Environmental Microbiology Reports (2009) doi:10.1111/j.1758-2229.2009.00014.x

Honeybee colony collapse due to *Nosema ceranae* in professional apiaries

Mariano Higes,[1]* Raquel Martín-Hernández,[1] Encarna Garrido-Bailón,[1] Amelia V. González-Porto,[2] Pilar García-Palencia,[3] Aranzazu Meana,[4] María J. del Nozal,[5] R. Mayo[5] and José L. Bernal[5]

[1]*Bee Pathology Laboratory and* [2]*Hive Products Laboratory, Centro Apícola Regional, JCCM, 19180 Marchamalo, Spain.*

[3]*Animal Medicine and Surgery Department and* [4]*Animal Health Department, Veterinary School, Universidad Complutense de Madrid, 28040 Madrid, Spain.*

[5]*Analitical Chemistry Department, Sciences School, Universidad de Valladolid, 47005 Valladolid, Spain.*

Summary

Honeybee colony collapse is a sanitary and ecological worldwide problem. The features of this syndrome are an unexplained disappearance of adult bees, a lack of brood attention, reduced colony strength, and heavy winter mortality without any previous evident pathological disturbances. To date there has not been a consensus about its origins. This report describes the clinical features of two professional bee-keepers affecting by this syndrome. Anamnesis, clinical examination and analyses support that the depopulation in both cases was due to the infection by *Nosema ceranae* (*Microsporidia*), an emerging pathogen of *Apis mellifera*. No other significant pathogens or pesticides (neonicotinoids) were detected and the bees had not been foraging in corn or sunflower crops. The treatment with fumagillin avoided the loss of surviving weak colonies. This is the first case report of honeybee colony collapse due to *N. ceranae* in professional apiaries in field conditions reported worldwide.

Introduction

In recent years, there has been a devastating loss of honeybee colonies (*Apis mellifera* spp.), initially evidenced in Europe but now affecting other continents (Stokstad, 2007; Molga, 2008). Indeed, this recurrent phenomenon has been named Colony Collapse Disorder (CCD) in America and Honey Bee Colony Depopulation Syndrome (CDS) in Europe. The consequences of this syndrome are evident as an unexplained disappearance of adult bees, a lack of attention to the brood, reduced colony vigour, and heavy winter mortality without any apparent pathological infection. In addition, it leads to decreased honey production and ultimately to colony mortality. While to date it has not been possible to reach a consensus about the origins of CCD or CDS, pathogens do seem to play a central role in this phenomenon (Higes *et al*., 2006; 2008a; Martín-Hernández *et al*., 2007; Blanchard *et al*., 2008; Van Ooij, 2008).

In this work, clinical features from two professional apiaries are described. Both apiaries are situated 750 km apart (Fig. 1) and they are subjected to quite different climatic, orographic and apicultural conditions (type of flora available to the bees, bee-keeping practices, etc). Nevertheless, in both apiaries high colony losses with disappearance of adult bees occurred in the winter of 2006 (Fig. 2).

Results and discussion

The results of these analyses indicated that the only pathogen present in all the honeybee samples analysed was *Nosema cerana*e (*Microsporidia*) (as summarized in Table 1). The gross overview of the ventriculus showed them to be oedematous and friable, due to a strong epithelial cell infection, and with similar features to those described previously in association with *N. ceranae* infection (Higes *et al*., 2007; 2008b). There was evidence of epithelial cell degeneration and extensive lysis (Figs 3 and 4). As described previously (Higes *et al*., 2008a), the heavily infected cells may be either dead or dying, which will eventually lead to the early death of the infected worker bees due to starvation (Liu, 1984). This leads to the result that some heavily infected honeybees (foragers preferably) do not return to the hive and finally the depopulation of colonies is evident when the queen cannot compensate for the loss of foragers, depopulation becomes evident and death is forthcoming (Higes *et al*., 2008a).

Only deformed wing virus (DWV) was detected in one pool bee sample ($n = 15$ colonies) from Apiary 1, but no clinical signs of viral illness were evident. The absence of clinical signs related to this virus suggests that infection was covert or latent. Deformed wing virus has been

Received 26 November, 2008; accepted 18 December 2008. *For correspondence. E-mail mhiges@jccm.es; Tel. (+34) 949 25 00 26; Fax (+34) 949 25 01 76.

Fig. 1. Location of sampling site areas. Northern apiary sited in Noia (A Coruña, Spain, 42°47′55.8″N, 8°51′55.08″W, Apiary 1) and southern apiary sited in Mirabueno (Guadalajara, Spain, 42°57′8.47″N, 2°43′7.39″W, Apiary 2). The northern apiary (Apiary 1) was composed of 75 colonies of *Apis mellifera iberiensis* in August 2006 at which point no clinical signs were evident. By the following December (2006), 60 colonies had died with clear symptoms of depopulation in the weeks prior to their death (disappearance of adult bees, unattended brood, reduced colony vigour, no dead or trembling bees around the hives), and the 15 survivors recovered were in heavily weakness (two to three combs with bees and 200–300 brood cells). Samples were sent for analysis in the same month (December 2006), which included 50 dead bees from a collapsed colony, as well as samples of workers bees, brood combs and stored pollen, from the surviving 15 colonies. The southern apiary (Apiary 2) initially contained 150 healthy hives in August 2006 but in December 2006, 50 colonies had died and the rest were not sufficiently crowded to be able to adequately survive the winter (Fig. 2). A total of 10 samples of around 30 bees from each of the dead colonies (CCD colonies) were analysed, as well as 10 samples of worker bees ($n = 100$ bees each), brood (around 500 cells surface each sample) and one sample of stored pollen from 10 weakened surviving colonies. After 3, 6 and 9 months of treatments with fumagillin, in the spring, summer and autumn, all the colonies from Apiary 1 and 2 were sampled (foragers honeybee workers) and analysed by PCR for *Nosema* spp.

proposed as a covert-infecting virus, needing a strong trigger, such as immunosuppression by *Varroa destructor* (Yue *et al*., 2007). The absence of mites in all samples clearly indicates a very low number or even the total absence due to *Varroa* control. No other viruses were detected, not even Israeli acute paralysis virus (IAPV). This virus was previously proposed as a CCD marker (Cox-Foster *et al*., 2007); however, its prevalence is very low (around 15%, data no show) and it has not been associated with colony collapse in Europe (Blanchard *et al*., 2008; Higes *et al*., 2008a).

The higher losses of colonies (80% in Apiary 1 and 34% in Apiary 2) and the only presence of DWV in Apiary 1 without clinical signs related to it clearly indicate that in both cases the viruses seem not to play an aetiological role.

The residue analysis of 40 compounds demonstrated that the bees from both apiaries were not exposed to any agricultural pesticides. The only micropollutants detected in the stored pollen were those applied as acaricides by

Fig. 2. Weakened honeybee colony with an evident disappearance of most of adult bees, lack of attention to the brood, reduced colony vigour, without any apparent pathological or toxicological problem (no crawling bees, no death bees or dysentery evidenced by the presence of faecal spots in the hive).

Table 1. Bee pathogens, main type of stored pollen and presence of micropollutants in affected apiaries (summarized results).

	Bee pathogens		Micropollutants in stored pollen			Main type of pollen in stored pollen	
Apiary	Main pathogens	Virus	Pesticide	Mean (μg kg^{-1})	Toxicity to bees	Taxon	%
1	*N. ceranae*	DWV	Fluvalinate[a]	125 (s.d. 96.1)	VNT[b]	*Castanea*	84 (s.d. 3.1)
			z-chlorfenvinphos	16 (s.d. 8.3)	HT[c]	*Rubus*	6 (s.d. 0.4)
						Leguninoseae	5 (s.d. 1.1)
2	*N. ceranae*	No	Fluvalinate[a]	225 (s.d. 119)	VNT[b]	*Cruciferae*	60 (s.d. 28.1)
						Cichorioideae	20 (s.d. 19.5)
						Lavandula latifolia	15 (s.d. 3)

a. Mean of the two isomers.
b. Classification of toxicity based on DL_{50} (μg bee^{-1}) from Johansen and Mayer (1990).
c. Classification of toxicity based on DL_{50} (μg bee^{-1}) from the pesticide manual (Tomlin, 1997).
> 100: virtually non-toxic (VNT)
11–100: slightly toxic (ST)
2–10.99: moderately toxic (MT)
< 2: highly toxic (HT).

Visual examination of worker bees (dead and living) and of the brood did not identify any abnormalities and thus, the presence of *Nosema* spores, *Malpinghamoeba* cyst, *Varroa destructor*, *Acarapis woodi*, *Ascosphaera apis*, *Paenibacillus larvae* ssp. *larvae* and *Melissococus plutonius* were analysed using standard Office International des Epizooties (OIE) methods (OIE, 2004). The presence of *Nosema* spores was also diagnosed by Phase Contrast Microscopy (PCM) and species confirmed by PCR as described previously (Higes *et al*., 2007). In some bees whose lesions might be related to infection by *Nosema*, the ventriculus was dissected and prepared for histological studies as described previously (Higes *et al*., 2007; 2008a). The virological analysis [deformed wing virus (DWV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), Kashmir bee virus (KBV), sacbrood virus (SBV) and Israeli acute paralysis virus (IAPV)] was performed by Dr Ribiere (Agence française de sécurité sanitaire des aliments) and Dr Sela (University of Jerusalem), and it was carried out in a pool sample of around 50 worker bees from survivors colonies ($n = 15$) from Apiary 1 and 10 samples from Apiary 2 (around 10 bees per hive) as described in Higes and colleagues (2008a). Stored pollen samples ($n = 15$ for Apiary 1, and $n = 10$ for Apiary 2) were also analysed, as described previously (Higes *et al*., 2008a), in order to confirm the type of foraging crop and the flowers from which the bees were collecting pollen (palynological analysis). Accordingly multiresidue analyses to determine 40 organic micropollutants were performed (including herbicides, insecticides, varroacides, organochloride compounds and polyaromatic hydrocarbons).

bee-keepers (probably as homemade formulas), such as z-clorfenvinphos (organophosphorus) and fluvalinate (pyrethroid). Only wild pollen was detected, and there was no evidence that the bees had visited crops such as sunflower or corn. Indeed, the absence of neonicotinoids (imidaclopride or fipronil) was confirmed.

All the colonies that remained alive in January 2007 in both apiaries received treatment with fumagillin (30 mg per colony, once weekly for 4 weeks). After 3, 6 and 9 months, in the spring, summer and autumn, all the colonies were still alive and evolved normally, and PCR analysis indicated that they were not infected with *N. ceranae* (15 colonies from Apiary 1 and 100 colonies from Apiary 2). In winter 2007, no colony collapse was detected in these apiaries.

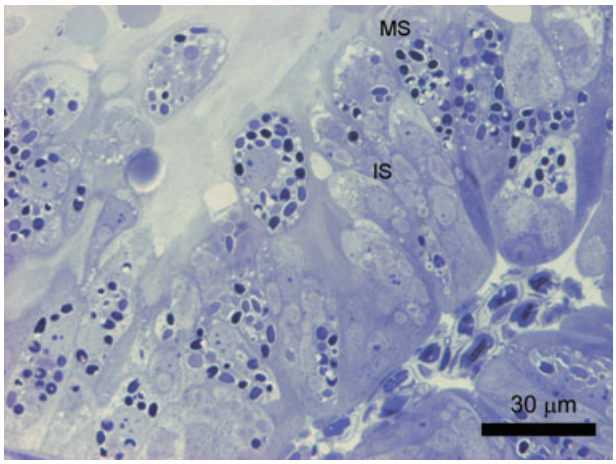

Fig. 3. Heavily infected tissue of alimentary canal of *Apis mellifera iberiensis*. Ventriculus epithelial cells showing intracellular parasite stages (MS, mature stage; IS, immature stage) of *Nosema ceranae*. Bar: 30 μm.

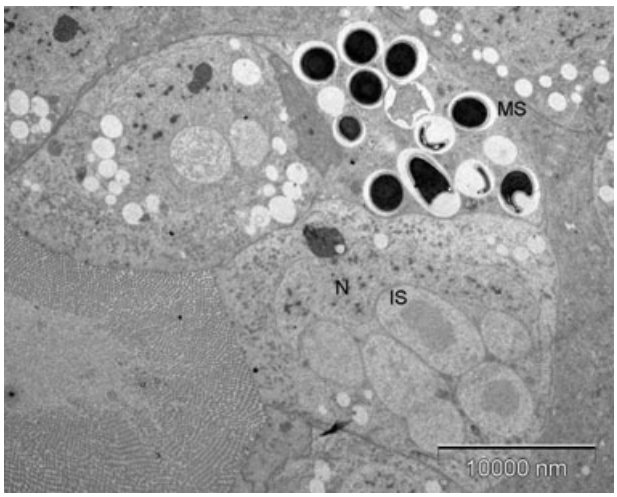

Fig. 4. Meronts (IS) and mature spores (MS) of *Nosema ceranae* located in invaginations of the nuclear membrane in apically displaced nuclei (N) of epithelial cells of ventriculus. Bar: 10 000 nm.

As described previously (Higes *et al*., 2008a), fumagillin has been proven to be effective in controlling *N. ceranae* infection in honeybee colonies and it reduces the risk of depopulation due to this microsporidium.

To the best of our knowledge, this is the first report of honeybee colony collapse in apiaries of professional beekeepers in field condition due to *N. ceranae* infection. In addition, these data provide evidence of the effectiveness of fumagillin to overcome this problem when *N. ceranae* is shown to be present.

Acknowledgements

We thank M. Ribière, F. Schurr and I. Sela for the virological analyses, and M.L. García and A. Fernández for image processing. R. Martín-Hernández and A.V. González-Porto were co-financed by the Junta de Comunidades de Castilla-La Mancha (JCCM) and the INIA-European Social Fund. The work reported here was supported by JCCM and Ministerio de Medio Ambiente y Medio Rural y Marino (API/FEGA-MAPYA FOUNDS).

References

Blanchard, P., Schurr, F., Celle, O., Cougoule, N., Drajnudel, P., Thiéry, T., *et al*. (2008) First detection of Israeli acute paralysis virus (IAPV) in France, a dicistrovirus affecting honeybees (*Apis mellifera*). *J Invert Pathol* **99:** 348–350.

Tomlin, C.D.S. (ed.) (1997) *Pesticide Manual*, 11th edn. Farnham, UK: British Crop Protection Council.

Cox-Foster, D.L., Conlan, S., Holmes, E., Palacios, G., Evans, J.D., Moran, N., *et al*. (2007) A metagenomic survey of microbes in honey bee Colony Collapse Disorder. *Science* **318:** 283–287.

Higes, M., Martín, R., and Meana, A. (2006) *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. *J Invert Pathol* **92:** 93–95.

Higes, M., García-Palencia, P., Martín-Hernández, R., and Meana, A. (2007) Experimental infection of *Apis mellifera* honeybees with the Microsporidia *Nosema ceranae*. *J Invert Pathol* **94:** 211–217.

Higes, M., Martín-Hernández, R., Botías, C., Garrido-Bailón, E., González-Porto, A.V., Barrios, L., *et al*. (2008a) How natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environ Microbiol* **10:** 2659–2669.

Higes, M., Martín-Hernández, R., Garrido-Bailón, E., Botías, C., García-Palencia, P., and Meana, A. (2008b) Regurgitated pellets of *Merops apiaster* as fomites of infective *Nosema ceranae* (Microsporidia) spores. *Environ Microbiol* **5:** 1374–1379.

Johansen, C.A., and Mayer, D.F. (1990) *Pollinator Protection. A Bee and Pesticide Handbook*. Cheshire, CT, USA: Wicwas Press.

Liu, T.P. (1984) Ultrastructure of the midgut of the worker honeybee *Apis mellifera* heavily infected with *Nosema apis*. *J Invertebr Pathol* **44:** 282–291.

Martín-Hernández, R., Meana, A., Prieto, L., Martínez Salvador, A., Garrido-Bailón, E., and Higes, M. (2007) Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Appl Environ Microbiol* **73:** 6331–6338.

Molga, P. (2008) *'La mort des abeilles met la planète en danger'. Les echos* [WWW document]. URL http://www.lesechos.fr/info/energie/4611614.htm.

Office International des Epizooties (OIE) (2004) *Manual of standards for diagnostic test and vaccines* [WWW document]. URL http://www.oie.int/eng/normes/mmanual/A_00123.htm.

Stokstad, E. (2007) The case of empty hives. *Science Magazine* [WWW document]. URL http://www.sciencemag.org/cgi/reprint/316/5827/970.pdf. doi:10.1126/science.316.5827.970.

Van Ooij, C. (2008) Buzzkill. *Nat Rev Microbiol* **6:** 650–651.

Yue, C., Schröder, M., Gisder, S., and Genersch, E. (2007) Vertical-transmission routes for deformed wing virus of honeybees (*Apis mellifera*). *J Gen Virol* **88:** 2329–2336.