Spanish Journal of Agricultural Research
16(2), e0502, 10 pages (2018)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2018162-11474
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria O. A., M. P. (INIA)

RESEARCH PAPER

OPEN ACCESS

Epidemiological study of honeybee pathogens in Europe: The results of Castilla-La Mancha (Spain)

María Buendía[1], Raquel Martín-Hernández[1, 2], Concepción Ornosa[3], Laura Barrios[4], Carolina Bartolomé[5] and Mariano Higes[1]

[1] Centro de Investigación Apícola y Agroambiental (CIAPA), IRIAF, Marchamalo, 19180 Guadalajara, Spain. [2] Fundación Parque Científico y Tecnológico de Albacete, INCRECYT, Albacete, Spain. [3] Universidad Complutense de Madrid, Fac. Ciencias Biológicas, Dept. Biodiversidad, Ecología y Evolución, 28040 Madrid, Spain. [4] CSIC, SGAI. Dept. de Estadística, 28006 Madrid. Spain. [5] Universidade de Santiago de Compostela, CIMUS, IDIS, Medicina Xenómica, 15782 Santiago de Compostela. Spain.

Abstract

As a part of a Pilot Monitoring Program of honey bee health coordinated by the EURL (European Union Reference Laboratory) and according to the criteria established for Spain, 14 apiaries in Castilla-La Mancha were selected at random and sampled during the autumns of 2012-2014 to identify the most prevalent nosogenic agents, potentially those related to the honey bee colony collapse phenomenon. In all the apiaries studied, *Nosema ceranae* was the most prevalent pathogen detected over the three years, confirming the worldwide spread of this microsporidian, a pathogen that negatively affects honey bee health at an individual and colony level. Trypanosomatids were also very prevalent in honey bee colonies, although the majority of Trypanosomatids detected were not *Crithidia mellificae* but rather the genetically distinct *Lotmaria passim* lineage. We also detected *Varroa destructor* mites, and the particularly high prevalence in 2014 suggests a possible problem regarding mite control in field conditions that requires attention. In agreement with data from other regions, the BQCV and DWV were the most prevalent viruses in honey bee colonies and thus, the *Varroa*-DWV interaction may be an important cause of bee colony mortality. While there was little evidence of a relationship between the BQCV virus and *N. ceranae* under field conditions during 2012, this was not the case in 2013 and 2014. Finally, the AKI-complex or LSV-complex was not detected. The information obtained in this study should help orientate future plans for honey bee disease control.

Additional key words: *Apis mellifera*; monitoring program; *Nosema ceranae; Varroa destructor; Lotmaria passim*; honey bee viruses.

Abbreviations used: AKI (Acute bee paralysis, Kashmir bee and Israeli acute paralysis complex viruses); BQCV (Black Queen Cell Virus); CLM (Autonomous Community Castilla-La Mancha); DWV (Deformed Wing Virus); EURL (European Union Reference Laboratory); LSV (Lake Sinai Virus); PCR (Polymerase Chain Reaction).

Authors' contributions: Coordinating the research project: MH. Conceived and designed the study: MH, RMH. Performed the experiments: MH, MB, RMH, CB. Statistical analysis: LB. Analysed the data and wrote the paper: MH, MB, CO, CB, RMH.

Citation: Buendía, M.; Martín-Hernández, R.; Ornosa, C.; Barrios, L.; Bartolomé, C.; Higes, M. (2018). Epidemiological study of honeybee pathogens in Europe: The results of Castilla-La Mancha (Spain). Spanish Journal of Agricultural Research, Volume 16, Issue 2, e0502. https://doi.org/10.5424/sjar/2018162-11474

Received: 29 Mar 2017. **Accepted:** 11 May 2018

Funding: INIA-FEDER (RTA2013-00042-C10-06 and E-RTA2014-00003-C03).

Competing interests: None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. The authors declare that they have no competing interests.

Correspondence should be addressed to Mariano Higes: mhiges@jccm.es

Introduction

The honey bee, *Apis mellifera* Linnaeus, 1758, plays an essential role in the developmental cycle of a large number of plant species (Breeze *et al.*, 2011) and it has produced food (honey and pollen) for human consumption for thousands of years (Chauzat *et al.*, 2013). In the last decade, honey bees have been affected by serious health threats that have contributed to a substantial loss of honey bee colonies around the world (VanEngelsdrop & Meixner, 2010; Van der Zee *et al.*, 2014). Persistent honey bee colony losses may be most problematic in areas like Spain, where professional beekeepers are essentially dedicated to honey production. Indeed, Spain is the country with the greatest honey production in Europe (more than 29 thousand tons: MAGRAMA 2012, 2013), the largest number of honey bee colonies (more than 2.5 million on 01/05/2014: MAGRAMA, 2013) and the highest percentage of professional beekeepers (Chauzat *et al.*, 2013). For this reason, honey bee colony losses are a major concern in this country. Castilla-La Mancha is

one of the autonomous Spanish regions with the highest number of honey bee colonies (>155,000 colonies), and it is the fifth largest producer of honey in Spain. Indeed, "La Alcarria Honey" is produced in this area, the first honey to be awarded a European Protected Designation of Origin, highlighting the beekeeping tradition in the region and its essential impact on the agrarian structure in Spain.

A multitude of factors (viruses, mites, microsporidia, pesticides, etc.), either acting alone or in combination, have been associated with the worldwide decline in pollinators that causes premature colony mortality (Potts *et al.*, 2010; Ratnieks & Carreck, 2010; Runckel *et al.*, 2011; Cornman *et al.*, 2012; Francis *et al.*, 2013; Chen *et al.*, 2014; Simon-Delso *et al.*, 2014; Staveley *et al.*, 2014; Porrini *et al.*, 2016). However, the main drivers of colony collapse might differ in distinct geographical locations (Cepero *et al.*, 2014, 2016). While the most prevalent nosogenic agents related to the phenomenon of honey bee colony collapse have already been studied in Spain (see for example: Martín-Hernández *et al.*, 2007, 2012; Higes *et al.*, 2009, 2010a; Bernal *et al.*, 2010; Antúnez *et al.*, 2012; Garrido-Bailón, 2012; Botías *et al.*, 2013), it is vital to maintain a surveillance system to detect changes in the prevalence of such agents. In the present work, we report the prevalence of the major honey bee pathogens in 14 apiaries situated in the region of Castilla-La Mancha, and we investigated their potential role in the decline of honey bee health experienced in this area with such an important beekeeping tradition.

Material and methods

Sampling protocol

As part of the Pilot Monitoring Program of honey bee health coordinated by the EURL (European Union Reference Laboratory), 14 apiaries located in Castilla-La Mancha were selected at random and sampled during the autumn of 2012, 2013 and 2014 (September to November). The number of apiaries to be studied in this region was determined by the MAGRAMA criteria. In each apiary, a minimum of 8 and a maximum of 13 bee colonies were selected at random, and a total of 164 (2012), 151 (2013) and 123 (2014) worker honey bee samples were sent to the Centro de Investigación Apícola y Agroambiental (CIAPA) Honey Bee Pathology Laboratory (see Table 1 & Fig. 1). Each sample consisted of more than 300 adult worker bees for analysis and they arrived at our laboratory in perfect conditions (alive or frozen). Samples were taken during the mandatory application period of acaricides to varroosis control in Spain.

Varroa destructor analysis

To assess the presence of *Varroa destructor* Anderson & Trueman, 2000, in all worker honey bee samples from each colony in each apiary, we followed the EURL recommendations for the Epilobee program, adapted from the OIE (2008) methods. Each mite detected was collected individually and analyzed macroscopically to confirm the species, differentiating it from *Braula coeca* Nitzsch, 1818 or *Tropilaelaps clareae* Delfinado & Baker, 1962. A honey bee colony was considered infested with *V. destructor* when at least one *Varroa* mite was found in the sample. The rate of infestation of the bee colony was estimated by assessing the number of *Varroa* mites in relation with the number of adult bees in each sample (more than 300 in all cases) and it was expressed as the number of *Varroa* mites/100 bees/sample.

Nucleic acid extraction for pathogen detection

Each of the honey bee colonies that were sampled during the study were analysed to detect the main pathogens. To obtain nucleic acids, a sub-sample of 120 worker honey bees from each honey bee colonies was used. The remaining bees (more than 180) were kept frozen at -80ºC. Each sub-sample was macerated in AL buffer 50% (Qiagen) as described previously (Antúnez *et al.*, 2012; Cepero *et al.*, 2014), using sterile bags with a filter (BA6040 STRAINER BAGS) in a Stomacher 80 blender (Biomaster). The macerated bees were centrifuged at 3,000 rpm for 10 min, and the resulting pellets were used for DNA extraction and the supernatants for RNA extraction. Both the pellets and supernatants were stored at -20ºC prior to nucleic acid (DNA or RNA) extraction.

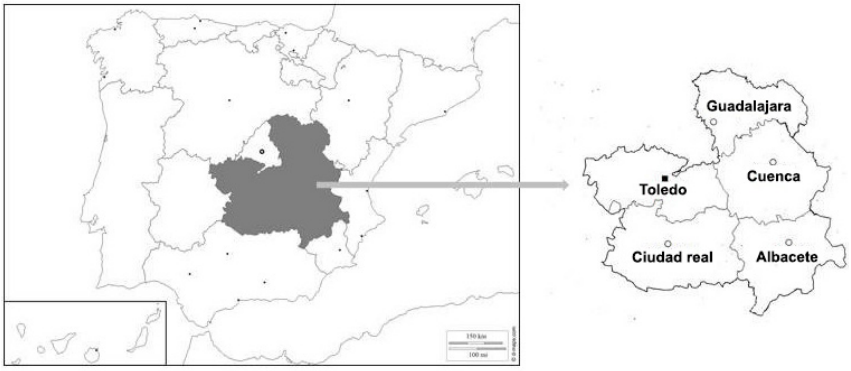
For DNA extraction, the pellets were resuspended in 3 mL milliQ H_2O and a 400 µL aliquot was transferred to a 96-well plate (Qiagen) with glass beads (2 mm diameter, Sigma) using disposable Pasteur pipettes. After overnight pre-incubation with Proteinase K (20 µL, Qiagen), the samples were then processed as described previously (Martín-Hernández *et al.*, 2012) and followed the BS96 DNA Tissue extraction protocol in a BioSprint station (Qiagen). The plates were then stored at -20°C.

For RNA extraction, 400 µL of the supernatant was incubated for 15 min with protease (20 µL, Qiagen) at 70 ºC and the nucleic acids were then extracted as described above (Biosprint 96 DNA; Qiagen and Bio-Sprint workstation). The nucleic acids recovered were then subjected to DNA digestion with DNase I (Qiagen) to completely remove any genomic DNA and the total

Table 1. Apiaries and colonies sampled in each region of Castilla-La Mancha (CLM) on 2012, 2013 and 2014.

Province	2012		2013		2014	
	Apiaries	Colonies	Apiaries	Colonies	Apiaries	Colonies
Albacete	5	62	4	52	4	52
Ciudad Real	4	45	0	0	2	22
Toledo	1	10	2	24	4	49
Cuenca	2	23	5	50	0	0
Guadalajara	2	24	2	25	0	0
Total CLM	14	164	13	151	10	123

Figure 1. Sampling zones in Castilla-La Mancha, Spain.



RNA recovered was used immediately to generate first strand cDNAs using the Quantitec Reverse Transcription Kit (Qi-agen) according to the manufacturer's instructions. The resultant cDNA was used for subsequent virus analysis (with no further dilution).

Negative and positive controls were run in parallel for each step: bee maceration, DNA and RNA extraction, and reverse transcription.

PCR and RT-PCR reactions

Broad pathogen screening (Table 2) was performed using published PCR assays to detect: *Acarapis woodi* (Rennie, 1921; Cepero *et al.*, 2015); *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries *et al.*, 1996) in triplex PCR with an internal control (Martín-Hernández *et al.*, 2012); Trypanosomatids and Neogregarines (Meeus *et al.*, 2010). Virus analysis was performed using RT-PCR published to detect Lake Sinai Virus complex (LSV1-LSV2-complex; Ravoet *et al.*, 2013); Acute Bee Paralysis Virus-Kashmir Bee Virus-Israeli Acute Paralysis (AKI-complex: Francis & Kryger, 2012); Black Queen Cell Virus (BQCV: Bailey & Woods, 1974) and Deformed Wing Virus (DWV: Bailey *et al.*, 1979) using qRT-PCR described by Chantawannakul *et al.*, 2006). Negative PCR controls were included in all analyses.

Trypanosomatid sequencing

To identify the infecting species, PCR products of all trypanosomatid positive isolated were sequenced. For this, the PCR products were purified (Gómez-Moracho *et al.*, 2015) and sequenced on an ABI3730XL Automatic Sequencer using Big Dye (Applied Biosystems, Foster City, CA, USA). The resulting sequences were assembled, aligned and checked for accura-te base calling using CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA). The species were identified based on a single nucleotide variant that allows the *18S rDNA* sequences of *L. passim* (Schwarz *et al.*, 2015) and *Crithidia mellificae* (Langridge & McGhee, 1967) to be discriminated ("*G*" for *L. passim* and "*A*" for *C. mellificae*, respectively: Cepero *et al.*, 2014, 2016), and also compared with sequences deposited in GenBank database.

Statistical analysis

Statistical analyses were performed using the SPSS 23 software, carrying out Fisher Exact Tests to determine if there was any association between the pathogens. Chi-square significance tests were also performed to check if the prevalence of each pathogen changed over the years and if there were any pathogen associations.

Table 2. Primers used for each pathogen in PCR reactions.

	Pathogen	Primer	Sequence	Amplicon size (bp)
PCR	*N. ceranae*	218 CER-F	5'-CGGCGACGATGTGATATGAAAATATTAA-3'	218-219
		218 CER-R	5'-CCCGGTCATTCTCAAACAAAAAACCG-3'	
	N.apis	218 CER-F	5'-GGGGGCATGTCTTTGACGTACTATGTA-3'	321
		218 CER-R	5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3'	
	Trypanosomatids	CRI-SEF	5'- CTTTTGGTCGGTGGAGTGAT- 3'	417
		CRI-SER	5'- GGACGTAATCGGCACAGTTT- 3'	
	Neogregarines	API-NEOF	5'- CCAGCATGGAATAACATGTAAGG- 3'	260
		API-NEOR	5'- GACAGCTTCCAATCTCTAGTCG- 3'	
	A. woodi	AW180-FOR	5'-GGAATATGATCTGGTTTAGTTGGTC-3'	180
		AW180-REV	5'- GAATCAATTTCCAAACCCACCAATC-3'	
RT-PCR	LSV-complex	LSVdeg-F	5'-GCCWCGRYTGTTGGTYCCCCC-3'	600
		LSVdeg-R	5'-GAGGTGGCGGCGCSAGATAAAGT-3'	
	AKI	AKI-F	5'-CTTTCATGATGTGGAAACTCC-3'	100
		AKI-R	5'-AAACTGAATAATACTGTGCGTA-3'	
RT-qPCR	BQCV	BQCV 9195F	5'-GGTGCGGGAGATGATATGGA-3'	305
		BQCV 265r	5'-GCCGTCTGAGATGCATGAATAC-3'	
		BQCV 8217T*	5'-FAM-TTTCCATCTTTATCGGTACGCCGCC-TAMRA-3'	
	DWV	DWV 9587F	5'-CCTGGACAAGGTCTCGGTAGAA-3'	250
		DWV 9711R	5'-ATTCAGGACCCCACCCAAAT-3'	
		DWV 9627T*	5'-FAM-CATGCTCGAGGATTGGGTCGTCGT-TAMRA-3'	

*5' (FAM, 6-carboxyfluorescein); 3' (TAMRA, tetramethylrhodamine)

Results

The prevalence of the major bee pathogens is shown in Table 3. The most prevalent pathogen over the three years of sampling was *N. ceranae*, with a mean prevalence of 52.6% in all the apiaries sampled (53.7%, 40% and 64.2% in each sampling period) and present in all apiaries studied). Trypanosomatids were also detected in all the apiaries analysed, with a mean prevalence of 32.6% (29.9%, 27.1% and 40.7%, in each year). The sequencing of all positive trypanosomatid isolates indicated that *L. passim* was the most abundant in this area since 80% of the analysed sequences corresponded with this species, whereas *C. mellificae* was far less frequent (20% of sequences studied).

V. destructor was the third parasite in terms of prevalence, with a mean of 18%. *V. destructor* was identified in three of the five provinces in 2012 (Albacete, Guadalajara and Toledo) but in only 4 of the 14 apiaries analysed (28.6%), with a mean prevalence of 9.1% and a mean percentage parasitization of 0.4% in this year. However, its prevalence increased significantly in the next two years, with an average prevalence of 11.9% in 2013 and 33% in 2014. *V. destructor* was detected in all the apiaries studied in 2013 and 2014. The mean percentage of parasitization increased in 2013 to 1%, and rose to 3% on average in 2014. *N. apis* was the fourth parasite in prevalence, with a mean of 5.6%. It was not detected in 2012, but in 2013 and 2014 its prevalence was 15.2% and 1.6% respectively. Neogregarines were the fifth parasite in prevalence, with a mean prevalence of 1.6% (0.6%, 4.6% and 0% in each year). *A. woodi* was not detected in any sample.

Of the seven viruses analyzed, only DWV and BQCV were found in all the apiaries analysed. The mean prevalence for BQCV was 25.1% (30.5% in 2012, 23.8% in 2013 and 21.1% in 2014). For DWV the mean prevalence was 18.6% (14.6% in 2012, 11.9% in 2013 and 29.3% in 2014). No LSV or AKI viruses were found.

Regarding associations, a higher presence of DWV was observed in the colonies where *V. destructor* was detected (Chi-squared; $p<0.0001$), whereas BQCV was less common in these colonies (Chi-squared; $p<0.05$). The detection of BQCV was associated with the presence of *L. passim* in honey bee colonies (Chi-squared; $p<0.05$) in 2012, and with *N. ceranae* in 2013 and 2014 (Chi-squared; $p<0.05$). The detection of *N. ceranae* in honey bee colonies was not significantly associated with the appearance of *L. passim* (Chi-squared; $p>0.05$).

Table 3. Prevalence of the main bee pathogens per year.

Pathogen	2012		2013		2014	
	Positive samples	%	Positive samples	%	Positive samples	%
V. destructor	15	9.1	18	11.9	41	33.3
N. ceranae	88	53.7	60	39.7	79	64.2
N. apis	0	0	23	15.2	2	1.6
Neogregarines	1	0.6	7	4.6	0	0
Tripanosomatids	47	29.9	41	27.2	50	40.7
A. woodi	0	0	0	0	0	0
BQCV	50	30.5	36	23.8	26	21.1
DWV	24	14.6	18	11.9	36	29.3
LSV1 and 2	0	0	0	0	0	0
AKI-complex	0	0	0	0	0	0

Discussion

As the EURL reports, the Epilobee project requires a strong effort to coordinate and collaborate with beekeepers in order to correctly sample and analyse honey bees. This study formed part of this project, delivering scientifically solid data on honey bee health in a particular geographical area within the EU. Indeed, we report here the prevalence of the major honey bee pathogens in Castilla-La Mancha that are potentially related to the weakness and mortality of honey bee colonies evident in Europe since the early years of the 21st century.

As reported previously for Spain (Higes *et al.*, 2009, 2010a; Botías *et al.*, 2012; Martín-Hernández *et al.*, 2012; Cepero *et al.*, 2014, 2016), *N. ceranae* was the most prevalent pathogen over the three years in all the apiaries studied. This high prevalence was also found in other parts of Europe (Stevanovic *et al.*, 2011; Ravoet *et al.*, 2013), in South (Martínez *et al.*, 2012) and North America (Runckel *et al.*, 2011; Martin *et al.*, 2013; Emsen *et al.*, 2016), and in Asia (Li *et al.*, 2012; Morimoto *et al.*, 2013; Yang *et al.*, 2013), confirming the worldwide spread of this microsporidian (Martín-Hernández *et al.*, 2007; Higes *et al.*, 2010a,b, 2013). The role of *N. ceranae* in honey bee colony loss remains controversial (Higes *et al.*, 2013). Yet despite this dispute, it is well accepted that *N. ceranae* negatively affects honey bee health at the individual and colony levels (Higes *et al.*, 2008, 2009; Martín-Hernández *et al.*, 2009; Dussaubat *et al.*, 2012, 2013; Ravoet *et al.*, 2013; Goblirsch *et al.*, 2013; Botías *et al.*, 2013; Alaux *et al.*, 2014; Cepero *et al.*, 2014, 2016; Vidau *et al.*, 2014; Wolf *et al.*, 2014; Maes *et al.*, 2016; Bordier *et al.*, 2017), causing colony collapse under certain conditions (Higes *et al.*, 2013; Betti *et al.*, 2014). Thus, this pathogen clearly represents a serious risk for professional beekeeping in temperate areas of the world (Higes *et al.*, 2009; Hatjina *et al.*, 2011; Nabian *et al.*, 2011; Martín-Hernández *et al.*, 2012; Lodesani *et al.*, 2014; Adjlane & Haddad, 2016). An additional problem with *N. ceranae* infection is that this pathogen can spill over from honey bees into bumble bee populations, causing fatal infection and contributing to bumble bee decline (Plischuk *et al.*, 2009; Graystock *et al.*, 2013; Fürst *et al.*, 2014). Therefore, *N. ceranae* poses a threat to managed and wild pollinators, and developing control strategies against *N. ceranae* is now a priority in contemporary bee research in order to save both honey bees and wild bees.

Trypanosomatids were also very prevalent in honey bee colonies, mainly *L. passim*. These pathogens are currently receiving much attention given that the presence of *C. mellificae* has been correlated with colony loses in the USA and Belgium, especially when detected in conjunction with *N. ceranae* (Runckel *et al.*, 2011; Cornman *et al.*, 2012; Ravoet *et al.*, 2013). Such co-infection was also reported in depopulated honey bee colonies (Cepero *et al.*, 2014), although the presence of *N. ceranae* was not significantly associated with the detection of *L. passim* in the honey bee colonies studied here, suggesting that this relationship may only occur in conjunction with colony collapse. When these Trypanosomatids were sequenced for species identification, we confirmed the previous data (Cepero *et al.*, 2014) indicating that the majority of Trypanosomatids detected in honey bees were not *C. mellificae* but rather, the genetically distinct lineage, *L. passim* (Schwarz *et al.*, 2015).

V. destructor mite is a serious and devastating ectoparasite of honey bees worldwide (OIE, 2008; Rosenkranz *et al.*, 2010) that is strongly suspected to participate in the collapse of bee colonies (Dainat *et al.*, 2012; Francis *et al.*, 2013). In the present study we detected *Varroa* mites in three of the five provinces in the first year (2012), yet its prevalence increased, especially in 2014. In Spain there is legislation on the management of varroosis (BOE, 2006) that requires the application of a veterinary product to control this mite during autumn. Based on the results obtained here, it appears that this control was effective in the area studied during 2012 and 2013, although the increase in prevalence detected in 2014 indicated a problem in mite control in field conditions, situation that has worsened from 2015 to the present, as our team has been able to confirm based on the data that our passive surveillance system shows in this regard.

Gregarines are a diverse group of apicomplexa protists that infect many invertebrate phyla (Stejskal, 1965). The neogregarine *A. bombi* (Liu, Macfarlane & Pengelly, 1974) is considered to be an infrequent parasite of *Bombus* species and although the use of a molecular detection method has led to its detection in honey bees (Meeus *et al.*, 2010; Plischuk *et al.*, 2011; Maharramov *et al.*, 2013; Morimoto *et al.*, 2013; Cepero *et al.*, 2014), the potential pathological effect of *A. bombi* in honey bees is not clear. Indeed, it has even been suggested that the PCR primers used to detect this protist (Meeus *et al.*, 2010) may also amplify other pathogens, which should be taken into account to avoid misdiagnosis (Cepero *et al.*, 2014). The results obtained here indicate that as reported previously (*e.g.*, Plischuk *et al.*, 2011; Marimoto et al, 2013), Neogregarines have a very low prevalence in Castilla-La Mancha and thus, they would not be a great threat for local bee colonies if pathogenic.

Honey bees can be infected by as many as twenty RNA viruses, of which twelve have been detected in Europe (Aubert *et al.*, 2008). The most frequent are the AKI-complex (Francis & Kryger, 2012; Francis *et al.*, 2013), BQCV and DWV (Runckel *et al.*, 2011; Mondet *et al.*, 2014), and LSV-complex mainly LSV1 and LSV2 (Runckel *et al.*, 2011; Ravoet *et al.*, 2013) which could therefore be related to bee colony collapse. In agreement with data from other regions (Chen & Siede, 2007; Teixeira *et al.*, 2008; Li *et al.*, 2012; Antúnez *et al.*, 2012; Morimoto *et al.*, 2013; Ravoet *et al.*, 2013; Rodriguez *et al.*, 2014), our results show that BQCV and DWV were the most prevalent viruses in Castilla-La Mancha. The *Varroa*-DWV interaction is thought to be an important cause of mortality in bee colonies (Rosenkranz *et al.*, 2010; Dainat *et al.*, 2012; Francis *et al.*, 2013) and our study confirmed this relationship as the presence of *V. destructor* was positively correlated with the occurrence of DWV.

In the field, BQCV disease outbreaks have been linked with infection with *N. apis*. The rapid multiplication of BQCV in adult bees infected with this microsporidian (Bailey, 1981, 1982) might be a secondary effect of such infection, either by increasing the susceptibility of the alimentary tract to infection by this virus (Chen & Siede, 2007) or by activating its replication in the gut where *N. apis* resides (Chen *et al.*, 2006). Given that the gut is also the target organ for infection by *N. ceranae*, the same relationship might be expected between this microsporidian and the BQCV virus. Such an association was indeed reported recently (Dainat *et al.*, 2012; Mendoza *et al.*, 2014; Francis *et al.*, 2014) and although our data did not support this relationship under field conditions in 2012, they did in 2013 and 2014. While a positive association between *Nosema* infection and BQCV has been found in some studies, the mechanism by which *Nosema* activates and transmits BQCV infection remains to be determined (Chen & Siede, 2007).

The AKI-complex and LSV-complex were not detected here and while IAPV has been related to honey bee colony collapse (Cox-Foster *et al.*, 2007; Chen *et al.*, 2014), its prevalence in Spain was very low (Garrido-Bailón *et al.*, 2010; Antúnez *et al.*, 2012) as confirmed here. The abundance of a new virus complex in honey bees (Lake Sinai viruses complex, LSV-complex: Runckel *et al.*, 2011; Granberg *et al.*, 2013; Ravoet *et al.*, 2013; Cepero *et al.*, 2014) suggests that this group may play significant role in colony health (Runckel *et al.*, 2011, Cornman *et al.*, 2012; Cepero *et al.*, 2014). However, this hypothesis is not always supported (Ravoet, *et al.*, 2013) and we did not detect this complex here.

In conclusion, in accordance with previous studies in Spain (Higes *et al.*, 2009; Garrido-Bailón *et al.*, 2012; Martín-Hernández *et al.*, 2012), the most prevalent pathogens in Castilla-la Mancha are *N. ceranae, L. passim*, BQCV and DWV. Future disease control should focus on these pathogens and the effects that the interactions between them could have on bee colony health. Another priority should be to monitor changes in prevalence to detect potential problems with pathogen control programs, such as those that possibly occurred in 2014 with the *Varroa* control program that was apparently effective in field conditions until then. As such, studies like this will help advance our understanding of pathogen interactions and bee health, therefore helping in the application of control measures to limit colony losses.

Acknowledgments

The authors wish to thank J. Almagro, J. García, V. Albendea, C. Uceta, M. Gajero and T. Corrales at Laboratorio de Patología Apícola, Centro de Investigación Apícola y Agroambiental (CIAPA), IRIAF, Junta de Comunidades de Castilla-La Mancha, for their technical support. Authors also thank the colleagues from the Consejería de Agricultura de Castilla-La Mancha, who coordinated the selection of apiaries and the sending of samples to our laboratory and, of course, the beekeepers participating in the study.

References

Adjlane N, Haddad N, 2016. Effect of some honeybee diseases on seasonal mortality of *Apis mellifera intermissa* in Algeria apiaries. Proc Zool Soc 71 (1): 83-87. https://doi.org/10.1007/s12595-016-0188-5

Alaux C, Crauser D, Pioz M, Saulnier C, Le Conte Y, 2014. Parasitic and immune modulation of flight activity in honey bees tracked with optical countes. J Exp Biol 217 (19): 3416-3424. https://doi.org/10.1242/jeb.105783

Anderson DL, Trueman JWH, 2000. *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Exp Appl Acarol 24 (3): 165-189. https://doi.org/10.1023/A:1006456720416

Antúnez K, Anido M, Garrido-Bailón E, Botías C, Zunino P, Martínez-Salvador A, Martín-Hernández R, Higes M, 2012. Low prevalence of honeybee viruses in Spain during 2006 and 2007. Res Vet Sci 93 (3): 1441-1445. https://doi.org/10.1016/j.rvsc.2012.03.006

Aubert M, Ball B, Fries I, Moritz R, Milani N, Bernardinelli I, 2008. Virology and the honey bee. European Commission Directorate-General for Research, Brussels, Belgium.

Bailey L, Woods RD, 1974. Three previously undescribed viruses from the honey bee. J Gen Virol 25 (2): 175-186. https://doi.org/10.1099/0022-1317-25-2-175

Bailey L, Carpenter JM, Woods RD, 1979. Egypt bee virus and Australian isolates of Kashmir bee virus. J Gen Virol 43 (3): 641-647. https://doi.org/10.1099/0022-1317-43-3-641

Bailey L, 1981. Honey bee pathology. Academic Press, London. 124 pp.

Bailey L, 1982. Viruses of honeybees. Bee World 63 (4): 165-173. https://doi.org/10.1080/0005772X.1982.11097891

Bernal J, Garrido-Bailon E, Del Nozal MJ, González-Porto AV, Martín-Hernández R, Diego JC, Jiménez JJ, Bernal JL, Higes M, 2010. Overview of pesticide residues in stored pollen and their potential effect on bee colony (*Apis mellifera*) losses in Spain. J Econ Entomol 103 (6): 1964-1971. https://doi.org/10.1603/EC10235

Betti MI, Wahl LM, Zamir M, 2014. Effects of infection on honey bee population dynamics: a model. PloS ONE 9 (10): e110237. https://doi.org/10.1371/journal.pone.0110237

BOE, 2006. Royal decree 608/2006, of 19 May, that establishes and regulates a national program for the control of honey bee diseases. Boletín Oficial del Estado (Spain) No. 131, 07/06/06.

Bordier C, Suchail S, Pioz M, Devaud JM, Collet C, Charreton M, Le Conte Y, Alaux C, 2017. Stress response in honeybees is associated with changes in task-related physiology and energetic metabolism. J. Insect Physiol 98: 47-54. https://doi.org/10.1016/j.jinsphys.2016.11.013

Botías C, Martín-Hernández R, Garrido-Bailón E, González-Porto A, Martínez-Salvador A, De La Rúa P, Meana A, Higes M, 2012. The growing prevalence of *Nosema ceranae* in honey bees in Spain, an emerging problem for the last decade. Res Vet Sci 93 (1): 150-155. https://doi.org/10.1016/j.rvsc.2011.08.002

Botías C, Martín-Hernández R, Barrios L, Meana A, Higes M, 2013. *Nosema spp*. infection and its negative effects on honey bees (*Apis mellifera iberiensis*) at the colony level. Vet Res 44 (1): 25. https://doi.org/10.1186/1297-9716-44-25

Breeze TD, Bailey AP, Balcombe KG, Potts SG, 2011. Pollination services in the UK: How important are honeybees? Agric Ecosyst Environ 142 (3): 137-143. https://doi.org/10.1016/j.agee.2011.03.020

Cepero A, Ravoet J, Gómez-Moracho T, Bernal JL, Del Nozal MJ, Bartolomé C, Maside X, Meana A, González-Porto AV, de Graaf DC, Martín-Hernández R, Higes M, 2014. Holistic screening of collapsing honey bee colonies in Spain: a case study. BMC Res Notes 7 (1): 649. https://doi.org/10.1186/1756-0500-7-649

Cepero A, Martín-Hernández R, Prieto L, Gómez-Moracho T, Martínez-Salvador A, Bartolomé C, Maside X, Meana A, Higes M, 2015.Is Acarapis woodi a single species? A new PCR protocol to evaluate its prevalence. Parasitol Res 114 (2): 651-658. https://doi.org/10.1007/s00436-014-4229-6

Cepero A, Martín-Hernández R, Bartolomé C, Gómez-Moracho T, Barrios L, Bernal J, Martín MT, Meana A, Higes M, 2016. Passive laboratory surveillance in Spain: pathogens as risk factors for honey bee colony collapse. J Apic Res 54 (5): 411-419.

Chantawannakul P, Ward L, Boonham N, Brown M, 2006. A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in *Varroa* mites collected from a Thai honeybee (*Apis mellifera*) apiary. J Invert Pathol 91 (1): 69-73. https://doi.org/10.1016/j.jip.2005.11.001

Chauzat MP, Cauquil L, Roy L, Franco S, Hendrikx P, Ribière-Chabert M, 2013. Demographics of the European Apicultural Industry. PLoS ONE 8 (11): e79018. https://doi.org/10.1371/journal.pone.0079018

Chen Y, Evans J, Feldlaufer M., 2006. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. J Invert Pathol 92 (3): 152-159. https://doi.org/10.1016/j.jip.2006.03.010

Chen YP, Siede R, 2007. Honey bee viruses. Adv Virus Res 70: 33-80. https://doi.org/10.1016/S0065-3527(07)70002-7

Chen YP, Pettis JS, Corona M, Chen WP, Li CJ, Spivak M, Delaplane K, 2014. Israeli acute paralysis virus: epidemiology, pathogenesis and implications for honey bee health. PLoS Pathog 10 (7): e1004261. https://doi.org/10.1371/journal.ppat.1004261

Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, vanEngelsdrop D, Evans JD, 2012. Pathogen webs in collapsing honey bee colonies. Plos ONE 7 (8): e43562. https://doi.org/10.1371/journal.pone.0043562

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan PL, Briese T, Hornig M, Geiser DM, *et al.*, 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318 (5848): 283-287. https://doi.org/10.1126/science.1146498

Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P, 2012. Dead or alive: deformed wing virus and *Varroa destructor* reduce the life span of winter honeybees. Appl Environ Microbiol 78 (4): 981-987. https://doi.org/10.1128/AEM.06537-11

Dussaubat C, Brunet JL, Higes M, Colbourne JK, Lopez J, Choi JH, Bonnet M, 2012. Gut pathology and responses to the microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*. PloS ONE 7 (5): e37017. https://doi.org/10.1371/journal.pone.0037017

Dussaubat C, Maisonnasse A, Crauser D, Beslay D, Costagliola G, Soubeyrand S, Le Conte Y, 2013. Flight behavior and pheromone changes associated to *Nosema ceranae* infection of honey bee workers (*Apis mellifera*) in field conditions. J Invert Pathol 113 (1): 42-51. https://doi.org/10.1016/j.jip.2013.01.002

Emsen B, Guzman-Novoa E, Hamiduzzaman MM, Eccles L, Lacey B, Ruiz-Pérez RA, Nasr M, 2016. Higher prevalence and levels of *Nosema ceranae* than *Nosema apis* infections in Canadian honey bee colonies. Parasitol Res 115 (1):175-181. https://doi.org/10.1007/s00436-015-4733-3

Francis R, Kryger P, 2012. Single assay detection of Acute Bee Paralysis Virus, Kashmir Bee Virus and Israeli Acute Paralysis Virus. J Apic Sci 56 (1): 137-146. https://doi.org/10.2478/v10289-012-0014-x

Francis RM, Nielsen SL, Kryger P, 2013. Varroa-virus interaction in collapsing honey bee colonies. PloS ONE 8 (3): e57540. https://doi.org/10.1371/journal.pone.0057540

Francis RM, Amiri E, Meixner MD, Kryger P, Gajda A, Andonov S, Berg S, 2014. Effect of genotype and environment on parasite and pathogen levels in one apiary-a case study. J Apic Res 53 (2): 230-232. https://doi.org/10.3896/IBRA.1.53.2.14

Fries I, Feng F, da Silva A, Slemenda SB, Pieniazek NJ, 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 (3): 356-365. https://doi.org/10.1016/S0932-4739(96)80059-9

Fürst MA, McMahon DP, Osborne JL, Paxton RJ, Brown MJF, 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506 (7488): 364-366. https://doi.org/10.1038/nature12977

Garrido-Bailón E, Martín-Hernández R, Bernal J, Bernal JL, Martínez- Salvador A, Barrios L, Meana A, Higes M, 2010. The detection of israeli acute paralysis virus (IAPV), fipronil and imidacloprid in professional apiaries are not related with massive honey bee colony loss in Spain. Span J Agric Res 8 (6): 58- 661. https://doi.org/10.5424/sjar/2010083-1262

Garrido-Bailón ME, 2012. Repercusión potencial en la cabaña apícola española de agentes nosógenos detectados en colonias de "*Apis mellifera iberiensis*". Doctoral thesis. Univ. Complutense de Madrid, Madrid, Spain.

Garrido-Bailón E, Botías C, Martín-Hernández R, Salvador AM, Meana A, Higes M, 2012. Prevalencia de los principales agentes patógenos de *Apis mellifera iberienses* en la cabaña apícola española. Anales de la Real Academia de Ciencias Veterinarias de Andalucía Oriental 25: 109-130.

Goblirsch M, Huang ZY, Spivak M, 2013. Physiological and behavioral changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection. PLoS ONE 8 (3): e58165. https://doi.org/10.1371/journal.pone.0058165

Gómez-Moracho T, Bartolomé C, Bello X, Martín-Hernández R, Higes M, Maside X, 2015. Recent worldwide expansion of *Nosema ceranae* (Microsporidia) in *Apis mellifera* populations inferred from multilocus patterns of genetic variation. Infect Genet Evol 31: 87-94. https://doi.org/10.1016/j.meegid.2015.01.002

Granberg F, Vicente-Rubiano M, Rubio-Guerri C, Karlsson OE, Kukielka D, Belák S, Sánchez-Vizcaíno JM, 2013. Metagenomic detection of viral pathogens in spanish honeybees: co-infection by aphid lethal paralysis, Israel acute paralysis and Lake Sinai viruses. PLoS ONE 8 (2): e57459. https://doi.org/10.1371/journal.pone.0057459

Graystock P, Yates K, Evison SE, Darvill B, Goulson D, Hughes WO, 2013. The Trojan hives: pollinator pathogens, imported and distributed in bumblebee colonies. J Appl Ecol 50 (5): 1207-1215. https://doi.org/10.1111/1365-2664.12134

Hatjina F, Tsoktouridis G, Bouga M, Charistos L, Evangelou V, Avtzis D, Meeus I, Brunain M, Smagghe G, de Graaf DC, 2011. Polar tube protein diversity among *Nosema ceranae* strains derived from a Greek honey bee health study. J Invertebr Pathol 108: 131-134. https://doi.org/10.101 6/j.ji p.2011.07.003

Higes M, Martín-Hernández R, Botías C, Bailón EG, González-Porto AV, Barrios L, del Nozal MJ, Jiménez JJ, Palencia PG, Meana A, 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse.

Environ Microbiol 10 (10): 2659-2669. https://doi.org/10.1111/j.1462-2920.2008.01687.x

Higes M, Martín-Hernández R, Garrido-Bailón E, González-Porto AV, García-Palencia P, Meana A, del Nozal MJ, Mayo R, Bernal JL, 2009. Honeybee colony collapse due to *Nosema ceranae* in professional apiaries. Environ Microbiol Rep 1 (2): 110-113. https://doi.org/10.1111/j.1758-2229.2009.00014.x

Higes M, Martín-Hernández R, Meana A, 2010a. *Nosema ceranae* in Europe: an emergent type C nosemosis. Apidologie 41 (3): 375-392. https://doi.org/10.1051/apido/2010019

Higes M, Martín-Hernández R, Martínez-Salvador A, Garrido-Bailón E, González-Porto AV, Meana A, Bernal JL, del Nozal MJ, Bernal J, 2010b. A preliminary study of the epidemiological factors related to honey bee colony loss in Spain. Environ Microbiol Rep 2 (2): 243-250. https://doi.org/10.1111/j.1758-2229.2009.00099.x

Higes M, Meana A, Bartolomé C, Botías C, Martín-Hernández R, 2013. *Nosema ceranae* (Microsporidia), a controversial 21st century honey bee pathogen. Environ Microbiol Rep 5 (1): 17-29. https://doi.org/10.1111/1758-2229.12024

Langridge DF, McGhee RB, 1967. *Crithidia mellificae* n. sp. an acidophilic trypanosomatid of the honey bee *Apis mellifera*. J Protozool 14 (3): 485-487. https://doi.org/10.1111/j.1550-7408.1967.tb02033.x

Li J, Chen W, Wu J, Peng W, An J, Schmid-Hempel P, Schmid-Hempel R, 2012. Diversity of *Nosema* associated with bumblebees (*Bombus* spp.) from China. Int J Parasitol 42 (1): 49-61. https://doi.org/10.1016/j.ijpara.2011.10.005

Lodesani M, Costa C, Besana A, Dall'Olio R, Franceschetti S, Tesoriero D, Giacomo D, 2014. Impact of control strategies for *Varroa destructor* on colony survival and health in northern and central regions of Italy. J Apic Res 53 (1): 155-164. https://doi.org/10.3896/IBRA.1.53.1.17

Maes PW, Rodrigues PA, Oliver R, Mott BM, Anderson KE, 2016. Diet-related gut bacterial dysbiosis correlates with impaired development, increased mortality and *Nosema* disease in the honeybee (*Apis mellifera*). Mol Ecol 25 (21): 5439-5450. https://doi.org/10.1111/mec.13862

MAGRAMA, 2012. Protocolo Programa de vigilancia piloto sobre las pérdidas de colonias de abejas. Ministerio de Agricultura, Ganadería y Medio Ambiente, Gobierno de España.

MAGRAMA, 2013. Protocolo Programa de Vigilancia Piloto sobre las pérdidas de colonias de abejas. Ministerio de Agricultura, Ganadería y Medio Ambiente, Gobierno de España.

Maharramov J, Meeus I, Maebe K, Arbetman M, Morales C, Graystock P, Zapata N, 2013. Genetic variability of the neogregarine *Apicystis bombi*, an etiological agent of an emergent bumblebee disease. PLoS ONE 8 (12): e81475. https://doi.org/10.1371/journal.pone.0081475

Martin SJ, Hardy J, Villalobos E, Martín- Hernández R, Nikaido S, Higes M, 2013. Do the honeybee pathogens *Nosema ceranae* and deformed wing virus act synergistically? Environ Microbiol Rep 5 (4): 506-510. https://doi.org/10.11 11/1758-2229.12052

Martínez J, Leal G, Conget P, 2012. *Nosema ceranae* an emergent pathogen of *Apis mellifer*a in Chile. Parasitol Res 111 (2): 601-607. https://doi.org/10.1007/s00436-012-2875-0

Martín-Hernández R, Meana A, Prieto L, Salvador AM, Garrido-Bailón E, Higes M, 2007. Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Appl Environ Microbiol 73 (20): 6331-6338. https://doi.org/10.1128/AEM.00270-07

Martín-Hernández R, Meana A, García-Palencia P, Marín P, Botías C, Garrido-Bailón E, Barrios L, 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 EG, 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 (8): 2127-2138. https://doi.org/10.1111/j.1462-2920.2011.02645.x

Meeus I, De Graaf DC, Jans K, Smagghe G, 2010. Multiplex PCR detection of slowly envolving Trypanosomatids and Neogregarines in bumblebees using broad-range primers. J Appl Microbiol 109 (1): 107-115.

Mendoza Y, Antúnez K, Branchiccela B, Anido M, Santos E, Invernizzi C, 2014. *Nosema ceranae* and RNA viruses in European and Africanized honeybee colonies (*Apis mellifera*) in Uruguay. Apidologie 45 (2): 224-234. https://doi.org/10.1007/s13592-013-0241-6

Mondet F, de Miranda JR, Kretzschmar A, Le Conte Y, Mercer AR, 2014. On the front line: Quantitative virus dynamics in honeybee (*Apis mellifera L.*) colonies along a new expansion front of the parasite *Varroa destructor*. PLoS Pathog 10 (8): e1004323. https://doi.org/10.1371/journal.ppat.1004323

Morimoto T, Kojima Y, Yoshiyama M, Kimura K, Yang B, Peng G, Kadowaki T, 2013. Molecular detection of protozoan parasites infecting *Apis mellifera* colonies in Japan. Environ Microbiol Rep 5 (1): 74-77. https://doi.org/10.1111/j.1758-2229.2012.00385.x

Nabian S, Ahmadi K, Shirazi MN, Sadeghian AG, 2011. First detection of *Nosema ceranae*, a microsporidian protozoa of European honeybees (*Apis mellifera*) in Iran. Iran J Parasitol 6 (3): 89.

OIE, 2008. Manual of standards for diagnostic test and vaccines. Office International des Epizooties. http://www.oie.int/eng/norms/mmanual/2008

Plischuk S, Martín-Hernández R, Prieto L, Lucía M, Botías C, Meana A, Abrahamovich AH, Lange C, Higes M,

2009. South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen of honeybees (*Apis mellifera*). Environ Microbiol Rep 1 (2): 131-135. https://doi.org/10.1111/j.1758-2229.2009.00018.x

Plischuk S, Meeus I, Smagghe G, Lange CE, 2011. *Apicystis bombi* (Apicomplexa: Neogregarinorida) parasitizing *Apis mellifera* and *Bombus terrestris* (Hymenoptera: Apidae) in Argentina. Environ Microbiol Rep 3 (5): 565-568. https://doi.org/10.1111/j.1758-2229.2011.00261.x

Porrini C, Mutinelli F, Bortolotti L, Granato A, Laurenson L, Roberts K, Sgolastra F, 2016. The status of honey bee health in Italy: results from the nationwide bee monitoring network. PloS ONE 11 (5): e0155411. https://doi.org/10.1371/journal.pone.0155411

Potts SG, Roberts SP, Dean R, Marris G, Brown MA, Jones R., Nemann P, Settele J, 2010. Declines of managed honey bees and beekeepers in Europe. J Apicult Res 49 (1): 15-22. https://doi.org/10.3896/IBRA.1.49.1.02

Ratnieks FL, Carreck NL, 2010. Clarity on honey bee collapse? Science 327 (5962): 152-153. https://doi.org/10.1126/science.1185563

Ravoet J, Maharramov J, Meeus I, De Smet L, Wenseleers T, Smagghe G, de Graaf DC, 2013. Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. PLoS ONE 8 (8): e72443. https://doi.org/10.1371/journal.pone.0072443

Rennie J, 1921. Isle of Wight disease in hive bees - Acarine disease: The organism associated with the disease Tarsonemus woodi, n. sp. T Roy Soc Edinburgh 52: 768-779. https://doi.org/10.1017/S0080456800016008

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 (2): 170-177. https://doi.org/10.4067/S0718-58392014000200007

Rosenkranz P, Aumeier P, Ziegelmann B, 2010. Biology and control of *Varroa destructor*. J Invertebr Pathol 103: 96-119. https://doi.org/10.1016/j.jip.2009.07.016

Runckel C, Flenniken ML, Engel JC, Ruby JG, Ganem D, Andino R, DeRisi JL, 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. PloS ONE 6 (6): e20656. https://doi.org/10.1371/journal.pone.0020656

Schwarz RS, Bauchan GR, Murphy CA, Ravoet J, Graaf DC, Evans JD, 2015. Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera: Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. gen., n. sp. J Eukaryot Microbiol 62 (5): 567-583. https://doi.org/10.1111/jeu.12209

Simon-Delso N, San Martin G, Bruneau E, Minsart LA, Mouret C, Hautier L, 2014. Honeybee colony disorder in crop areas: the role of pesticides and viruses. PloS ONE 9 (7): e103073. https://doi.org/10.1371/journal.pone.0103073

Staveley JP, Law SA, Fairbrother A, Menzie CA, 2014. A causal analysis of observed declines in managed honey bees (*Apis mellifera*). Hum Ecol Risk Assess 20 (2): 566-591. https://doi.org/10.1080/10807039.2013.831263

Stejskal M, 1965. Eine neue Gregarine, *Fusiona geusi*, n. sp., n. gen., n. fam., n. superfam., aus der Sudamerikanischen Schabe *Pycnoscelus surinamensis* L. 1758. Z Parasitenkd 26 (3): 215-220.

Stevanovic J, Stanimirovic Z, Genersch E, Kovacevic SR, Ljubenkovic J, Radakovic M, Aleksic N, 2011. Dominance of *Nosema ceranae* in honey bees in the Balkan countries in the absence of symptoms of colony collapse disorder. Apidologie 42: 49- 58. https://doi.org/10.1051/apido/2010034

Teixeira EW, Chen Y, Message D, Pettis J, Evans JD, 2008. Virus infections in Brazilian honey bees. J Invert Pathol 99 (1): 117-119. https://doi.org/10.1016/j.jip.2008.03.014

Van der Zee R, Gómez- Moracho T, Pisa L, Sagastume S, García- Palencia P, Maside X, Bartolomé C, Martín-Hernández R, Higes M, 2014. Virulence and polar tube protein genetic diversity of *Nosema ceranae* (Microsporidia) field isolates from Northern and Southern Europe in honeybees (*Apis mellifera iberiensis*). Environ Microbiol Rep 6 (4): 401-413. https://doi.org/10.1111/1758 -2229.12133

VanEngelsdorp D, Meixner MD, 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J Invertebr Pathol 103: 80-95. https://doi.org/10.1016/j.jip.2 009.06.011

Vidau C, Panek J, Texier C, Biron DG, Belzunces LP, Le Gall M, El Alaoui H, 2014. Differential proteomic analysis of midguts from *Nosema ceranae*-infected honeybees reveals manipulation of key host functions. J Invertebr Pathol 121: 89-96. https://doi.org/10.1016/j.jip.2014.07.002

Wolf S, McMahon DP, Lim KS, Pull CD, Clark SJ, Paxton RJ, Osborne JL, 2014. So near and yet so far: harmonic radar reveals reduced homing ability of *Nosema* infected honeybees. PLoS ONE 9 (8): e103989. https://doi.org/10.1 371/journal.pone.0103989

Yang B, Peng G, Li T, Kadowaki T, 2013. Molecular and phylogenetic characterization of honey bee viruses, *Nosema* microsporidia, protozoan parasites, and parasitic mites in China. Ecol Evol 3 (2): 298-311. https://doi.org/10.1002/ece3.464

Zander E, 1909. Tierische Parasiten als Krankenheitserreger bei der Biene. Münch 31: 196-204.