International Journal for Parasitology 49 (2019) 657–667

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

The levels of natural *Nosema* spp. infection in *Apis mellifera iberiensis* brood stages

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ARTICLE INFO

Article history:
Received 25 January 2019
Received in revised form 8 April 2019
Accepted 10 April 2019
Available online 3 June 2019

Keywords:
Apis mellifera
Nosema ceranae
Nosema apis
Microsporidia
Epidemiology
Brood infection

ABSTRACT

Nosema ceranae is the most prevalent endoparasite of *Apis mellifera iberiensis* and it is a major health problem for bees worldwide. The infective capacity of *N. ceranae* has been demonstrated experimentally in honey bee brood, however no data are available about its prevalence in brood under natural conditions. Thus, brood combs from 10 different hives were analyzed over two consecutive years, taking samples before and after winter. A total of 1433 larvae/pupae were analyzed individually and *N. ceranae* (3.53%) was the microsporidian most frequently detected, as opposed to *Nosema apis* (0.42%) which was more frequently detected in conjunction with *N. ceranae* (0.71%). The active multiplication of both microsporidians was confirmed by the expression (real-time-PCR) of the *N. ceranae* polar tube protein 3 gene and/or the *N. apis* RNA polymerase II gene in 24% of the brood samples positive for *Nosema* spp. Both genes are related to microsporidian multiplication. As such, *N. ceranae* multiplication was confirmed in 1.06% of the samples, while *N. apis* multiplication was only observed in co-infections with *N. ceranae* (0.07%). Brood cells were analyzed for the presence of *Nosema* spp., as those are the immediate environment where the brood stages develop. The brood samples infected by *Nosema* spp. were in brood cells in which that microsporidians were not detected, while brood cells positive for *N. ceranae* hosted brood stages that were not apparently infected, indicating that this is unlikely to be the main pathway of infection. Finally, the colonies with brood infected by *N. ceranae* showed higher levels (numbers) of infected adult bees, although the differences were not significant before ($P = 0.260$), during ($P = 0.055$) or after ($P = 0.056$) brood sampling. These results show that *N. ceranae* is a bee parasite ubiquitous to all members of the colony, irrespective of the age of the bee. It is also of veterinary interest and should be considered when studying the epidemiology of the disease.

1. Introduction

Microsporidians are a truly diverse lineage of extremely reduced parasites that are related to the Order Rozellida (Rozellosporidia = Rozellomycota = Cryptomycota) or that diverged as the branch below the fungi, within the Holomycota clade of Opisthokonta (James et al., 2013; Bass et al., 2018; Galindo et al., 2018). These organisms are obligate intracellular parasites that infect a wide range of vertebrates and invertebrates (Didier et al., 2004). They are frequently found in domestically managed aquatic and terrestrial hosts, many of which are vulnerable to epizootics and all of which are crucial for the stability of the animal–human food chain (Stentiford et al., 2016). In economically relevant insects such as the silkworm (*Bombyx mori*) and honey bee (*Apis mellifera*), microsporidians can cause serious diseases and, as a result, important financial losses.

Some insects play a vital role as pollinators, regulating natural ecosystems. Globally, nearly 90% of wild flowering plant species to some extent depend on the transfer of pollen by animals, especially bees (IPBES, 2016, https://www.ipbes.net/system/tdf/spm_

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https://doi.org/10.1016/j.ijpara.2019.04.002
0020-7519/

deliverable_3a_pollination_20170222.pdf?file=1&type=node&id=15248; European Commission, 2017, http://ec.europa.eu/environment/nature/pdf/roadmap-for-the-eu-pollinators-initiative.pdf).

To date, three microsporidian species have been described in *A. mellifera* honey bees: *Nosema apis* (Zander, 1909), *Nosema ceranae* (Fries et al., 1996), and *Nosema neumanni* (Chemurot et al., 2017). Due to its more recent identification, there is currently little information available about the effects of *N. neumanni* infection, while the other two *Nosema* spp. (*N. apis* and *N. ceranae*) have different clinical consequences in honey bee colonies. *Nosema apis* was first described over a century ago (Zander, 1909) and the consequences of its infection in honey bee colonies were first described many decades ago. Dysentery and bees crawling around the hive are among the most recognizable clinical signs of *N. apis* infection and, indeed, this disease is thought to cause significant economic losses to beekeepers around the world. By contrast, *N. ceranae* was first described in 1996 as a parasite of the eastern bee *Apis cerana* (Fries et al., 1996), although by the beginning of this century it was considered to be an epidemic for its new host, *A. mellifera* (Higes et al., 2006; Martín-Hernández et al., 2007; Paxton et al., 2007). The disease provoked by *N. ceranae* has clinical signs distinct from those caused by *N. apis*, and *N. ceranae* infection can be particularly unsettling for beekeepers because the infected colonies do not generally show outward manifestations of infection until their health is severely compromised (Higes et al., 2008a; Botías et al., 2012a). Because the diseases caused by both microsporidians are well differentiated, that produced by *N. apis* is now referred to as Nosemosis type A and that caused by *N. ceranae* as Nosemosis type C (Higes et al., 2010). Indeed, Nosemosis Type C has been associated with altered polyethism and a gradual loss of workers bees, ultimately exhausting the colony and causing its death (Higes et al., 2008a; Hatjina et al., 2011; Khoury et al, 2011, 2013; Botías et al., 2012a,b; Ravoet et al., 2013; Lodesani et al., 2014; Bekele et al., 2015; Lecocq et al, 2016).

Both *Nosema* spp. have now been reported across the five continents where apiculture practices involve the use of sub-species and variants of *A. mellifera*: Africa (Fries et al., 2003; Higes et al., 2009a); Asia (Huang et al., 2008; Chen et al., 2009); North America (Chen et al., 2008, Williams et al., 2008) and South America (Calderón et al., 2008; Invernizzi et al., 2009); Europe (Higes et al., 2006; Paxton et al., 2007); and Oceania (Giersch et al., 2009). In fact, globalization has provided parasites/pathogens opportunities to cross geographic boundaries and expand to new hosts in apiculture (Chen et al., 2009; Chen and Huang, 2010; Li et al., 2012; Vanbergen et al., 2013). Despite its more recent identification, *N. ceranae* is now not only more widespread but it is even more predominant in many regions (Klee et al., 2007; Martín-Hernández et al., 2007; Giersch et al., 2009; Invernizzi et al., 2009; Fries, 2010; Copley et al., 2012; Porrini et al., 2016; Gisder et al., 2017; Khezri et al., 2018; Tokarev et al., 2018). In fact, the prevalence of *N. ceranae* is not only on an upward trend (Cox-Foster et al., 2007; Botías et al., 2012b; Cepero et al., 2016; Gisder et al., 2017) but since its prevalence could be favored at higher temperatures (Martín-Hernández et al., 2009; Paxton, 2010; Sánchez Collado et al., 2014; Özgör et al., 2015; Gisder et al., 2017), it represents an important challenge in the face of global warming.

Nosema infection occurs after the ingestion of mature spores in food or water, and it is mainly transmitted by trophalaxis (food sharing: Smith, 2012) or the fecal-oral route (fecal waste cleaning) via an infected nest mate (Webster, 1993; Higes et al., 2009b). The target tissue for both *N. apis* and *N. ceranae* is the bees' ventriculum (Higes et al., 2007; Huang and Solter, 2013), infecting the epithelial cells in which these microsporidian species produce their proliferative stages.

All *A. mellifera* castes (workers, queen and drones) are susceptible to *Nosema* spp. infection (Higes et al., 2007; Webster et al., 2008; Higes et al., 2009a,b; Traver and Fell, 2011, 2012; Martín-Hernández et al., 2012). Although Nosemosis has traditionally been considered an adult disease, drone and queen pupae were seen to be weakly infected by *N. ceranae* in naturally infected colonies (Traver and Fell, 2011, 2012), and both *N. apis* and *N. ceranae* were detected in spring workers and queen larvae and/or pupae in Chile (Rodríguez et al., 2012. A later study confirmed that pre-pupae of *A. mellifera* workers could be successfully infected under laboratory conditions (Eiri et al., 2015), producing a range of pathological problems in the adults that emerged from them after metamorphosis, shortening their life, delaying normal age polyethism and disrupting the balance of the colony (Eiri et al., 2015; Benvau and Nieh, 2017). Immature stages of other hymenopterans have previously been reported to be susceptible to experimental infection by microsporidians, eg *Bombus terrestris* to *Nosema bombi* (van den Eijnde and Vette, 1993; Schmid-Hempel and Loosli, 1998). Similarly, larvae of *Loxostege sticticalis* (Lepidoptera: Crambidae) were successfully infected with fresh *N. ceranae* spores isolated from *A. mellifera* (Malysh et al., 2018), and *N. apis* and *N. ceranae* DNA was also recently isolated from the larvae of the honey bee pest *Galleria mellonella* (Lepidoptera: Pyralidae) collected from honey bee hives in Turkey (Ozgor et al., 2017).

Due to the high prevalence of *N. ceranae* in Spain (Botías et al., 2012b; Cepero et al., 2014, 2016), the aim of this study was to determine if brood infection occurs naturally in *Nosema* spp. infected colonies, assessing two well-defined beekeeping moments: before and after winter cluster formation. Additionally, correlations with the levels of infection at different developmental bee stages were studied, as was the presence of the microsporidians in the brood cells, to evaluate this as a possible route of bee brood exposure.

2. Materials and methods

2.1. Colonies

Ten *Apis mellifera iberiensis* colonies located at an experimental apiary of the Centro de Investigación Apícola y Agroambiental de Marchamalo (CIAPA) Research Center (Marchamalo, Spain) were used for this study. Two consecutive studies were carried out between 2015 and 2017, using five colonies in each assay. The colonies H1 to H5 were included in the first study (Assay 1: 2015–2016) and H6 to H10 in the second one (Assay 2: 2016–2017). All of them were positive for *N. ceranae* infection from previous DNA analyses.

2.2. Bee brood (BB) sampling

The sampling times are indicated in Table 1. In order to analyze bee brood (BB) samples before and after winter bee cluster formation, one comb with a BB was collected from each colony in October 2015 and February 2016 (Assay 1; colonies H1–H5), or in November 2016 and March 2017 (Assay 2; colonies H6–H10). Despite using different sampling months in the two assays, the samples were collected at the same beekeeping moment ie prior to the wintering cluster formation (pre-clustering; October/November) and after winter cluster formation (post-clustering; February/March). Brood combs were collected at the experimental apiary and immediately transported to the laboratory, where they were frozen at −80 °C until analysis.

The total number of brood samples (including recently capped larvae, pupae and pre-emerging adult bees) is shown in Table 2.

Table 1
Study sampling design: months in which bee broods, adult bees and brood-cells were collected between September 2015 and July 2017.

	Assay 1							Assay 2					
	Pre-clustering			Post-clustering				Pre-clustering			Post-clustering		
	Sep 15	Oct 15	Nov 15	Jan 16	Feb 16	Mar 16	Apr 16	Oct 16	Nov 16	Dec 16	Mar 17	May 17	Jul 17
Bee brood (BB)		x			x				x		x		
Interior bees (IB)	x	x	x	x	x	x	x	x	x	x	x	x	x
Forager bees (FB)	x	x	x	x	x	x	x	x	x	x	x	x	x
Cells									x		x		
Hive identification	H1–H5							H6–H10					

At each sampling time, 90 capped BBs were analyzed from each colony when possible; post-clustering in Assay 1, three colonies (H1, H3 and H4) had died and thus brood samples were only taken from the remaining colonies. Similarly, the H7 colony was very weak in November 2016 (Assay 2), with no consistent brood, so no brood comb was taken from it.

To collect brood samples, frozen brood combs were thawed sufficiently to be able to carefully cut them and individually extract the different developmental bee stages (larvae and pupae) from their cells. Brood samples were selected randomly from the BB area, trying to include most of the developmental phases present in the combs. Each sample was aseptically removed from the cell one by one, with the aid of a sterile wooden stick and sterile tweezers to first completely open the operculum and break the cell wall. The samples were then extracted, avoiding any external contamination with *Nosema* spp. spores outside their own cells. Samples were discarded when larvae or pupae came into contact with the external surface of the cell or when there was any contact with an adjacent brood. To remove any spores on the brood surface, the brood sample was washed after it was taken out of the cell with MilliQ® H_2O (MilliQ® system, Merck, Damstadt, Germany) using a sterile disposable Pasteur pipette.

The brood samples were then visually classified into seven groups according to their developmental post-capping time (pct) stage (Human et al., 2013): L (capped stretched larva; 2–3 days pct); P1 (white eye pupa stage; 4 days pct); P2 (pink eye stage; 5–6 days pct); P3 (dark pink-purple eye pupa stage; 7–8 days pct); P4 (dark purple eye pupa stage; 9 days pct); P5 (black eye pupa; 10–11 days pct) and P6 (resting adult pre-emergence stage; 12 days pct).

2.3. Brood cell sampling

The cells were examined during the second assay to evaluate the presence of *Nosema* spp. DNA in the area where the brood develops (Table 1). Having carefully removed the brood samples (Section 2.2), approximately 20 cells per brood comb were sampled by dripping 100 µl of doubled-distilled water (dd-H_2O; PCR quality) slowly onto the cell walls and subsequently recovering it from the bottom of the cell with a disposable sterile Pasteur pipette. A total of 164 cells were analyzed: 77 cells from four colonies pre-clustering and 87 cells from all five colonies post-clustering (Table 2).

2.4. Adult bee sampling

Details of both interior bee (IB) and forager bee (FB) sampling collection can be seen in Table 1. In Assay 1, 25 IBs plus 25 FBs were sampled per honey bee colony at each of seven time points between September 2015 and April 2016. During Assay 2, in order to increase the detection limit of *Nosema* spp., the sample size was increased and 100 IBs plus 100 FBs were collected at each of six time points between September 2016 and July 2017, except in November 2016 when only 25 IBs were collected. The total number of worker bees sampled is shown in Table 2. The IBs were sampled from the brood chamber, brushing away the bees from the first combs with no brood, while the FBs were collected at the hive entrance at midday, blocked as described previously (Meana et al., 2010). During bee clustering in the coldest months of autumn and winter, no FBs were sampled since no foraging activity was observed (H1 November 2015 and January 2016; H4: November 2015; H6–H10: November and December 2016).

2.5. Sample processing and nucleic acid extraction

2.5.1. Brood samples

Each immature stage was placed individually into a well of a 96-well collection plate (Deltalab, Barcelona, Spain, No. 409004) over an ice surface. Every well contained 180 µl of 60% AL lysis buffer (Qiagen, Hilden, Germany No. 1014600), 1 µg/ml of RNA carrier (Qiagen, 148037710) and four 2 mm glass beads (Sigma®), as described previously (Cepero et al., 2014). One well in every 20 brood samples was left empty as a negative control (with milliQ® H_2O and the reagents only). The plates were sealed and shaken at 30 Hz for 6 min (TyssueLyser II, Qiagen®), and 180 µl of the resulting macerate were transferred to a new multi-well plate (Eppendorf® AG, Hamburg, Germany, No. 0030128.575), mixed with 200 µl of 60% AL buffer and 20 µl of protease (supplied with the BioSprint® 96 DNA Blood Kit, Qiagen®), and incubated for 10 min at 70 °C and 300 rpm. Total nucleic acid extraction was performed using the BioSprint® 96 DNA Blood Kit (Qiagen®, No. 940057) and the BS96 DNATissue® protocol (Qiagen®), in a

Table 2
Number of samples analyzed in this study.

Year	Hive ID	Season	Bee brood	Cells	Interior bees	Foragers	Total adult
2015–2016	H1–H5	Pre-clustering	447	–	350	300	650
		Post-clustering	179	–	225	175	400
2016–2017	H6–H10	Pre-clustering	360	77	1125	500	1625
		Post-clustering	447	87	1625	1400	3025
Total	H1–H10		1433	164	3325	2375	5700

Biosprint® 96 workstation (Martín-Hernández et al., 2012), with a final elution volume of 100 µl. Immediately after extraction, 25 µl of the 100 µl of the eluted nucleic acid sample was treated with RNase-free DNase I (Qiagen®, No. 79254) and used to generate first strand cDNAs using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California, No. 1708891), according to the manufacturer's instructions. The cDNA was generated by maintaining the reaction mix at 25 °C for 5 min, 46 °C for 20 min and finally, 95 °C for 1 min (Mastercycler® ep gradient S; Eppendorf), storing the generated cDNA at −20 °C until use. Negative controls were run in parallel for each step (DNA and RNA extraction, and reverse transcription controls).

2.5.2. Brood cell samples

The double distilled (dd)-H_2O (100 µl) recovered from the bottom of the cells (Section 2.3) was placed individually into a well of a Deepwell-96 plate with 100 µl of 50% AL buffer and 1 µg/ml of RNA carrier (Cepero et al., 2014). The plates were then processed in the same way as those with the BB samples (Section 2.5.1) and again, one well with no sample was included with every 20 samples as a negative control.

2.5.3. Adult bee samples

Similarly, the abdomen of each adult bee (IB and FB) was placed individually into a well of a Deepwell-96 plate on ice, which contained 180 µl of 50% AL buffer and 1 µg/ml of RNA carrier (Cepero et al., 2014). The plates were then stored at −80 °C until further processing and again, one in every 20 wells was left with no sample and processed in parallel as a negative control. After maceration, the DNA was extracted from the adult samples collected in Assay 1 as indicated in Section 2.5.1. However, DNA was extracted from the adult bees from Assay 2 using a different method, transferring 50 µl of the bee macerate to a new multiwell plate with 50 µl of a Tris–HCl solution (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and 15 µl of Proteinase K (Qiagen®, No. 1019499), and incubating for 20 min at 95 °C. This method has a similar limit of detection for *Nosema* spp. as that used for the samples from Assay 1 but it is quicker and cheaper (Rodríguez-García, C., Higes, M., De La Rua, P., Martín-Hernández, R., 2014. Comparative analysis of different methods for gDNA extraction and detection of *Nosema ceranae*, *Nosema apis* and Nosema bombi. In: Pilar de la Rúa (Ed.), Proceedings of the Sixth European Conference of Apidologie, Universidad de Murcia, Spain pp. 211–212. http://www.eurbee.org/Files/book%20of%20abstracts%20Eurbee6.pdf). Negative controls were run in parallel for each step of the two assays.

2.6. Molecular detection of Nosema spp. (PCR): BB, adult bees and brood cell samples

The presence of *N. apis* and *N. ceranae* spp. was assessed in all the samples using a triplex conventional PCR as described previously (Martín-Hernández et al., 2012), including the detection of *A. mellifera* DNA as an internal control (IPC) for the PCRs. All the PCRs were carried out in a volume of 25 µL containing the HotSplit DNA polymerase (1 U/µl: Biotools®, Madrid, Spain, cod. 10.531–10.533), 0.4 µM of each primer, 0.2 mg/ml of BSA, 0.1% Triton X-100 and 2.5 µL of DNA template in customized gelified plates (Biotools®). These reaction conditions had the same sensitivity as that described previously (Martín-Hernández et al., 2012): 2.5 spores of *N. ceranae* and/or 25 spores of *N. apis* in 150 µl of bee macerate. All reactions were carried out in a Mastercycler® ep gradient S (Eppendorf) and analyzed in a QIAxcel Advanced System (Qiagen®) using a QIAxcel DNA Screening Kit (Qiagen®, No. 929004). The AM420 method was applied (sample uptake 10 s at 5 KV, separation 420 s at 5 KV), with QX Alignment Markers 15 bp/1 kb (Qiagen®, No. 929521) and QX Size Markers 50–800 pb at 20 ng/ µl (Qiagen®, No. 929556). The electropherograms were analyzed with BioCalculator 3.0 (Qiagen®), and all samples that generated a fragment of the expected length and yielded ≥0.1 relative fluorescent units (RFU) were considered to be positive. Positive controls and no template controls were analyzed in parallel to detect possible contamination or amplification failures.

2.7. Multiplication of Nosema spp. in a BB (real-timePCR (RT-PCR))

The brood samples that proved positive for *Nosema* spp. by conventional tripex-PCR were analyzed by real-time-PCR (RT-PCR) to determine whether the detection did indeed correspond to an active infection (*Nosema* spp. multiplication) or if it was merely due to the presence of inactive spores or contamination. The cDNA from *Nosema* spp. positive brood samples was analyzed to detect the expression of genes from these microsporidians. The single-copy gene selected for *N. ceranae* encodes the polar tubule protein 3 (*PTP3*) and for *N. apis*, the largest subunit of RNA polymerase II (*RPB1*). Both genes were selected since they have been related to sporogonial phases (Brosson et al., 2006) or transcription (Katinka et al., 2001) and protein maturation processes (Belkorchia et al., 2017), and are therefore related to microsporidian multiplication.

To select the conserved areas for primer design, the sequences available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) were aligned using BioEdit® Sequence Alignment Editor software (Hall, 1999), and the primers and probes for *N. ceranae-PTP3* and *N. apis-RPB1* were designed using the Universal ProbeLibrary Assay Design Center (https://lifescience.roche.com/en_es/brands/universal-probe-library.html#assay-design-center). The specificity of primers was initially in silico verified by the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines were followed for the RT-PCR design (Bustin et al., 2009). The honey bee *β-actin* gene was also assayed in each sample as an internal control of mRNA preservation and cDNA synthesis, using the primers and probes described previously (Martín-Hernández et al., 2017). Controls with no template control (NTC) or with no reverse transcription (RTC) were run in parallel. The sequences of the primers and probes used in this study and the amplicon sizes are shown in Table 3.

All RT-PCRs were performed on a Roche LightCycler® 480 thermocycler using 20 µl reaction mixes with the LightCycler® 480 Probes Master Mix (Roche), according to the manufacturer's instructions: primers at 500 nM and universal probes at 10 nM (UPL, Roche®). The PCR program consisted of an initial step of 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s each, and a final cooling step at 40 °C for 30 s. Each amplification cycle was analyzed with the LightCycler® 480 software v1.5.1 (Roche Diagnostics) and the crossing point (Cp) was determined by the second derivative maximum of each curve, and it was recorded for all samples. Any Cp value over the threshold level was interpreted as multiplication of either *N. ceranae* or *N. apis*, while nil Cp values were considered to indicate uninfected BBs and the absence of microsporidian replication. Negative and positive controls were also included.

2.8. Validation of Nosema spp. multiplication

To test the reliability of the technique detecting the *Nosema* spp. multiplication (active infection; see Section 2.7), environmental spores of *N. ceranae* ($n = 6$) and *N. apis* ($n = 5$) were analysed. The *N. ceranae* spores came from naturally infected experimental colonies and *N. apis* spores came from the stocks kept at 4 °C in the CIAPA. Spores were purified by 95% isotonic-Percoll® and the

Table 3
Real Time PCR primers and probes used in this study to detect active multiplication of *Nosema* spp. in naturally infected bee brood samples: *PTP3*, the polar tubule protein 3 gene of *Nosema ceranae*; *RPB1*, the largest subunit of the RNA *polymerase II* gene of *Nosema apis*.

Target organism	Primer	Sequence (5′–3′)	Reference	Amplicon length	GenBank reference
N. ceranae	PTP3	F: TGCTGATGTTATGGCTACAGAAG R: TACAGATTGCGCTGCTTTAA P: GCCAGGAA (UPL #72)	This study	90 bp	XM_002996713.1. *Nosema ceranae* BRL01 hypothetical protein (NCER_100083) mRNA, complete coding sequence
N. apis	RPB1	F: CCCGAATGGTTAATTTTGACTG R: TCCCTCCATAACAATAGAAGGTCT P: CTCCTCCT (UPL #73)	This study	71 bp	DQ996230.1. *Nosema apis* RNA polymerase II largest subunit (RPB1) gene, partial coding sequence
Apis mellifera	β-Actin	F: GTATGCCAACACTGTCCTTTCTG R: AAGAATTGACCCACCAATCCA P: CCTAGCACCATCCACCATGAAAATTAAGATCATC	Martín-Hernández et al., 2017		

species confirmed by PCR (Martín-Hernández et al., 2012). Spores were pelleted by centrifugation and they were processed for DNA extraction, DNase treatment and cDNA generation as described in Section 2.5.1. Controls for the extraction step were processed in parallel. In this case, all the samples were also processed with no reverse transcriptase enzyme (RTC) to later verify the DNase treatment had worked efficiently and no genomic DNA was present. All samples were analysed in duplicate and RT-PCRs were performed as described in Section 2.7. Additionally, the specificity of newly designed primers was tested in vitro by cross-analysis of *N. apis* DNA with NC-PTP3 primers and *N. ceranae* DNA with NA-RPB1 primers.

2.9. Parasitization levels and statistical analysis

The prevalence of BBs and adult bees (IBs and FBs) infected by *Nosema* spp. were analyzed for each assay (Assay 1 and 2) and sampling point (season: pre-clustering and post-clustering). The average infection in adult bees was calculated by determining the mean of the IBs and FBs in each colony. The distribution of the prevalence of BB microsporidians at each point analyzed (four levels: year and season) was compared, employing non-parametric tests (Kruskal–Wallis test), since the basal assumptions of parametric tests (normality and constant variance) were not satisfied.

The colonies were divided in two groups according to whether the colony had been detected with BBs positive or negative to *N. ceranae* infection. The level of infection in adult bees was compared between both groups using the Mann–Whitney test. $P < 0.05$ was considered significant and all statistical analyses were carried out using IBM SPSS Statistics software V24.

3. Results

3.1. Prevalence of Nosema spp. in brood

Nosema ceranae was found more frequently than *N. apis* in brood of *A. mellifera iberiensis*.

3.1.1. Detection by conventional PCR

Brood samples from all stages of development were analyzed in this study ($n = 1,433$, Table 4), from recently sealed larvae (L; 2–3 days pct) up to pre-emergent adults (P6; 12 days pct). All NTC controls analyzed by conventional PCR were negative. The distribution of the samples for each developmental phase differed due to the random selection of the brood. Of the 1433 brood samples analyzed, amplification of the IPC indicated good DNA extraction and conservation in 1418 and thus, only these samples were analyzed further. Of these 1418 samples, *Nosema* spp. were detected in 66 by triplex-PCR (see Tables 4 and 5), most frequently *N. ceranae*. This species was found in all immature stages ($n = 50$, Table 4), and pre- and post-clustering, with the highest prevalence at the L stage (6.41%). *Nosema apis* was detected alone in L and P2 stages ($n = 6$), although at levels below 1%, whereas *N. ceranae* and *N. apis* were detected together in L, P1 and P6 stages ($n = 10$), with the highest prevalence at P6 (1.72%).

The annual prevalence was assessed according to clustering (pre- or post-clustering, Table 5) and the relative detection of *N. ceranae* pre-clustering was very low in both assays. No immature stages were positive for *Nosema* spp. post-clustering in Assay 1 and only two colonies were still alive. However, the highest prevalence for *N. ceranae* was detected post-clustering in Assay 2, with a positive result in 10.45% of the BBs. This sampling point was also the only point when *N. apis* was detected, either alone (1.36%) or together with *N. ceranae* (1.82%).

3.1.2. Nosema spp. multiplication (RT-PCR)

Table 4 shows the number of positives and the prevalence of *Nosema* infection according to the larvae/pupae age. All brood samples analyzed by RT-PCR showed a correct amplification of the honey bee *β-actin* gene, showing the integrity of mRNA. The expression of the *N. ceranae-PTP3* or *N. apis-RPB1* gene was analyzed in the 66 brood samples in which *Nosema* spp. had been detected by conventional PCR. Multiplication of microsporidians was confirmed in only 16 of these samples (24%), of which 15 were positive for *N. ceranae* at a prevalence which was always lower than 2% (L, P1, P3, P4, P5 and P6 stages; range 1.12–1.60%; Table 4; Supplementary Table S1). Conversely, active multiplication of *N. apis* was only detected in one larva that was co-infected by *N. ceranae* (L stage, 0.18%, Table 4). Indeed, this was the only confirmed case of *N. apis* multiplication in immature stages in the 2 years of study, and only when accompanied by *N. ceranae* in a co-infection. All samples showed high Cp values (>34 cycles; Supplementary Table S1) although the extraction controls, RTC and NTC were negative, indicating the absence of any contamination or non-specific DNA amplification.

The annual prevalence of *Nosema* spp. in BBs (Table 5) was also calculated according to the total number of BBs analyzed in each season (pre- and post-clustering). The highest multiplication rate (2.27%) was observed for *N. ceranae* post-clustering in Assay 2, when the highest level of detection (10.45%) was registered. The only larvae positive to *N. apis* and *N. ceranae* (Table 4) corresponded to Assay 2 in the post-clustering season.

Six out of 10 beehives were positive for *Nosema* spp. in BBs at some point during the study (four colonies out of nine were found infected in pre-clustering and three out of six post-clustering: Supplementary Table S2) and the infection was only persistent in the BBs of a single colony (H8) in Assay 2.

Table 4
Number of positive samples and percentage (in brackets) to *Nosema* spp. detected by conventional PCR+ (detection) and by Real Time (RT) PCR (multiplication), according to the developmental phase of the bee brood: L (capped stretched larva; 2–3 days pct); P1 (white eye pupa stage; 4 days pct); P2 (pink eye stage; 5–6 days pct); P3 (dark pink-purple eye pupa stage; 7–8 days pct); P4 (dark purple eye pupa stage; 9 days pct); P5 (black eye pupa; 10–11 days pct) and P6 (resting adult pre-emergence stage; 12 days pct). Co-infection indicates brood with *N. ceranae* and *N. apis* together.

		Bee brood stage							
		L	P1	P2	P3	P4	P5	P6	Total
	n	562	179	233	210	117	59	58	1418
Detection (PCR+)	*N. ceranae*	36 (6.41)	6 (3.35)	1 (0.43)	3 (1.43)	1 (0.85)	1 (1.69)	2 (3.45)	**50 (3.53)**
	N. apis	5 (0.89)	0 (0.00)	1 (0.43)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	**6 (0.42)**
	Co-infection	8 (1.42)	1 (0.56)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (1.72)	**10 (0.71)**
Multiplication (RT-PCR+)	*N. ceranae*	9 (1.60)	2 (1.12)	0 (0.00)	1 (0.48)	1 (0.85)	1 (1.69)	1 (1.72)	**15 (1.06)**
	N. apis	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	**0 (0.00)**
	Co-infection	1 (0.18)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	**1 (0.07)**

pct, post capping time.

Table 5
Percentage of brood samples positive for *Nosema* spp. detected by conventional PCR (detection) and by real time (RT) PCR (multiplication) according to the season (pre- and post-clustering). Co-infection indicates a brood infected with both *Nosema ceranae* and *Nosema apis*.

			Bee brood samples								
				Detection (% PCR+)				Multiplication (% RT-PCR+)			
Year	Hive ID	Season	*n*	*N. ceranae*	*N. apis*	Co-infection	No. positive samples	*N. ceranae*	*N. apis*	Co-infection	No. positive samples
2015–2016	H1–H5	Pre-clustering	447	0.45	0.00	0.00	2	0.45	0.00	0.00	2
		Post-clustering	172	0.00	0.00	0.00	0	–	–	–	0
2016–2017	H6–H10	Pre-clustering	359	0.56	0.00	0.56	4	0.84	0.00	0.28	4
		Post-clustering	440	10.45	1.36	1.82	60	2.27	0.00	0.00	10
2015–2017	**H1–H10**	**Study**	**1418**	**3.53**	**0.42**	**0.71**	**66**	**1.06**	**0.00**	**0.07**	**16**

The analysis of the prevalence in BBs pre-clustering and post-clustering adopted a non-normal distribution. The frequency of the samples displaying active multiplication of *N. apis* (co-infected with *N. ceranae)* was very low (one sample) so no further statistical analyses were performed. The non-parametric Kruskal–Wallis test proved that there were no significant differences in the prevalence of natural *N. ceranae* infections of BBs in the two assays (year of study), or seasonally (pre- or post-clustering, $P > 0.05$).

3.2. Detection of Nosema spp. DNA in the brood cells

A total of 164 cells were studied, corresponding to 20.32% of the total brood analyzed in Assay 2. As the results on the BBs lodged in those cells were also available, both sets of data were represented together (Fig. 1). Of 164 cell samples analyzed, *Nosema* spp. were only detected in 26 cells (15.9%), of which *N. ceranae* was found in 17 cells (10.4%) and *N. apis* in four cells (2.4%), with both species detected in five cells (3.1%). Moreover, 12 cells (7.3%) that were negative for *Nosema* spp. DNA had hosted eight larvae/pupae in which *N. ceranae* was detected only by conventional PCR (4.9%), and another four brood samples (2.4%) were positive following RT-PCR which indicated the multiplication of this microsporidian. All controls (extraction and NTC) were negative; *N. apis* and *N. ceranae* positive controls showed amplicons of the expected size.

3.3. Nosema spp. prevalence in adult bees

A total of 5700 adult worker bees were analyzed individually to determine the number of infected bees in each colony and at each sampling time point (Tables 1 and 2). As only 5650 adult bees (3348 IBs and 2302 FBs) were positive for the IPC, only these samples were analyzed further. Extraction controls PCR-NTC were negative, showing the absence of any contamination; *N. apis* and *N. ceranae* positive controls showed amplicons of the expected size. The mean prevalence of ABs in the colonies with BBs infected by *N. ceranae* was higher than in the colonies with BBs not infected, and these results were always observed, irrespective of whether they were analyzed pre- and post-clustering or if they were analyzed before, after or at the same time as brood comb sampling (Fig. 2). However those results were not statistically significant ($P > 0.5$).

3.4. Validation of Nosema spp. multiplication assay

Table 6 shows the results on DNA and cDNA obtained from *N. apis* and *N. ceranae* environmental spores and analyzed by RT-PCR. All the cDNA samples were negative, showing that the environmental spores of *N. apis* or *N. ceranae* demonstrated no activity of *RPB1* or *PTP3* genes, respectively. In addition, the same samples that had been processed with no reverse transcriptase enzyme (RTC) did not show any amplification, thus demonstrating that the DNase treatment had been efficient and no genomic DNA remained. Conversely, the DNA obtained from the same samples showed low amplification Cp values, thus confirming good preservation of spores (CIAPA stocks) used for this assay.

On the other hand, no cross-reaction was observed and NC-PTP3 primers specifically detected *N. ceranae* samples while NA-RPB1 primers detected only *N. apis* samples. NTCs were negative in all the analyses performed and positive controls indicated the right amplification in their respective reactions.

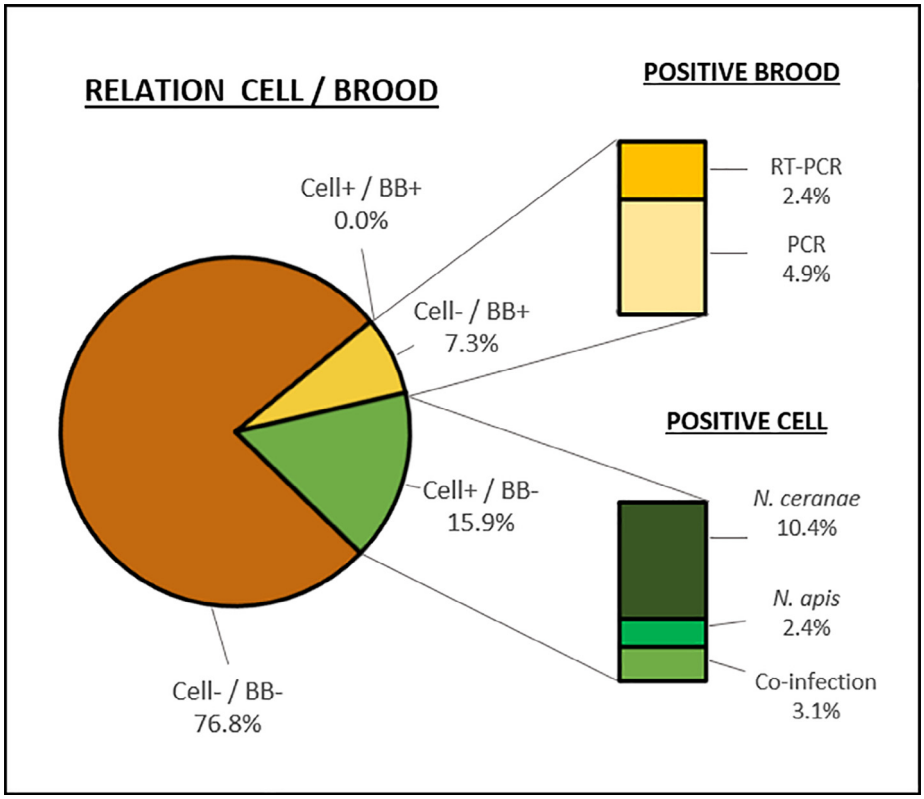

Fig. 1. Percentage of brood cells positive for *Nosema* spp. relative to the detection of *Nosema* spp. in the bee brood (BB). +, positive result; −, negative result. Positive brood: percentage of brood cells that were negative for *Nosema* spp., that were occupied with brood positive by PCR (*Nosema* spp. DNA detection) or by real-time-PCR (*Nosema* spp. multiplication). Positive cell: percentage of brood cells positive that were occupied with bee immature stages that had been found negative (uninfected brood).

4. Discussion

This work confirms that *Nosema* spp. infection occurs naturally in immature honey bees, as proposed previously based on laboratory studies (Eiri et al., 2015; Benvau and Nieh, 2017). Although nosemosis has been traditionally considered an adult disease since it was first described in *A. mellifera* (Zander, 1909; Bailey, 1955; Fries, 1988; Higes et al., 2006; Huang et al., 2007; Fries et al., 1996), there were a few descriptions of infection at immature stages that had been overlooked. The first report of microsporidians in brood was the detection of *N. apis* spores in the hemolymph of larvae (Gilliam and Shimanuki, 1967). Some years later "brood *Nosema*" infection of late larval or pupal honey bees was described in an apiary in South Africa (Buys, 1972, 1977; cited in Clark, 1980), believed to be a species distinct from *N. apis* which had not yet been confirmed. Many years later, *N. ceranae* was detected by PCR in immature drones, queens and workers (Traver and Fell, 2011, 2012; Rodríguez et al., 2014) and *N. apis* in worker pupae (Rodríguez et al., 2014), although data about their prevalence were not provided and their multiplication was not confirmed. Thus, to our knowledge, this is the first study reporting the prevalence of *N. ceranae* and *N. apis* infection in immature worker stages under natural conditions.

As expected, *N. ceranae* (3.53%) was found more frequently than *N. apis*, the latter being more frequent together with *N. ceranae* infection (0.71%) than alone (0.42%). The detection of RNAm of *N. ceranae-PTP3* or *N. apis-RPB1* genes proved to be a useful tool to confirm the multiplication of these microsporidians. Environmental spores are considered dormant stages with very low or even absent cellular activity (reviewed in Williams et al., 2014). In this work, spores failed to show any amplification by RT-PCR and consequently, the positive results obtained in BBs confirm the active multiplication of both *Nosema* spp., although the level of expression was low as reflected by the high Cp values observed (Supplementary Table S2). Indeed, *N. ceranae* multiplication was observed in 1.06% of the immature stages examined, while *N. apis* was only confirmed when the two species were co-infecting hosts (0.07%). *PTP3* gene expression, as with other spore-specific proteins, has been reported to be synthesized during the second half of sporogony during *Encephalitozoon cuniculi* infection (Brosson et al., 2006; Taupin et al., 2006; Grisdale et al., 2013). On the other hand, *RPB1* is directly related to the transcription of protein-coding genes (Katinka et al., 2001; Chymkowitch and Enserink, 2013) during the S phase of the cell cycle and to the production of non-coding RNA (Belkorchia et al., 2017) involved in various crucial cellular processes.

It was previously shown that *N. ceranae* spores could develop intracellularly in midgut cells at the pre-pupal stage when the brood was experimentally infected (Eiri et al., 2015). However, *N. apis* had only been considered to develop successfully in adult bees (van den Eijnde and Vette, 1993, https://www.nev.nl/pages/publicaties/proceedings/nummers/04/205-208.pdf). Nevertheless, our data confirm that both microsporidians can develop an active infection in brood under natural conditions, although at low levels for *N. ceranae* and almost anecdotally for *N. apis*. In fact, microsporidian multiplication was evident from the youngest (L) to the oldest stages (P6) studied, and no relationship was found between the detection or transcription of *N. ceranae* and/or *N. apis* genes and the stage of bee development. Similarly, *N. ceranae* completed its full life cycle inside brood midgut cells of larvae of the same age as those tested here (L: Eiri et al., 2015), the tissue that will become the adult ventriculum (Carreck et al., 2013). This fact was also confirmed for *N. apis* via the transcription of the *RPB1* gene, as for the *N. ceranae PTP3* gene. Nevertheless, multiplication

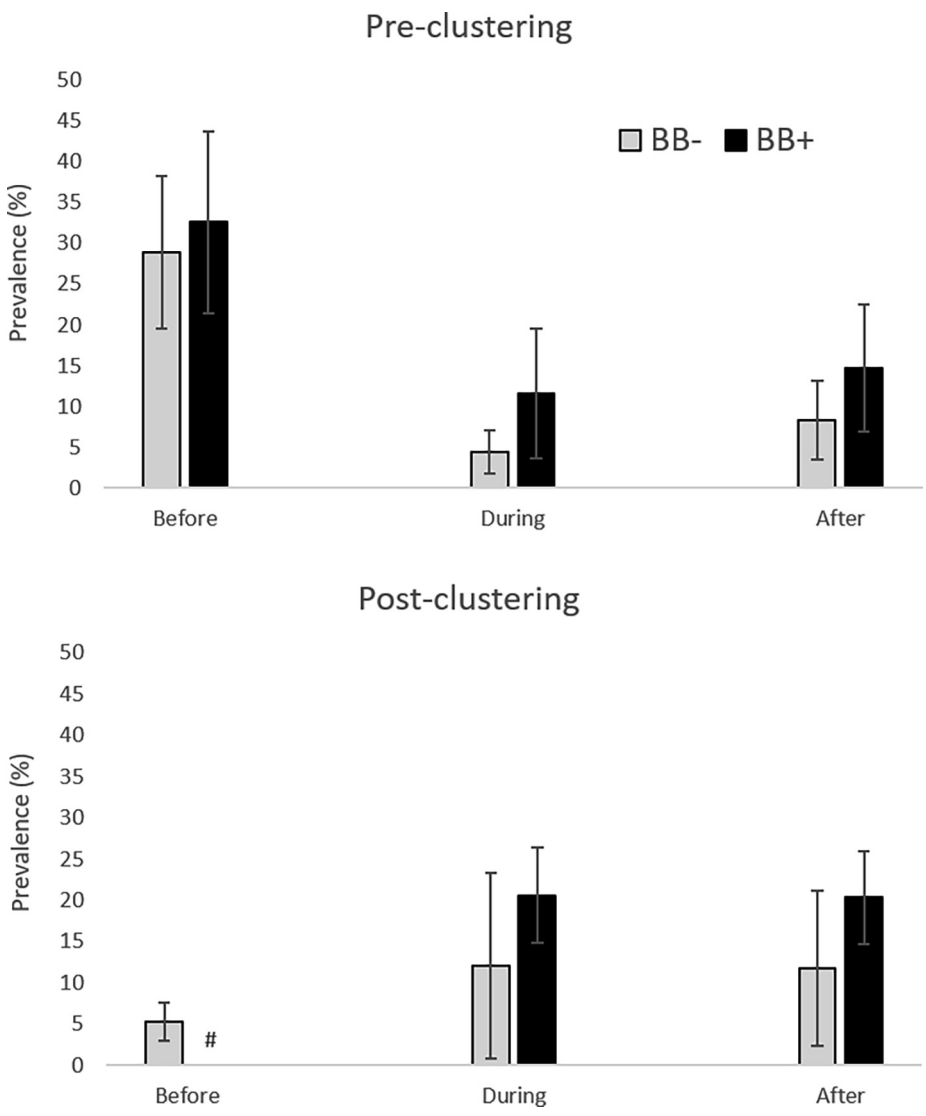

Fig. 2. Prevalence of *Nosema ceranae* infection in adult bees comparing colonies in which the bee brood (BB) was positive (+) or negative (−), grouped pre-clustering or post-clustering. a, no significant difference ($P > 0.05$). #, number of colonies available with infected BBs. Vertical lines indicate S.D.

Table 6
Validation of *Nosema ceranae* and *Nosema apis* multiplication assay by real-time-PCR (RT-PCR). DNA and RNA were extracted from environmental spores of *N. apis* and *N. ceranae*. *Cp [Crossing point]* values were obtained for DNA, cDNA and with no-retrotransciptase enzyme (RTC). O value indicates no amplification detected. Efficiency for the β-actin gene (91.4%) was determined using a previously described methodology (Martín-Hernández et al., 2017).

Gene	Sample	Cp values			RT-PCR efficiency
		DNA	cDNA	RTC	
PTP3	NC 1.1	27.24 ± 0.01	0	0	99.8%
	NC 1.2	27.11 ± 0.09	0	0	
	NC 1.3	32.02 ± 0.40	0	0	
	NC 1.4	32.18 ± 0.03	0	0	
	NC 3.1	24.89 ± 0.11	0	0	
	NC 3.3	29.81 ± 0.01	0	0	
RPB1	NA 1.2	21.09 ± 0.19	0	0	102%
	NA 1.3	22.65 ± 0.13	0	0	
	NA 1.4	21.51 ± 0.07	0	0	
	NA 3.2	27.60 ± 0.04	0	0	
	NA 3.3	35.19 ± 1.43	0	0	

C+, C−, positive and negative controls.

was only confirmed in the 24% of the samples in which *Nosema* spp. were identified by conventional PCR, indicating that infection by many of the microsporidians was apparently not successful (as the exhaustive method to process the brood ruled out surface contamination). This might have been because the germination of spores ingested by a brood did not actually occur (Bailey, 1955)

or because the *Nosema* spp. life cycle was stopped at some point, which could result in infection latency or spore dormancy (BenVau and Nieh, 2017). Some pathogenic consequences of brood infection on resulting mature adult bees have been reported (Eiri et al., 2015; Benvau and Nieh, 2017) that are detrimental to colony viability. These consequences of larval infection may include reduced longevity (Eiri et al., 2015), an altered sting morphology, and an increase in vitellogenin titers that could affect normal age polyethism and disturb the colony balance (Goblirsch et al., 2013; Lecocq et al., 2016; Benvau and Nieh, 2017). Microsporidian infection of brood stages is not rare as it has been confirmed for other species such as *N. bombi* infection of *B. terrestris* larvae (van den Eijnde and Vette, 1993, https://www.nev.nl/pages/publicaties/proceedings/nummers/04/205-208.pdf; Schmid-Hempel and Loosli, 1998) or *Nosema furnacalis* and *Nosema granulosis* infection of *L. sticticalis* larvae (Malysh et al., 2018). In fact, immature stages may be the only stages susceptible to infection by some species (van de Eijnde and Vette, 1993; Malysh et al., 2018), although once acquired, *N. ceranae* (Malysh et al., 2018) and *N. bombi* infection (van den Eijnde and Vette, 1993, https://www.nev.nl/pages/publicaties/proceedings/nummers/04/205-208.pdf) can also develop during adult stages.

Once brood infection was established, the brood cells were analyzed to test them as a possible means of transmission. The presence of *Nosema* spp. DNA was detected at low levels (15.9%), confirming the cells were contaminated and mainly with *N. ceranae*. However, none of the contaminated cells had been occupied by an infected brood, whereas some cells (7.3%) in which *Nosema* spp. DNA was not detected had hosted brood infected by *N. ceranae*, although only a few of these (2.7%) had a confirmed and active infection (positive by RT-PCR). As such, it would appear that contamination of the brood's immediate environment was not the main pathway of transmission, even when the prevalence of infected adult bees was quite high (reflecting a more intense contamination of the colony). *Nosema ceranae* spores can remain viable in wax cells (MacInnis C.I., 2017. *Nosema ceranae*: A sweet surprise? Investigating the viability and infectivity of the honey bee (*Apis mellifera* L.) parasite *N. ceranae*. MSc Thesis. University of Alberta, Canada. https://era.library.ualberta.ca/items/7b26607f-08fb-4e85-9f7a-0fbac0afee68/view/ccb5adfe-2b24-47c9-9ef6-79b69e598637/MacInnis_Courtney_I_201705_MSc.pdf) and they have been detected by PCR in royal jelly (Cox-Foster et al., 2007; Traver and Fell, 2012), which could be the main means of transmission to immature bees following exposure to the spores through brood food (Traver and Fell, 2012; Goblirsch, 2017).

Horizontal transmission of microsporidians is possible through different routes that include fecal–oral and oral–oral transmission, and the ingestion of contaminated food and water (Didier et al., 2004). The ingestion of spores via contaminated food, water and pollen by honey bees has already been confirmed (Fries, 1993; Higes et al., 2008b), through the exchange of food between bees or when they perform their cleaning duties (Bailey, 1955; Fries, 1993; Higes et al., 2009b; Smith, 2012). In fact, food exchange by nurse bees was probably the main source of infection here as they play a pivotal role in brood feeding (Crailsheim, 1990). Other routes have been suggested, such as vertical transmission of spores from the queen to BBs (Traver and Fell, 2011), and this possibility cannot be ruled out as no data about queen infection was collected here.

The prevalence of *Nosema* spp. infection in adult bees was assessed to determine whether the more infected adult bees, the greater the probability of infection of the brood. This effect might have been expected since the infected immature stages can survive to adulthood, their infection persisting after metamorphosis and when they become adults (Eiri et al., 2015; Benvau and Nieh, 2017). However, although Fig. 2 shows the higher level of infection in colonies where a BB was positive, this relationship could not be statistically confirmed. Consequently, more studies including a higher number of colonies should be developed to demonstrate this apparent pattern. Moreover, the spread of the pathogen from the newborn infected bees to their healthy nest mates may be very variable, depending on whether they engage in intensive interactions or are socially isolated, which influences the health status of the whole colony at different times of the year (Biganski et al., 2017).

According to the results presented here, *N. ceranae* and *N. apis* are not only an adult honey bee parasite, although there is little risk of infection of the worker brood under field conditions even though the level of infection in adult bees is relatively high. The consequences of brood infection are of veterinary interest and it will be necessary to determine the temporal evolution of such an infection, as well as the effect it may have on the epidemiology of the disease and on the evolution of the colonies.

Acknowledgements

The authors wish to thank V. Albendea, T. Corrales, M. Gajero, C. Uceta, M. Buendía, J. Almagro and J. García of the Honey Bee Pathology Laboratory, Spain for their technical support. A. Urbieta was supported by grant RTA2012-00076-C02-01 from The National Institute for Agricultural and Food Research and Technology (INIA, Spain). This work was supported by INIA; (Spain) and Fondo Europeo de Desarrollo Regional, European Union (FEDER) funds (RTA2015-00013-C03-01 and RTA2012-00076-C02-01). This funding had no influence on the content of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.04.002.

References

Bailey, L., 1955. The infection of the ventriculus of the adult honey bee by *Nosema apis* (Zander). Parasitology 45, 86–94. https://doi.org/10.1017/S0031182000027451.

Bass, D., Czech, L., Williams, B.A.P., Berney, C., Dunthorn, M., Mahé, F., Torruella, G., Stentiford, G.D., Williams, T.A., 2018. Clarifying the relationships between microsporidia and cryptomycota. J. Eukaryot. Microbiol. 65 (6), 773–782. https://doi.org/10.1111/jeu.12519.

Bekele, A.Z., Mor, S.K., Phelps, N.B.D., Goyal, S.M., Armién, A.G., 2015. A case report of *Nosema ceranae* infection in honey bees in Minnesota, USA. Vet. Q. 35, 48–50. https://doi.org/10.1080/01652176.2014.981766.

Belkorchia, A., Pombert, J.F., Polonais, V., Parisot, N., Delbac, F., Brugère, J.F., Peyret, P., Gaspin, C., Peyretaillade, E., 2017. Comparative genomics of microsporidian genomes reveals a minimal non-coding RNA set and new insights for transcription in minimal eukaryotic genomes. DNA Res. 24, 251–260. https://doi.org/10.1093/dnares/dsx002.

Benvau, L.R., Nieh, J.C., 2017. Larval honey bees infected with *Nosema ceranae* have increased vitellogenin titers as young adults. Sci. Rep. 7, 1–8. https://doi.org/10.1038/s41598-017-14702-4.

Biganski, S., Kurze, C., Müller, M.Y., Moritz, R.F.A., 2017. Social response of healthy honeybees towards *Nosema ceranae*-infected workers: care or kill? Apidologie. https://doi.org/10.1007/s13592-017-0557-8.

Botías, C., Martín-Hernández, R., Meana, A., Higes, M., 2012a. Critical aspects of the Nosema spp. diagnostic sampling in honey bee (*Apis mellifera* L.) colonies. Parasitol. Res. 110, 2557–2561. https://doi.org/10.1007/s00436-011-2760-2.

Botías, C., Martín-Hernández, R., Garrido-Bailón, E., González-Porto, A., Martínez-Salvador, A., De La Rúa, P., Meana, A., Higes, M., 2012b. The growing prevalence of *Nosema ceranae* in honey bees in Spain, an emerging problem for the last decade. Res. Vet. Sci. 93, 150–155. https://doi.org/10.1016/j.rvsc.2011.08.002.

Brosson, D., Kuhn, L., Delbac, F., Garin, J., Vivarès, C.P., Texier, C., 2006. Proteomic analysis of the eukaryotic parasite *Encephalitozoon cuniculi* (microsporidia): a reference map for proteins expressed in late sporogonial stages. Proteomics 6, 3625–3635. https://doi.org/10.1002/pmic.200500796.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Buys, B., 1977. A nosema disease aVecting honeybee brood. In: Colibaba, S. (Ed.), Apimondia Symposium of Bee Biology and Pathology. Merelbeke, Belgium, pp. 73–76.

Calderón, R.A., Sanchez, L.A., Yañez, O., Fallas, N., 2008. Presence of *Nosema ceranae* in africanized honey bee colonies in costa rica. J. Apic. Res. 47, 328–329. https://doi.org/10.1080/00218839.2008.11101485.

Carreck, N.L., Andree, M., Brent, C.S., Cox-Foster, D., Dade, H.A., Ellis, J.D., Hatjina, F., VanEnglesdorp, D., 2013. Standard methods for Apis mellifera anatomy and dissection. In V. Dietemann, J.D. Ellis, P. Neumann (Eds) The COLOSS BEEBOOK, Volume I: standard methods for Apis melliferaresearch. J. Apic. Res. 52, 1–40. https://doi.org/10.3896/IBRA.1.52.4.03.

Cepero, A., Ravoet, J., Gómez-Moracho, T., Bernal, J.L., Del Nozal, M.J., Bartolomé, C., Maside, X., Meana, A., González-Porto, A.V., De Graaf, D.C., Martín-Hernández, R., Higes, M., 2014. Holistic screening of collapsing honey bee colonies in Spain: a case study. BMC Res. Notes 7. https://doi.org/10.1186/1756-0500-7-649.

Cepero, A., Martín-Hernández, R., Bartolomé, C., Gómez-Moracho, T., Barrios, B., Bernal, J., Martín, M.T., et al., 2016. Passive laboratory surveillance in Spain: pathogens as risk factors for honey bee colony collapse. J. Apicult. Res. 54, 525–531. https://doi.org/10.1080/00218839.2016.1162978.

Chemurot, M., De Smet, L., Brunain, M., De Rycke, R., de Graaf, D.C., 2017. Nosema neumanni n. sp. (Microsporidia, Nosematidae), a new microsporidian parasite of honeybees, *Apis mellifera* in Uganda. Eur. J. Protistol. 61, 13–19. https://doi.org/10.1016/j.ejop.2017.07.002.

Chen, Y., Evans, J.D., Smith, I.B., Pettis, J.S., 2008. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J. Invertebr. Pathol. 97, 186–188. https://doi.org/10.1016/j.jip.2007.07.010.

Chen, Y., Evans, J.D., Zhou, L., Boncristiani, H., Kimura, K., Xiao, T., Litkowski, A.M., Pettis, J.S., 2009. Asymmetrical coexistence of *Nosema ceranae* and *Nosema apis* in honey bees. J. Invertebr. Pathol. 101, 204–209. https://doi.org/10.1016/j.jip.2009.05.012.

Chen, Y.P., Huang, Z.Y., 2010. *Nosema ceranae*, a newly identified pathogen of *Apis mellifera* in the USA and Asia. Apidologie 41, 364–374. https://doi.org/10.1051/apido/2010021.

Chymkowitch, P., Enserink, J.M., 2013. The cell cycle rallies the transcription cycle Cdc28/Cdk1 is a cell cycle-regulated transcriptional CDK. Transcription 4, 3–6. https://doi.org/10.4161/trns.22456.

Clark, T.B., 1980. A second microsporidian in the honeybee. J. Invertebr. Pathol. 35, 290–294. https://doi.org/10.1016/0022-2011(80)90164-0.

Copley, T.R., Chen, H., Giovenazzo, P., Houle, E., Jabaji, S.H., 2012. Prevalence and seasonality of *Nosema* species in Québec honey bees. Can. Entomol. 144, 577–588. https://doi.org/10.4039/tce.2012.46.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V., VanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S.K., Simons, J.F., Egholm, M., Pettis, J.S., Lipkin, W.I., 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318, 283–287. https://doi.org/10.1126/science.1146498.

Crailsheim, K., 1990. The protein balance of the honey bee worker. Apidologie 21, 417–429. https://doi.org/10.1051/apido:19900504.

Didier, E.S., Vossbrinck, C.R., Stovall, M.E., Green, L.C., Bowers, L., Fredenburg, A., Didier, P.J., 2004. Diagnosis and epidemiology of microsporidia infections in humans. Southeast Asian. J. Trop. Med. Public Health 35, 65–81 https://www.scopus.com/record/display.uri?eid=2-s2.0-40149108497&origin=inward.

Eiri, D.M., Suwannapong, G., Endler, M., Nieh, J.C., 2015. *Nosema ceranae* can infect honey bee larvae and reduces subsequent adult longevity. PLoS One 10, 1–17. https://doi.org/10.1371/journal.pone.0126330.

European Commission, 2017. EU Pollinators Initiative: Roadmap 1–3. Ref. Ares (2017)5895634 – 01/12/2017.

Fries, I., 1988. Infectivity and multiplication of *Nosema apis* Z. in the ventriculus of the honey bee. Apidologie 19, 319–328. https://doi.org/10.1051/apido:19880310.

Fries, I., 1993. *Nosema apis*—A parasite in the honey bee colony. Bee World 74 (1), 5–19. https://doi.org/10.1080/0005772X.1993.11099149.

Fries, I., 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). J. Invertebr. Pathol. 103, S73–S79. https://doi.org/10.1016/j.jip.2009.06.017.

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. https://doi.org/10.1016/S0932-4739(96)80059-9.

Fries, I., Slemenda, S.B., da Silva, A., Pieniazek, N.J., 2003. African honey bees (*Apis mellifera* scutellata) and nosema (*Nosema apis*) infections. J. Apic. Res. 42, 13–15. https://doi.org/10.1080/00218839.2003.11101080.

Galindo, L.J., Torruella, G., Moreira, D., Timpano, H., Paskerova, G., Smirnov, A., Nassonova, E., López-García, P., 2018. Evolutionary genomics of *Metchnikovella incurvata* (Metchnikovellidae): an early branching microsporidium. Genome Biol Evol. 10 (10), 2736–2748. https://doi.org/10.1093/gbe/evy205.

Giersch, T., Berg, T., Galea, F., Hornitzky, M., 2009. *Nosema ceranae* infects honey bees (*Apis mellifera*) and contaminates honey in Australia. Apidologie 40, 117–123. https://doi.org/10.1051/apido/2008065.

Gilliam, M., Shimanuki, H., 1967. In vitro phagocytosis of *Nosema apis* spores by honeybee hemocytes. J. Invertebr. Pathol. 9, 387–389. https://doi.org/10.1016/0022-2011(67)90074-2.

Gisder, S., Schüler, V., Horchler, L.L., Groth, D., Genersch, E., 2017. Long-term temporal trends of Nosema spp. infection prevalence in Northeast Germany: continuous spread of *Nosema ceranae*, an emerging pathogen of honey bees (*Apis mellifera*), but no general replacement of *Nosema apis*. Front. Cell. Infect. Microbiol. 7, 1–14. https://doi.org/10.3389/fcimb.2017.00301.

Goblirsch, M., 2017. *Nosema ceranae* disease of the honey bee (*Apis mellifera*). Apidologie. https://doi.org/10.1007/s13592-017-0535-1.

Goblirsch, M., Huang, Z.Y., Spivak, M., 2013. Physiological and behavioral changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection. PLoS One 8. https://doi.org/10.1371/journal.pone.0058165.

Grisdale, C.J., Bowers, L.C., Didier, E.S., Fast, N.M., 2013. Transcriptome analysis of the parasite *Encephalitozoon cuniculi*: an in-depth examination of pre-mRNA splicing in a reduced eukaryote. BMC Genomics 14, 207. https://doi.org/10.1186/1471-2164-14-207.

Hall, A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98. https://doi.org/10.12691/ajidm-4-3-3. http://brownlab.mbio.ncsu.edu/JWB/papers/1999Hall1.pdf.

Hatjina, F., Tsoktouridis, G., Bouga, M., Charistos, L., Evangelou, V., Avtzis, D., Meeus, I., Brunain, M., Smagghe, G., De Graaf, D.C., 2011. Polar tube protein gene diversity among *Nosema ceranae* strains derived from a Greek honey bee health study. J. Invertebr. Pathol. 108, 131–134. https://doi.org/10.1016/j.jip.2011.07.003.

Higes, M., Martín, R., Meana, A., 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J. Invertebr. Pathol. 92, 93–95. https://doi.org/10.1016/j.jip.2006.02.005.

Higes, M., García-Palencia, P., Martín-Hernández, R., Meana, A., 2007. Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). J. Invertebr. Pathol. 94, 211–217. https://doi.org/10.1016/j.jip.2006.11.001.

Higes, M., Martín-Hernández, R., Garrido-Bailón, E., García-Palencia, P., Meana, A., 2008b. Detection of infective *Nosema ceranae* (Microsporidia) spores in corbicular pollen of forager honeybees. J. Invertebr. Pathol. 97, 76–78. https://doi.org/10.1016/j.jip.2007.06.002.

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E.G., González-Porto, A.V., Barrios, L., Del Nozal, M.J., Bernal, J.L., Jiménez, J.J., Palencia, P.G., Meana, A., 2008a. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ. Microbiol. 10, 2659–2669. https://doi.org/10.1111/j.1462-2920.2008.01687.x.

Higes, M., Martín-Hernández, R., Garrido-Bailón, E., Botías, C., Meana, A., 2009a. The presence of *Nosema ceranae* (Microsporidia) in North African honey bees (*Apis mellifera* intermissa). J. Apic. Res. 48, 217–219. https://doi.org/10.3896/IBRA.1.48.3.12.

Higes, M., Martín-Hernández, R., García-Palencia, P., Marín, P., Meana, A., 2009b. Horizontal transmission of *Nosema ceranae* (Microsporidia) from worker honeybees to queens (*Apis mellifera*). Environ. Microbiol. Rep. 1, 495–498. https://doi.org/10.1111/j.1758-2229.2009.00052.x.

Higes, M., Martín-Hernández, R., Meana, A., 2010. *Nosema ceranae* in Europe: an emergent type C nosemosis. Apidologie 41, 375–392. https://doi.org/10.1051/apido/2010019.

Huang, W.F., Bocquet, M., Lee, K.C., Sung, I.H., Jiang, J.H., Chen, Y.W., Wang, C.H., 2008. The comparison of rDNA spacer regions of *Nosema ceranae* isolates from different hosts and locations. J. Invertebr. Pathol. 97, 9–13. https://doi.org/10.1016/j.jip.2007.07.001.

Huang, W.-F., Jiang, J.-H., Chen, Y.-W., Wang, C.-H., 2007. A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. Apidologie 38, 30–37. https://doi.org/10.1051/apido.

Huang, W.F., Solter, L.F., 2013. Comparative development and tissue tropism of *Nosema apis* and *Nosema ceranae*. J. Invertebr. Pathol. 113, 35–41. https://doi.org/10.1016/j.jip.2013.01.001.

Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellis, J., Forsgren, E., Fries, I., Hatjina, F., Hu, F.L., Jaffé, R., Köhler, A., Pirk, C W W., Rose, R., Strauss, U., Tanner, G., Tarpy, D R., Van Der Steen, J J M., Vejsnæs, F., Williams, G R., Zheng, H.Q., 2013. Miscellaneous standard methods for Apis mellifera research. In V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS BEEBOOK, Volume I: standard methods for Apis mellifera research. Journal of Apicultural Research 52, 1-53. http://dx.doi.org/10.3896/IBRA.1.52.4.10.

Invernizzi, C., Abud, C., Tomasco, I.H., Harriet, J., Ramallo, G., Campá, J., Katz, H., Gardiol, G., Mendoza, Y., 2009. Presence of *Nosema ceranae* in honeybees (*Apis mellifera*) in Uruguay. J. Invertebr. Pathol. 101, 150–153. https://doi.org/10.1016/j.jip.2009.03.006.

IPBES (Intergovernmental Platform on Biodiversity and Ecosystem Services), 2016. Summary for policymakers of the assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production. S.G. Potts, V. L. Imperatriz-Fonseca, H. T. Ngo, J. C. Biesmeijer, T. D. Breeze, L. V. Dicks, L. A. Garibaldi, R. Hill, J. Settele, A. J. Vanbergen, M. A. Aizen, S. A. Cunningham, C. Eardley, B. M. Freitas, N. Gallai, P. G. Kevan, A. Kovács-Hostyánszki, P. K. Kwapong, J. Li, X. Li, D. J. Martins, G. Nates-Parra, J. S. Pettis, R. Rader, and B. F. Viana (Eds.). Secretariat of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, Bonn, Germany. 36 pages.

James, T.Y., Pelin, A., Bonen, L., Ahrendt, S., Sain, D., Corradi, N., Stajich, J.E., 2013. Shared signatures of parasitism and phylogenomics unite Cryptomycota and microsporidia. Curr. Biol. 23 (16), 1548–1553. https://doi.org/10.1016/j.cub.2013.06.057.

Katinka, M.D., Duprat, S., Cornillott, E., Méténler, G., Thomarat, F., Prensier, G., Barbe, V., Peyretaillade, E., Brottier, P., Wincker, P., Delbac, F., El Alaoul, H., Peyret, P., Saurin, W., Gouy, M., Weissenbach, J., 2001. Genome sequence and gene compaction of the eukaryote parasite Encephalitozoon cuniculi. Nature 414, 450–453. https://doi.org/10.1038/35106579.

Khezri, M., Moharrami, M., Modirrousta, H., Torkaman, M., Salehi, S., Rokhzad, B., Khanbabai, H., 2018. Molecular detection of *Nosema ceranae* in the apiaries of Kurdistan province, Iran. Vet Res Forum. 9 (3), 273–278 http://vrf.iranjournals.ir/article_32086.html.

Khoury, D.S., Myerscough, M.R., Barron, A.B., 2011. A quantitative model of honey bee colony population dynamics. PLoS One 6,. https://doi.org/10.1371/journal.pone.0018491 e18491.

Khoury, D.S., Barron, A.B., Myerscough, M.R., 2013. Modelling food and population dynamics in honey bee colonies. PLoS One 8, (5). https://doi.org/10.1371/journal.pone.0059084 e59084.

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. https://doi.org/10.1016/j.jip.2007.02.014.

Lecocq, A., Jensen, A.B., Kryger, P., Nieh, J.C., 2016. Parasite infection accelerates age polyethism in young honey bees. Sci. Rep. 6, 1–11. https://doi.org/10.1038/srep22042.

Li, J., Qin, H., Wu, J., Sadd, B.M., Wang, X., Evans, J.D., Peng, W., Chen, Y., 2012. The prevalence of parasites and pathogens in Asian honeybees Apis cerana in China. PLoS One 7, 1–12. https://doi.org/10.1371/journal.pone.0047955.

Lodesani, M., Costa, C., Besana, A., Dall'Olio, R., Franceschetti, S., Tesoriero, D., Vaccari, G., 2014. Impact of control strategies for *Varroa destructor* on colony survival and health in northern and central regions of Italy. J. Apic. Res. 53, 155–164. https://doi.org/10.3896/IBRA.1.53.1.17.

Malysh, J.M., Ignatieva, A.N., Artokhin, K.S., Frolov, A.N., Tokarev, Y.S., 2018. Natural infection of the beet webworm Loxostege sticticalis L. (Lepidoptera: Crambidae) with three Microsporidia and host switching in *Nosema ceranae*. Parasitol. Res. 117, 3039. https://doi.org/10.1007/s00436-018-5987-3.

Martín-Hernández, R., Meana, A., Prieto, L., Martínez-Salvador, A., Garrido-Bailón, E., Higes, M., 2007. Outcome of colonization of *A. mellifera* by *N. ceranae*. Appl. Environ. Microbiol. 73 (20), 6331–6338. https://dx.doi.org/10.1128%2FAEM.00270-07.

Martín-Hernández, R., Meana, A., García-Palencia, P., Marín, P., Botías, C., Garrido-Bailón, E., Higes, M., 2009. Effect of temperature on the biotic potential of honeybee microsporidia. Appl. Environ. Microbiol. 75 (8), 2554–2557. https://doi.org/10.1128/AEM.02908-08.

Martín-Hernández, R., Botías, C., Bailón, E.G., Martínez-Salvador, A., Prieto, L., Meana, A., Higes, M., 2012. Microsporidia infecting *Apis mellifera*: coexistence or competition. Is *Nosema ceranae* replacing *Nosema apis*? Environ. Microbiol. 14, 2127–2138. https://doi.org/10.1111/j.1462-2920.2011.02645.x.

Martín-Hernández, R., Higes, M., Sagastume, S., Juarranz, Á., Dias-Almeida, J., Budge, G.E., Meana, A., Boonham, N., 2017. Microsporidia infection impacts the host cell's cycle and reduces host cell apoptosis. PLoS One 12, (2). https://doi.org/10.1371/journal.pone.0170183 e0170183.

Meana, A., Martín-Hernández, R., Higes, M., 2010. The reliability of spore counts to diagnose *Nosema ceranae* infections in honey bees. J. Apic. Res. 49 (2), 212–214. https://doi.org/10.3896/IBRA.1.49.2.12.

Ozgor, E., Celebier, I., Ulusoy, M., Keskin, N., 2017. First detection of *Nosema ceranae* and *Nosema apis* in greater wax moth *Galleria mellonella*. J. Apic. Sci. 61 (1). https://doi.org/10.1515/jas-2017-0015.

Özgör, E., Güzerin, E., Keskin, N., 2015. Determination and comparison of *Nosema apis* and *Nosema ceranae* in terms of geographic and climatic factors. Hacettepe J. Biol. Chem. 43, 9–15 https://doi.org/10.15671/HJBC.20154311200.

Paxton, R.J., 2010. Does infection by *Nosema ceranae* cause "Colony Collapse Disorder" in honey bees (*Apis mellifera*)? J. Apic. Res. 49, 80–84. https://doi.org/10.3896/IBRA.1.49.1.11.

Paxton, R.J., Klee, J., Korpela, S., Fries, I., 2007. *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie 38, 558–565. https://doi.org/10.1051/apido.

Porrini, C., Mutinelli, F., Bortolotti, L., Granato, A., Laurenson, L., Roberts, K., Gallina, A., Silvester, N., Medrzycki, P., Renzi, T., Sgolastra, F., Lodesani, M., 2016. The status of honey bee health in Italy: Results from the nationwide bee monitoring network. PLoS One 11, (5). https://doi.org/10.1371/journal.pone.0155411 e0155411.

Ravoet, J., Maharramov, J., Meeus, I., De Smet, L., Wenseleers, T., Smagghe, G., de Graaf, D.C., 2013. Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. PLoS One 8. https://doi.org/10.1371/journal.pone.0072443.

Rodríguez, M., Vargas, M., Antúnez, K., Gerding, M., Ovídio Castro, F., Zapata, N., 2014. Prevalence and phylogenetic analysis of honey bee viruses in the Biobío Region of Chile and their association with other honey bee pathogens. Chil. J. Agric. Res. 74, 170–177. https://doi.org/10.4067/S0718-58392014000200007.

Rodríguez, M., Vargas, M., Gerding, M., Navarro, H., Antúnez, K., 2012. Viral infection and Nosema ceranae in honey bees (Apis mellifera) in Chile. J. Apic. Res. 51, 285–287. https://doi.org/10.3896/IBRA.1.51.3.12.

Sánchez Collado, J.G., Higes, M., Barrio, L., Martín-Hernández, R., 2014. Flow cytometry analysis of *Nosema* species to assess spore viability and longevity. Parasitol. Res. 113, 1695–1701. https://doi.org/10.1007/s00436-014-3814-z.

Schmid-Hempel, P., Loosli, R., 1998. A contribution to the knowledge of *Nosema* infections in bumble bees, *Bombus* spp. Apidologie. 29, 525–535. https://doi.org/10.1051/apido:19980605.

Smith, M.L., 2012. The honey bee parasite Nosema ceranae: transmissible via food exchange? PLoS One 7 (8). https://doi.org/10.1371/journal.pone.

Stentiford, G.D., Becnel, J.J., Weiss, L.M., Keeling, P.J., Didier, E.S., Williams, B.A.P., Bjornson, S., Kent, M.L., Freeman, M.A., Brown, M.J.F., Troemel, E.R., Roesel, K., Sokolova, Y., Snowden, K.F., Solter, L., 2016. Microsporidia – emergent pathogens in the global food Chain. Trends. Parasitol. 32, 336–348. https://doi.org/10.1016/j.pt.2015.12.004.

Taupin, V., Méténier, G., Delbac, F., Vivarès, C.P., Prensier, G., 2006. Expression of two cell Wall proteins during the intracellular development of *Encephalitozoon cuniculi*: an immunocytochemical and *in situ* hybridization study with ultrathin frozen sections. Parasitology 132, 815–825. https://doi.org/10.1017/S0031182005009777.

Tokarev, Y.S., Zinatullina, Z.Y., Ignatieva, A.N., Zhigileva, O.N., Malysh, J.M., Sokolova, Y.Y., 2018. Detection of two microsporidia pathogens of the European honey bee *Apis mellifera* (Insecta: Apidae) in Western Siberia. Acta Parasitol. 63 (4), 728–732. https://doi.org/10.1515/ap-2018-0086.

Traver, B.E., Fell, R.D., 2011. *Nosema ceranae* in drone honey bees (*Apis mellifera*). J. Invertebr. Pathol. 107, 234–236. https://doi.org/10.1016/j.jip.2011.05.016.

Traver, B.E., Fell, R.D., 2012. Low natural levels of *Nosema ceranae* in *Apis mellifera* queens. J. Invertebr. Pathol. 110, 408–410. https://doi.org/10.1016/j.jip.2012.04.001.

Vanbergen, A.J., Initiative, I.P., 2013. Threats to an ecosystem service: pressures on pollinators. Front. Ecol. Environ 11, 251–259. https://doi.org/10.1890/120126.

van den Eijnde, J., Vette, N., 1993. Nosema infection in honey bees (Apis mellifera L.) and bumble bees (Bombus terrestris L.). Proc. Sect. Exp. Appl. Entomol. Neth. Entomol. Soc. 4, 205–208 https://www.nev.nl/pages/publicaties/proceedings/nummers/04/205-208.pdf.

Webster, T.C., 1993. *Nosema apis* spore transmission among honey bees. Am. Bee J. 133, 869–870.

Webster, T.C., Thacker, E.M., Pomper, K., Lowe, J., Hunt, G., 2008. *Nosema apis* infection in honey bee (*Apis mellifera*) queens. J. Apic. Res. 47, 53–57. https://doi.org/10.1080/00218839.2008.11101423.

Williams, G.R., Shafer, A.B.A., Rogers, R.E.L., Shutler, D., Stewart, D.T., 2008. First detection of *Nosema ceranae*, a microsporidian parasite of European honey bees (*Apis mellifera*), in Canada and central USA. J. Invertebr. Pathol. 97, 189–192. https://doi.org/10.1016/j.jip.2007.08.005.

Zander, E., 1909. Tierische Parasiten als Krankheitserreger bei der Biene. Leipziger Bienenztg 24, 147–150.