–2017 were stored at −20 °C and subsequently subjected to mPCR analysis (the samples from 1992 to 2009 were not suitable for analysis, because technical possibilities.

2.3. DNA extraction

The commercial DNA extraction kit (QIAamp DNA Kit, USA) was used to obtain DNA from macerated honeybee abdomens using a procedure described by manufacturer. Extracted DNA was stored at −20 °C until mPCR.

2.4. PCR methodology

The primers used are shown in Table 1.

The thermocycler program consisted of 94 °C for 15 s followed by 35 cycles of 15 s at 94 °C, 30 s at 61.8 °C and 45 s at 72 °C and a final extension step at 72 °C for 7 min. Positive and negative controls were included in PCR runs. Amplicons were separated on 2% agarose gel in Mini-Sub Cell GT (Bio-Rad, USA) and products were visualized using Gel Doc XR system (Bio-Rad, USA). The positive controls used were from AFSSA, Sophia-Antipolis, Anses (France). The genomes are Clone plasmidique K3, *Nosema apis* APIS-FOR/APIS-REV (321 bp) ds pGEM-T-easy (Isolate AFSSA, September 15, 2009) and Clone plasmidique L2, *Nosema ceranae* MITOC-FOR/MITOC-REV (218 bp) ds pGEM-T-easy (Isolate Higes, August 26, 2009) from Reference Laboratory OIE AFSSA. The PCR products were purified with a QIA quick PCR purification Kit (QIAGEN, Germany).

3. Results and discussion

A total 7386 samples were submitted for light microscopic examination (Table 2). These samples were primarily from individual beekeepers from widely dispersed geographical locations within the 15 regions studied in Serbia. The results of this 25-year survey of *Nosema* species incidence (Table 2) revealed the continual high frequency of *Nosema*-positive bee colonies within each investigated year, ranging from 14.4% (2013) to 65.4% (1992). Lower, but still considerable percentages of bee colonies infected with *Nosema* species were detected in 2014 (18.8%) and 2015 (26.4%) (see Figure 2).

Based on the data reported by Republic Hydrometeorological Service of Serbia in the last 25 years (1992–2017), the average temperature in Serbia increased by 0.05 °C, annually (total 1.25 °C for the period). During the same period, the highest measured temperature was 44.9 °C, in 2007, the lowest was −34.8 °C, in 2000. Annually number of days with a temperature below 0 °C ranged from 59 (2014) to 111 (1994). Such temperature fluctuations may have influenced the incidence of nosmosis, which is maintained or even increased in Serbia. Previous studies have shown that the incidence of *N. apis* and *N. ceranae* significantly different in years, is possible also as climate change in Serbia (e.g. 1994 and 2000) (Stevanovic et al., 2013). After light microscopy, a total of 138 samples from different localities and years (2009–2011, 2015, 2017) were selected and investigated by mPCR for the presence of *N. apis* and *N. ceranae*. PCR amplicons from representative bee samples, plus negative and matching positive controls are presented in Table 3. *N. ceranae* was detected more frequently than *N. apis* in the monitored bee colonies during the years examined. Also, mixed *N. apis/N. ceranae* infections were detected using mPCR during 2017 but not in previous years.

N. ceranae-infected bees were found in honey bee samples collected from 2009 to 2017, in 15 examined regions and in all climatic areas (mountain and lowland zones) of Serbia that were included for study by mPCR. Also, the results showed that both examined bee samples that were positive for *N. apis* were also positive for *N. ceranae* during 2017.

In the last few years, infection of honey bees by *Nosema* spp. has been reported in numerous European countries, including Spain, France, Greece, Italy, Germany, Switzerland, Denmark, Finland and in our neighborhood (Hungary, Slovenia, Bosnia and Herzegovina) (Fries et al., 2006; Klee et al., 2007; Topolska and Kasprzak, 2007; Paxton et al., 2007; Tapaszti et al., 2009; Higes et al., 2010; Papini et al., 2017), as well as in other continents (Guerrero-Molina et al., 2016; Ansari et al., 2017). The exact date of entry of *N. ceranae* into European countries has not been reported

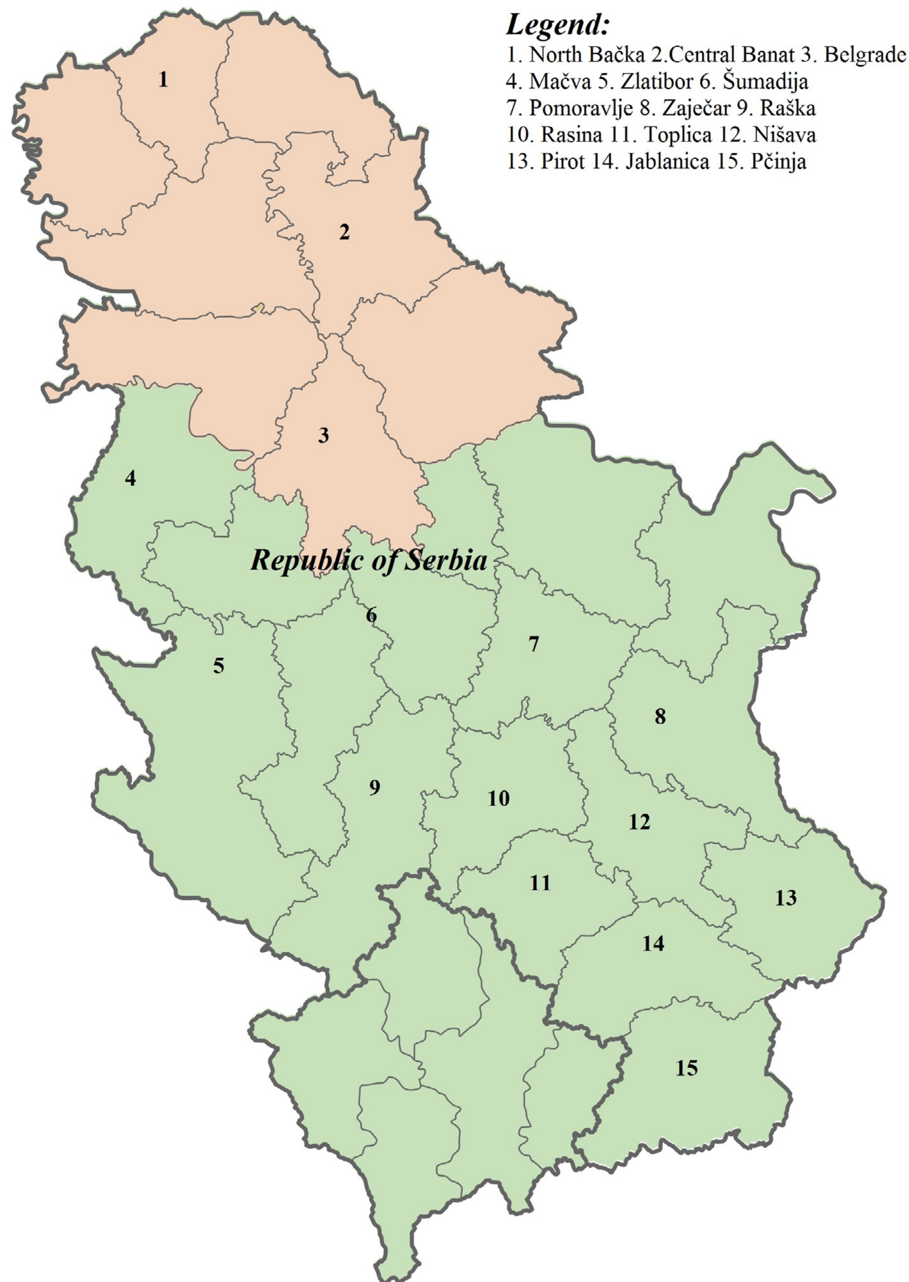

Fig. 1. Map of bee samples collected in regions in Serbia.

Table 1
Primers selected for detection of *N. ceranae* and *N. apis* (OIE Manual 2008, Chapter 2.2.4).

Primer	Sequence	PCR product size (bp)	Specificity
218MITOC-FOR	5′-CGGCGACGATGTGATATGAAAATATTAA-3′	218–219	*N. ceranae*
218MITOC-REV	5′-CCCGGTCATTCTCAAACAAAAAACCG-3′	218–219	*N. ceranae*
321APIS-FOR	5′-GGGGGCATGTCTTTGACGTACTATGTA-3′	321	*N. apis*
321APIS-REV	5′-GGGGGGCGTTTAAAATGTGAAACAACTATG-3′	321	*N. apis*

(Higes et al., 2010). Our results show that *N. ceranae* was in Serbia in 2009, in agreement with earlier findings by Stevanovic et al. (2011), who discovered the parasite in Serbia, in 2000. By implication, *N. ceranae* has probably been in Europe for at least the past two decades. It seems clear that *N. ceranae* is an pathogen of *A. mellifera*, having become distributed across the world, possibly within the last decade of the last century (Paxton et al., 2007; Klee et al., 2007; Guerrero-Molina et al., 2016). The detection of only *N. ceranae* in 2015 and in prior years in this study is in agreement with previously reports on *Nosema* in honey bees from Serbia by Stevanovic et al. (2011, 2013). In 2017, *N. apis* was detected in our study using a PCR method commonly used for molecular identification of *Nosema* species (Martín-Hernández et al., 2007, 2009; Higes et al., 2010; Hedtke et al., 2011). This is the first time they

Table 2
Number and percentage of bee samples and colonies in Serbia positive for *Nosema* spp. spores after light microscopic examination (1992–2017).

Year	Number of examined bee samples	Number of municipalities where the infection was detected	Number of infected apiaries	Number of positive bee samples	% of *Nosema*-positive bee colonies
1992	130	38	68	85	65.4
1993	107	34	34	34	31.8
1994	160	40	80	90	56.3
1995	127	7	34	38	29.9
1996	183	16	98	90	49.2
1997	140	21	57	62	44.3
1998	171	38	44	60	35.1
1999	217	24	76	93	42.9
2000	132	15	51	86	65.2
2001	176	18	45	60	34.1
2002	330	34	132	93	28.2
2003	337	8	42	155	46.0
2004	253	25	56	113	44.7
2005	384	16	58	157	40.9
2006	415	34	48	225	54.2
2007	719	25	78	230	32.0
2008	657	27	60	208	31.7
2009	245	21	41	148	60.4
2010	457	27	99	263	57.5
2011	504	21	66	242	48.0
2012	522	14	70	176	33.7
2013	355	12	17	51	14.4
2014	207	8	23	39	18.8
2015	148	8	29	39	26.4
2016	162	13	27	64	39.5
2017	148	10	11	45	30.4
Total	7386			2946	

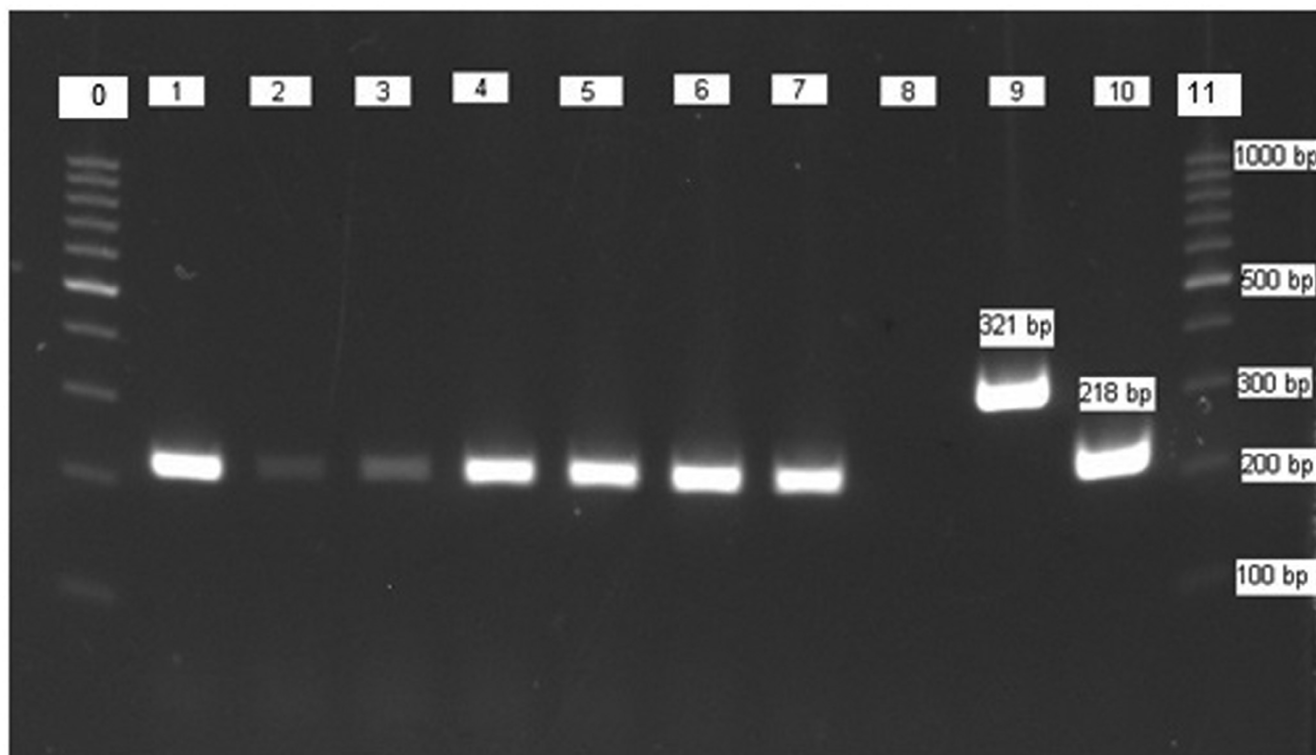

Fig. 2. Typical PCR products of *N. apis* and *N. ceranae*: Lane 0 and 11, molecular weight markers (DNA Ladder 10 × 100 bp); lanes 1–7, PCR products of positive samples; lane 8 negative control; lane 9 positive control for *N. apis*; lane 10 positive control for *N. ceranae*.

have *N. apis* and *N. cerana* detected in bee samples at the same time/co-infection, in the Republic of Serbia. This is the second time that *N. apis* has been molecular detected in Serbian bees. The present study showed that *Nosema* infections in Serbian honey bees can be caused by *N. ceranae*, either alone or in association with *N. apis*. In most of the examined beehives from the honey bee societies of infected European countries were also primarily *N. ceranae* (Higes et al., 2006, Stevanovic et al., 2011, 2013; Odnosum, 2017; Papini et al., 2017).

The detection of microsporidian infections in honey bees may not be related to its increased prevalence in the insects but rather to the development of the new, highly sensitive and specific molec-

Table 3
PCR results in relation to *Nosema* spp. genome presence in adult bee samples.

Year	Number of bee samples examined by PCR	*N. apis*	*N. ceranae*
2009	107	0	107
2010	21	0	21
2011	7	0	7
2015	1	0	1
2017	2	2	2
Total	138	2	138

ular technique, multiplex PCR. Using this molecular technique, the percent of positive samples is higher than is obtained using microscopic examination. Given that the spores of *N. apis* and *N. cerana* microscopy is not easily distinguishable, the use of molecular technique for species identification has played an important role in the study studies (Klee et al., 2007; Papini et al., 2017).

The world trade of honey bee products (honey, propolis and royal jelly) and beekeeping materials could also play an important role in the expansion of infective spores of *N. ceranae* from apiary to apiary over different geographical regions (Klee et al., 2007; Ansari et al., 2017). Commerce of queens and trade of worker bees can be a source of infection in some regions (Giersch et al., 2009). Education of beekeepers and good beekeeping practice are necessary in apiculture (Pacini et al., 2016). All these means of transmitting infective *N. ceranae* spores should be taken into account to explain the presence of *N. ceranae* in honey bees in remote geographical sites theoretically isolated from any source of infective spores (Colin et al., 2009; Ansari et al., 2017). The queen is susceptible to most of the diseases that attack her offspring, and *N. ceranae* can be transmitted horizontally from infected worker honey bees to queens by feeding (Higes et al., 2009). The behavioural changes described in confined honey bees could modify this mode of transmission since infected bees are less inclined to share their available sucrose solution with other bees (Naug and Gibbs, 2009). One of the reservoirs of *N. ceranae* is pollen stored in honeycombs. The presence of infective *N. ceranae* spores in pollen must be due to self-contamination during the process of pollen collection. The effect of pollen fermentation (to bee bread) on *Nosema* spore viability has yet to be evaluated. Honey and royal jelly have been reported as sources of *N. ceranae* spores (Giersch et al., 2009; Cox-Foxter et al., 2007). *N. ceranae*-infected colonies have a long incubation period that usually appears to be asymptomatic, and which is usually passed over by apiarists. This includes a longer breeding period during cold months and diminished honey production. Finally, colonies become weakened and depleted of adult bees, and they collapse in a period of 1.5 to 2 years (Higes et al., 2010). Higes et al. (2009) found a close connection between *N. ceranae* infection and colony loss in professional apiaries in Spain. These findings were not present in Serbia, according to research by Stevanovic et al. (2013) (Brodschneider et al., 2018). In other European countries, the presence of *N. ceranae* has not been related to colony death, including the reported massive colony loss in North Europe (Paris et al., 2018; Papini et al., 2017). The largest losses of bee colonies in Serbia were in 2014 and 2016, in the years without clinical symptoms of the disease and laboratory slightly confirmed cases of nosemosis (Brodschneider et al., 2018). Based on these researches, the greatest winter losses and disappearances of bee colonies are not always related to the presence of *N. ceranae* (Ansari et al., 2017; Brodschneider et al., 2018; Paris et al., 2018).

In other studies, infection by *N. ceranae* does not seem to have any effect on colony loss (Guerrero-Molina et al., 2016; Ansari et al., 2017; Papini et al., 2017). Thus, many different views on the consequences of *N. ceranae* infection in European colonies have been reported. Huang (2011) found that infection with this parasite can affect the physiology, behavior and survival of bees. *N. ceranae* infection certainly disrupts the integrity of the central midgut epithelial cells and changes the energy needs of bees (Martín-Hernández et al., 2011; Mayack and Naug, 2010; Paris et al., 2018). The infection can also significantly affect the immune response of the bees and modify the production of pheromones, leading to a disorder of bee nutrition (Antúnez et al., 2009; Paris et al., 2018; Sinpoo et al., 2018). This can also be explained by other stress factors (pesticide poisoning, parasites, poor nutrition, flaws in bee technology, heavy metal contamination, etc.) (Alaux et al., 2010; Ansari et al., 2017; Vidau et al., 2011; Cox-Foxter et al., 2007; Genersch et al., 2010; Gisder et al., 2010; Ruschioni et al., 2013; Odnosum, 2017; OIE, 2013; Paris et al., 2018; Papini et al., 2017; Sinpoo et al., 2018).

It has been reported that *N. ceranae* is more prevalent in warmer climates than is *N. apis* (Fries, 2010). It appears that *N. ceranae* is better adapted to complete its endogenous cycle with a higher biotic index in a greater temperature range (Martín-Hernández et al., 2007). This difference in temperature sensitivity between parasite species has epidemiological implications and could mean decreased transmission opportunities for *N. ceranae*. Also, it is generally accepted that the earth's temperature is progressively increasing, with great effect on parasites' life cycles (Brooks and Hoberg, 2007). Changes in climate could affect the distribution and seasonality of nosemosis in honey bees (De la Rocque et al., 2008). Thus, more research will be needed to establish the epidemiological field characteristics of nosemosis in different European countries (Van der Zee et al., 2009). In conclusion, beekeeping management of disease prevention, such as replacement of combs and queens and hygienic handling of colonies are useful in the prevention of *Nosema* species infection (Pacini et al., 2016; OIE, 2013).

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Disclosure statement

No potential conflict of interest was reported by the authors.

Compliance with ethics requirements

The manuscript does not contain clinical studies or patient data.

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