# How natural infection by *Nosema ceranae* causes honeybee colony collapse

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## Summary

**In recent years, honeybees (*Apis mellifera*) have been strangely disappearing from their hives, and strong colonies have suddenly become weak and died. The precise aetiology underlying the disappearance of the bees remains a mystery. However, during the same period, *Nosema ceranae*, a microsporidium of the Asian bee *Apis cerana*, seems to have colonized *A. mellifera*, and it's now frequently detected all over the world in both healthy and weak honeybee colonies. For first time, we show that natural *N. ceranae* infection can cause the sudden collapse of bee colonies, establishing a direct correlation between *N. ceranae* infection and the death of honeybee colonies under field conditions. Signs of colony weakness were not evident until the queen could no longer replace the loss of the infected bees. The long asymptomatic incubation period can explain the absence of evident symptoms prior to colony collapse. Furthermore, our results demonstrate that healthy colonies near to an infected one can also become infected, and that *N. ceranae* infection can be controlled with a specific antibiotic, fumagillin. Moreover, the administration of 120 mg of fumagillin has proven to eliminate the infection, but it cannot avoid reinfection after 6 months. We provide Koch's postulates between *N. ceranae* infection and a syndrome with a long incubation period involving continuous death of adult bees, non-stop brood rearing by the bees and colony loss in winter or early spring despite the presence of sufficient remaining pollen and honey.**

## Introduction

As a bee colony can be considered as a complex living system of individuals that functions as a whole, disease pathology of an individual bee is different to the pathology at the colony level. Indeed, a particular pathogen can be lethal to bees but the colony may be able to compensate for their loss. In this sense, the queen is essential to maintain the population of the colony stable. Depopulation syndrome or honeybee colony collapse has recently been the focus of many reports that have tried to determine why strong colonies suddenly, or at least that's the way it seems, become weak and occasionally die (Faucon *et al.*, 2002; Higes *et al.*, 2005; Ortiz, 2005; González, 2007; Stokstad, 2007; Molga, 2008). Many colonies are lost completely without any evident signs of a disorder. Yet these observations could be just the tip of the iceberg as colony breakdown may just be the final phase of a long process or of chronic infection by a silent pathogen. During the same period that this syndrome has been detected all over the world, *Nosema ceranae* seems to have colonized *Apis mellifera* (Martín-Hernández *et al.*, 2007), and it is now frequently detected in both healthy and weak honeybee colonies (Cox-Foster *et al.*, 2007).

*Nosema ceranae* is a widespread microsporidium that seems to have recently jumped from its host, the Asian honeybee *Apis cerana*, to the worldwide honey producer, *A. mellifera. As* it was first detected outside of Asia (Higes *et al.*, 2006), its presence has been confirmed in four continents, although there is no available data from Africa (Chauzat *et al.*, 2007; Cox-Foster *et al.*, 2007; Klee *et al.*, 2007; Martín-Hernández *et al.*, 2007; Chen *et al.*, 2008). *Nosema ceranae* is very pathogenic when experimentally inoculated into *A. mellifera* (Higes *et al.*, 2007; Paxton

**Fig. 1.** Clinical monitoring of *N. ceranae* natural infection.
A. Dynamics of natural *N. ceranae* infection of a model colony and the phases of nosemosis. Percentage of *N. ceranae*-infected bees (*n* = 30), mean spore count (*n* = 30) and number of combs covered by brood or bees in the B255 colony.
B. Dynamics of the natural infected *N. ceranae* B111 colony and the phases of nosemosis.
C. Colony collapse of untreated colonies (B255, B111) and nuclei (CN1–CN5, NSN1–NSN4) could occur in the cold or warm season. Box plots display differences in the percentage of infection and spore counts between dead bees collected when colonies collapsed during the warm (April to May, *n* = 6) or cold (November to February, *n* = 5) months in the Spanish climatic conditions. The horizontal line within each box marks the median. ✴ indicates collapse of the colony. FB, forager bees; IB, interior bees.

*et al.*, 2007), and natural infection has been associated with a syndrome of gradual depopulation, copious colony death in autumn or winter and poor honey production. In fact, the risk of colony depopulation is six times higher in colonies infected with *N. ceranae* than in uninfected ones (Martín-Hernández *et al.*, 2007).

Here, we show the pathological repercussion of *N. ceranae* infection of colonies and its clinical evolution until the colony dies. We have not only analysed the natural infection of *N. ceranae*, but also demonstrate the transmission of the disease to nearby uninfected hives and the effect of fumagillin (as Fumidil B) in controlling the infection.

## Results and discussion

### Clinical monitoring

Once the presence of spores from *Nosema* spp. was detected microscopically in a productive honeybee colony (B255) in the experimental apiaries of CAR, the species was identified as *N. ceranae* and this was confirmed throughout the study (Accession Number DQ 329034). Infection was transmitted to another uninfected colony (B111) and 13 uninfected nuclei within 3 months, while another nucleus was infected after 5 months of exposure.

From the outset, it was difficult to define the health status of a colony following infection as no classic signs of nosemosis were evident (OIE, 2004). As the mean spore count fluctuates greatly from the start to the end of the disease in interior bees (Fig. 1A), this is not a reliable measure of a colony's health when bees are infected with *N. ceranae* (CATPCA analysis, Fig. S1). Indeed, *Nosema* infection cannot be assessed at the colony level on the basis of bees sampled from the brood nest (El-Shemy and Pickard, 1989). In fact, the proportion of forager bees infected with *N. ceranae* was the only useful indicator of the extent of disease in the colony (Pearson *r* = −0.8, *P* = 0.001) and like the spore count, foragers were always more infected than interior bees. The more foragers infected, the smaller the number of brood combs and the fewer frames full of bees (Pearson, *P* = 0.001, CATPCA, Fig. S1). This relationship did not hold for the proportion of infected interior bees or the spore counts. The dynamics of bees and brood combs were related to the proportion of infected foragers and the maximum mean temperatures (regression stepwise:

adjusted R2: 0.702). No statistical differences could be established with other meteorological parameters.

Four Phases of natural *N. ceranae* infection were determined (Fig. 1A). Each of the phases of clinical nosemosis were observed in the whole colonies (number of combs = 10 or higher, if honey is going to be collected and an upper box is on the brood chamber box then *n* = 20), although they were milder in the B111 hive probably because of its infection in summer and not in spring. Nuclei only displayed three phases probably because of the fact that the nuclei had fewer combs and consequently, a smaller bee population. At the outset, infected colonies looked similar to those of other uninfected hives, at least from the spring to early autumn months, without any sign of disease. This was defined as Phase 1 or the 'asymptomatic' phase. Fewer than 60% of foragers were infected (*n* = 30) and mean spore counts never rose above one million. Next phase was considered as Phase 2 or the 'replacement' phase and started when unusual colony behaviours were detected (for this region) when the queen continued to lay eggs throughout the winter months (brood area around 125 cm$^2$). The fact that this phase took place in winter when the number of newborn bees is low could explain why the percentage of foragers and mean spore counts were always higher than in Phase 1.

The third phase started the following spring when the colonies built up rapidly and the queens laid so many eggs that almost every comb was full of brood. Clinical parameters (forager infection, spore counts) were similar to Phase 1 and as a result of the increasing bee population, this Phase 3 was named as the 'false recovery' phase. The bee population had built up in such a way that swarming seemed imminent but it never happened. The egg-laying 'fever' went on until the autumn when sudden depopulation (fewer than 40% of frames covered with bees) was observed and the brood reached a minimum, defining the fourth 'depopulation' phase. In this phase, the bees were very active but 2 months later, the queen was found dead and surrounded by only young bees that probably died because of the cold (breakdown or collapse, Table 1). Pollen and honey stores were present and even then, a tiny brood spot of capped brood cells was detected in most colonies.

Only in the fourth phase were more than 40% of the interior bees infected with a much higher proportion of infected foragers (like Phase 2).
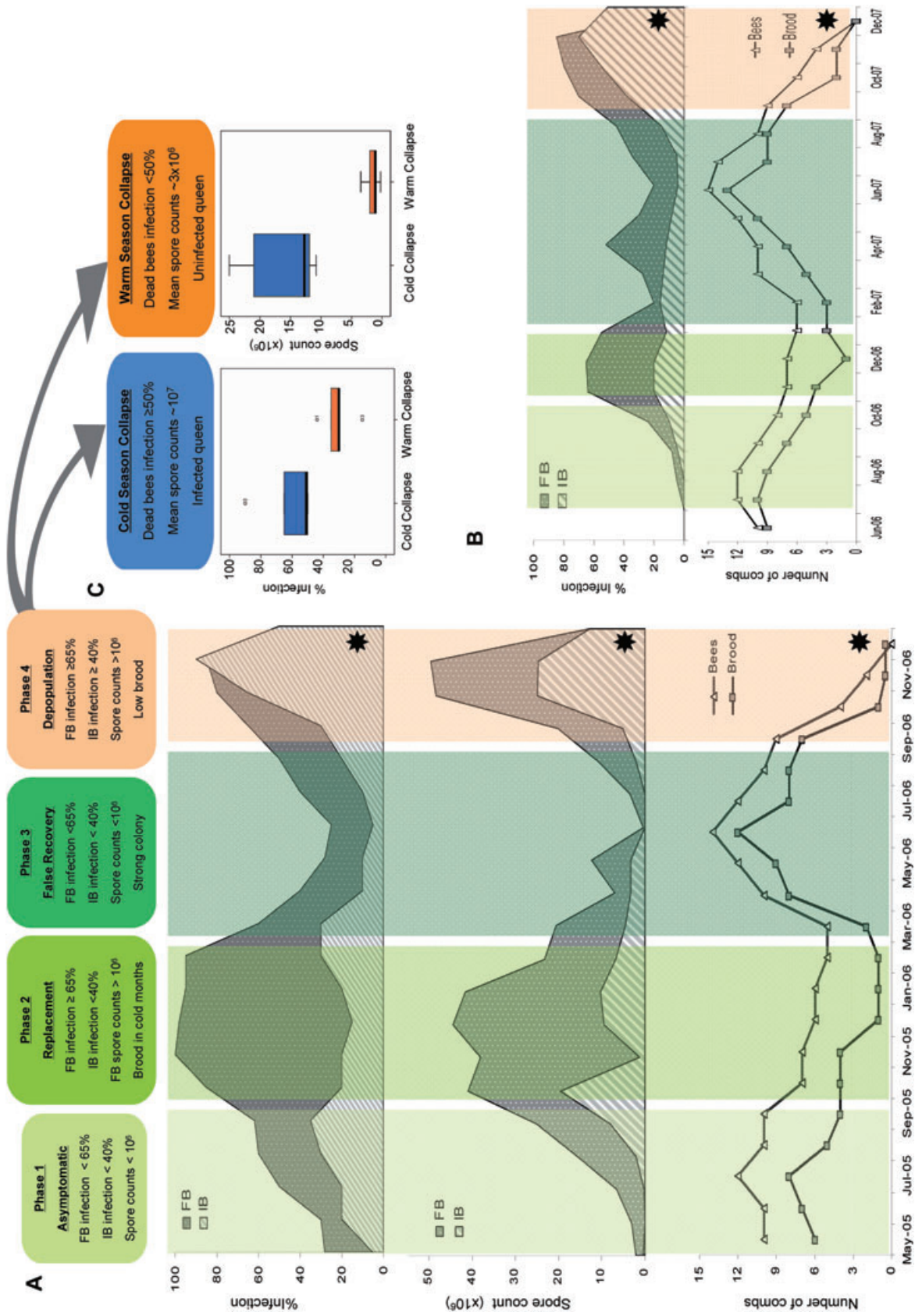
**Table 1.** Characteristics of colony breakdown in the cold or warm season.

| Colony | Brood cells | Bees | % Infection on worker bees | *N. ceranae* (spores/bee) Worker bees (mean) | Queen |
|---|---|---|---|---|---|
| **Cold season breakdown** | | | | | |
| B255 | 100 | 133 | 50 | $12.75 \times 10^6$ | $0.2 \times 10^6$ |
| B111 | 241 | 231 | 51 | $10.8 \times 10^6$ | $0.1 \times 10^6$ |
| CN6 | 50 | 13 | 90 | $21 \times 10^6$ | $0.21 \times 10^6$ |
| CN9 | 250 | 33 | 65 | $25 \times 10^6$ | Not found |
| CN10 | 350 | 65 | 50 | $11.9 \times 10^6$ | $0.4 \times 10^6$ |
| **Warm season breakdown** | | | | | |
| CN7 | 0 | 350 | 44 | $1.1 \times 10^6$ | < 50 000 (PCR+) |
| CN8 | 400 | 150 | 30 | $1 \times 10^6$ | Negative |
| NSN1 | 200 | 350 | 15 | $0.25 \times 10^6$ | < 50 000 (PCR+) |
| NSN2 | 1200 | 1081 | 35 | $3.5 \times 10^6$ | Negative |
| NSN3 | 500 | 512 | 30 | $2 \times 10^6$ | Negative |
| NSN4 | 0 | 0 | Not found | Not found | Not found |

Cold season breakdown from December to February; warm season breakdown from April to June. B255 and B111 hives and CN6–CN10 nuclei were treated and sampled monthly, while NSN1–NSN4 were nuclei not treated and not sampled till they were dead.

The dynamics of the B111 and B255 colonies were very similar. Hive B111 (Fig. 1B) was infected in summer and so, the first asymptomatic phase lasted for the 3 months of autumn and with fewer than 50% of the foragers infected, the second phase started in winter with fewer foragers infected than in hive B255 and more brood (three brood combs *versus* 1 in B255). This delayed infection coupled with enhanced replacement could explain the lower proportion of infected interior or forager bees. During the next spring and early summer (about a year after infection), false recovery was evident through the presence of brood in the combs of the upper box in June. However, again the colony did not swarm and from this 3rd phase of false recovery, the colony displayed clear signs of depopulation (Phase 4) and death was imminent.

All the untreated nuclei (CN6–CN10, NSN1–NSN4) had brood combs throughout the study until they collapsed (Fig. 1C) and at least one comb with brood was found even during the coldest months (December–February, Fig. 2A). Breakdown occurred in all the untreated nuclei (CN6–CN10, NSN1-NSN4) and none of the nine nuclei wintered. Maybe because of the smaller population (the nuclei had only 5 combs instead of 10), Phase 3 of false recovery was not seen and from Phase 2 they went directly to Phase 4 of depopulation and death. While some died in the winter, the remaining nuclei did so in the early spring, generally after a much shorter incubation period than in the whole colonies and reaching the depopulation phase 3–5 months after infection. Like whole colonies, collapse occurred in a 2 month period and again, more than 80% of the foragers (Table S1) were infected and fewer than 40% of the combs (*n* = 5) were full of bees.

Two ways of collapse were established that could be related to the moment in which the colonies or nuclei died
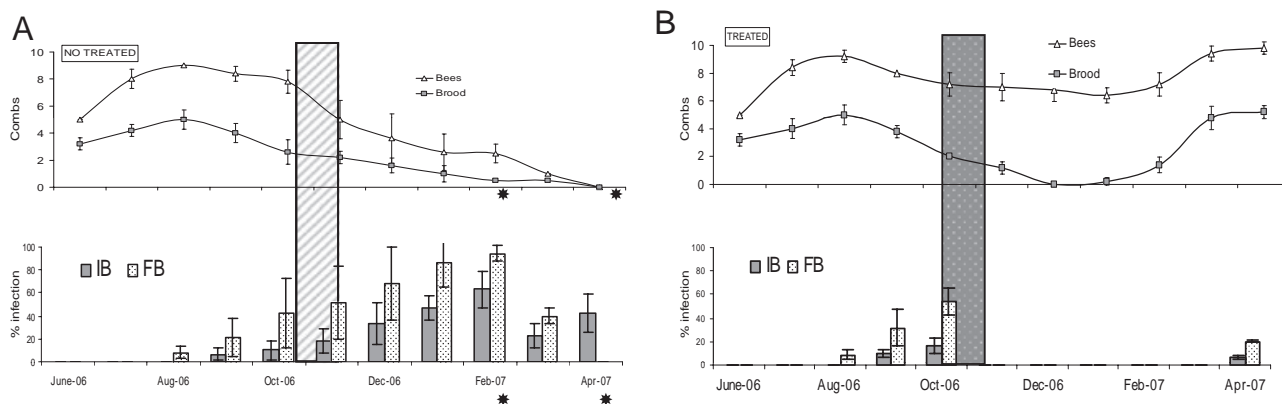


**Fig. 2.** Experiment 1: response trials with Fumidil B treatment in natural *N. ceranae*-infected nuclei. Mean dynamics of brood combs and bee combs, and the mean percentage infection of interior bees (*n* = 30) and foragers (*n* = 30) in (A) untreated (*n* = 5) and (B) fumagillin-treated (*n* = 5) nuclei (error bars as mean ± SD). Shaded areas indicate the moment of application (fumagillin in treated nuclei and syrup in untreated ones). ✱ indicates collapse of the untreated colonies.
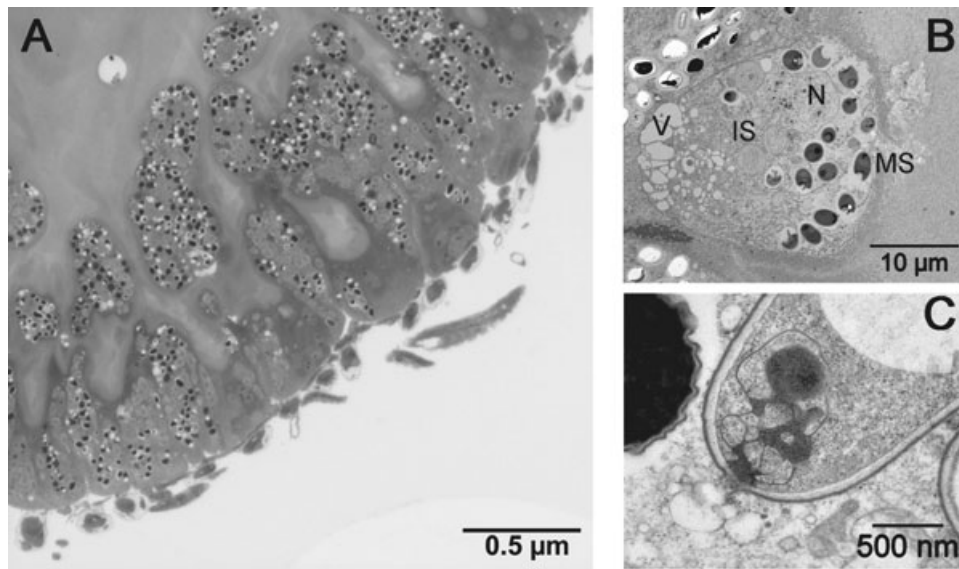
**Fig. 3.** Microscopy images of an infected bee ventriculus and *N. ceranae* stages.
A. Representative example of infected epithelial cells filled with different stage parasites. A predominance of basophilic mature spores in the apical cytoplasm is seen, the bottom of the cytoplasm is full of more vegetative parasites (MS, mature spore; N, nucleus; IS, immature stage; V, vacuole). Toluidine blue staining (20× magnification).
B. Infected epithelial cells with breakage of the cell plasma membrane at the base of the brush border. Brightly stained nucleus displaced apically and vacuolar basal degeneration were evident in the enlarged parasitized cells (4000× magnification).
C. Detail of an extruded spore inside an infected cell indicating that intracellular germination of spores takes place (60 000× magnification).

(Fig. 1C, Table 1). When collapse occurred during the cold months, more than 50% of the bees found dead inside the hive were infected, the mean spore count in these bees was always higher than 10 million, and the queens (when found) were infected. However, when collapse occurred later in early spring, the percentage of infection and mean spore counts were lower. Moreover, under these circumstances, the queens were usually uninfected or infection could only be detected by PCR. Probably, the differences between these two cases reflect the quantity of old and young bees in each season. In early spring, the proportion of newborn uninfected bees will reduce the infection parameters, thereby delaying the infection of the queen (Table 1).

Similar pathological lesions were seen in samples from dead or dying bees collected from the ground of a *Lavandula latifolia* crop and in infected bees collected from hives throughout the year, even in the summer months. Cells all along the ventriculus epithelium were infected (Fig. 3), showing comparable alterations to those described experimentally (Higes *et al.*, 2007), and intracellular germination was also seen. Indeed, there was evidence of epithelial cell degeneration and extensive lysis. The heavily infected cells may either be dead or dying, which will eventually lead to the early death of the infected worker bees as a result of starvation (Liu, 1984). Although pathological features due to *N. ceranae* closely resembled those of *N. apis*, some clinical signs usually associated with *N. apis* infection were not observed, such as crawling

bees, dysentery evidenced by the presence of fecal spots in the hive or supersedure of the queen. Foragers collected in lavender crop were infected with a mean of 21 million *N. ceranae* spores per bee. This demonstrates that some heavily infected foragers did not return to the hive.

The age at which worker honeybees begin foraging varies under different colony conditions and the age at which foraging commences seems to be delayed in the presence of foragers (Huang and Robinson, 1996). Thus, when the number of foragers is depleted, the age of onset of this behaviour drops (Huang and Robinson, 1996; Amdam and Omholt, 2003). Infestation by *Varroa destructor* mite was recently proposed to influence the flight behaviour of forager bees such that they might not return to the colony (Kralj and Fuchs, 2006). This effect was interpreted as an adaptative behaviour of the bees to expel the pathogens from the colony, a process known as 'suicidal pathogen removal'. In a other interpretation of data, the death due to *N. ceranae* could be a sudden event or a debilitating process by which bees cannot return to the hive. This population must be replaced by interior bees, which presumably start flying some days earlier than usual, as previously associated to *N. apis* infection (Hassanein, 1953). Once the queen cannot compensate for the loss of foragers, depopulation becomes evident and death is forthcoming.

Throughout the period studied, no adult or brood diseases were diagnosed, no other parasites were detected and Israeli acute paralysis virus (IAPV), a virus that was

**Table 2.** Samples from untreated nuclei were taken on the collapse date and samples from the treated ones were collected when the last untreated nuclei collapsed (April 2007).

| Colony | CBPV[a] | ABPV | DWV | SBV | KBV | BQCV | IAPV |
|---|---|---|---|---|---|---|---|
| CN1 | ND | ND | ND | ND | ND | ND | ND |
| CN2 | ND | ND | Positive | ND | ND | ND | ND |
| CN3 | Positive $7.26 \times 10^6$ | ND | ND | ND | ND | ND | ND |
| CN4 | Positive $2.01 \times 10^7$ | ND | ND | ND | ND | ND | ND |
| CN5 | ND | ND | ND | ND | ND | ND | ND |
| CN6 | Positive $6.05 \times 10^6$ | ND | Positive | ND | ND | Positive | ND |
| CN7 | ND | ND | ND | ND | ND | ND | ND |
| CN8 | ND | ND | Positive | ND | ND | ND | ND |
| CN9 | ND | ND | Positive | ND | ND | ND | ND |
| CN10 | ND | ND | ND | ND | ND | ND | ND |
| B255 | | | | | | | |
| July 2006 | Positive | ND | Positive | ND | ND | ND | ND |
| October 2006 | Positive $2.29 \times 10^5$ | ND | Positive | ND | ND | ND | ND |
| December 2006 | Positive $6.05 \times 10^6$ | ND | Positive | ND | ND | ND | ND |
| B111 | | | | | | | |
| October 2006 | ND | ND | ND | ND | ND | ND | ND |
| June 2007 | ND | ND | ND | ND | ND | ND | ND |
| November 2007 | ND | ND | ND | ND | ND | ND | ND |

| Colony | NSN1 | NSN2 | NSN3 | NSN4 |
|---|---|---|---|---|
| IAPV | ND | Positive | ND | ND |

**a.** Quantitatively analysed.
Samples were taken from B255 and B111 at different times.
ND, not detected; KBV, Kashmir bee virus; SBV, sacbrood virus; ABPV, acute bee paralysis virus; BQCV, black queen cell virus.

only identified in one of the nuclei studied, was not isolated in B255 bees, (Table 2). Indeed, this is the first report of IAPV in Spain. This virus was previously proposed as a CCD marker (Cox-Foster *et al.*, 2007); however, it is not prevalent in our studies and it was not associated with colony collapse. The microsporidium may have some influence on other viruses, as the epithelial lesions caused by the parasite reduce the natural resistance that the intestinal barrier provides against viral infection. Many viruses have been associated with *N. apis* infection (revised by Chen and Siede, 2007), possibly because of the cytopathogenic effect that facilitates transenteric viral infection. The absence of clinical signs related to viruses suggests that infection was covert or latent.

Although some viruses were detected by PCR in B255 and in six nuclei, B111 and four nuclei were negative and no relationship could be established between the presence of these viruses and collapse (Table 2). Chronic bee paralysis virus (CBPV) and deformed wing virus (DWV) viruses were always detected in B255, although there were no phenotypic signs of these infections at any moment. Indeed, the genomic load of the CBPV virus after colony death was about 20 million times lower than that necessary to produce symptoms in the nervous system (Blanchard *et al.*, 2007). Furthermore, CBPV, DWV and BQCV were also detected in another six nuclei

studied. While gut tissue may be a major reservoir for DWV (Chen *et al.*, 2006), virus particles were not detected in any intestinal sample studied by transmission electron microscopy (TEM).

The residue analysis of 40 compounds (Table S2) demonstrated that the bees from all the colonies were not exposed to any pesticides. The pollen availability, its diversity and the presence of more than enough stored pollen at the time of breakdown (Table S3) were demonstrated by palynological studies. Moreover, neither sunflower nor corn crops, previously related with large decreases in the population of bee colonies (Laurent and Rathahao, 2003), were close to the location of the hive, as reflected by the complete absence of these pollens in the samples analysed (Table S3). The presence of pollen is directly related with the beekeeping flora and indicates the botanic resources available monthly throughout the study. Twenty different taxa were found indicating high pollen diversity and availability in foraging areas. The blooming period of the different taxa identified indicated that the bees had access to sufficient flowers throughout the year, except in January.

*Experiment 1: response trial in nuclei*

In Experiment 1, fumagillin proved to be 100% effective in controlling *N. ceranae* when the bee colonies are nuclei.

**Table 3.** Experiment 2: colony response trial.

| Group | n | Intervention | Colonies positives to *N. ceranae* or death | | | | Nosemosis status 11.11.07 |
|---|---|---|---|---|---|---|---|
| | | | 30.10.06[a] | 15.12.06 | 26.04.07 | 26.09.07 | |
| Positive treated | 18 | Fumagillin (120 mg) | 18 | 0 | 5 | 18 | 18 in Phase 1 |
| Positive untreated | 15 | Syrup | 15 | 13[b] | 6 | 2 | 1 in Phase 2 |
| | | | | 2 death | 7 death[c] | 4 death[c] | 1 in Phase 4 |
| Negative untreated | 17 | Syrup | 0 | 5 | 17 | 14 | 4 in Phase 2 |
| | | | | | | | 5 death |

**a.** Pre-treatment.
**b.** Two colonies infected by *N. ceranae* and *N. apis*.
**c.** One colony infected by *N. ceranae* and *N. apis*.
Treatments (fumagillin as Fumidil· B in treated colonies and syrup in non-treated ones) were applied as described in the *Experimental procedures*. The first sampling of apiaries was made on the 30 October 2006, a day before the application of treatment. Nosemosis status represents the number of colonies according to the phases described in Fig. 1.

All treated nuclei (Fig. 2B) wintered in good health and the queens stopped laying eggs for 1 or 2 months. Indeed, these nuclei became colonies with 10 combs full of bees that developed until the next spring when they were again infected (6 months after treatment). By contrast, all the untreated nuclei had brood combs throughout the study until they died (CN6–CN10, Fig. 2A). Breakdown occurred in all the untreated nuclei (CN6–CN10, NSN1–NSN4). Furthermore, although the queen continued laying eggs during the cold months, these nuclei never developed into a whole colony. Significant differences were observed from November 2006 (brood combs) and December 2006 (bee combs). None of the treated nuclei had brood for a month or two during the cold months.

*Experiment 2: colony response trial*

In the Experiment 2 (Table 3), fumagillin treatment again controlled *N. ceranae* infection in standard size colonies. Treated hives were infected a year after the treatment had been applied and in that moment, they were clinically in Phase 1 (Fig. S2). Fumagillin treatment significantly reduced the risk of depopulation (Chi-squared, $P < 0.001$) in a 1 year period and untreated colonies were always in a more advanced clinical phase or dead (Chi-squared, $P < 0.001$).

Koch's postulates have been shown here for colonies, as previously confirmed in individual bees. In essence, we have extracted the pathogen, confirmed that it can be transmitted to healthy colonies inducing the disease and colony breakdown, and we have recovered *N. ceranae* from these newly infected colonies. Multiplication of the parasite occurs throughout the year with no standstill in its life cycle. Moreover, no differences in the pathological alterations to infected bees were observed in different seasons. Fumagillin treatment was a useful method to avoid colony collapse although it doesn't prevent reinfection.

Bee biology and colony dynamics are undoubtedly influenced by multiple facts, such as regional beekeeping management, health status or climate. The increasing prevalence of *N. ceranae* in the last 20 years, its spread (Cox-Foster *et al.*, 2007; Klee *et al.*, 2007; Martín-Hernández *et al.*, 2007; Chen *et al.*, 2008) and its capacity to cause colony collapse lead us to consider it as an emergent disease. Moreover, for the first time, our data provide experimental evidence of its aetiological role in the death of bees and colonies under field conditions.
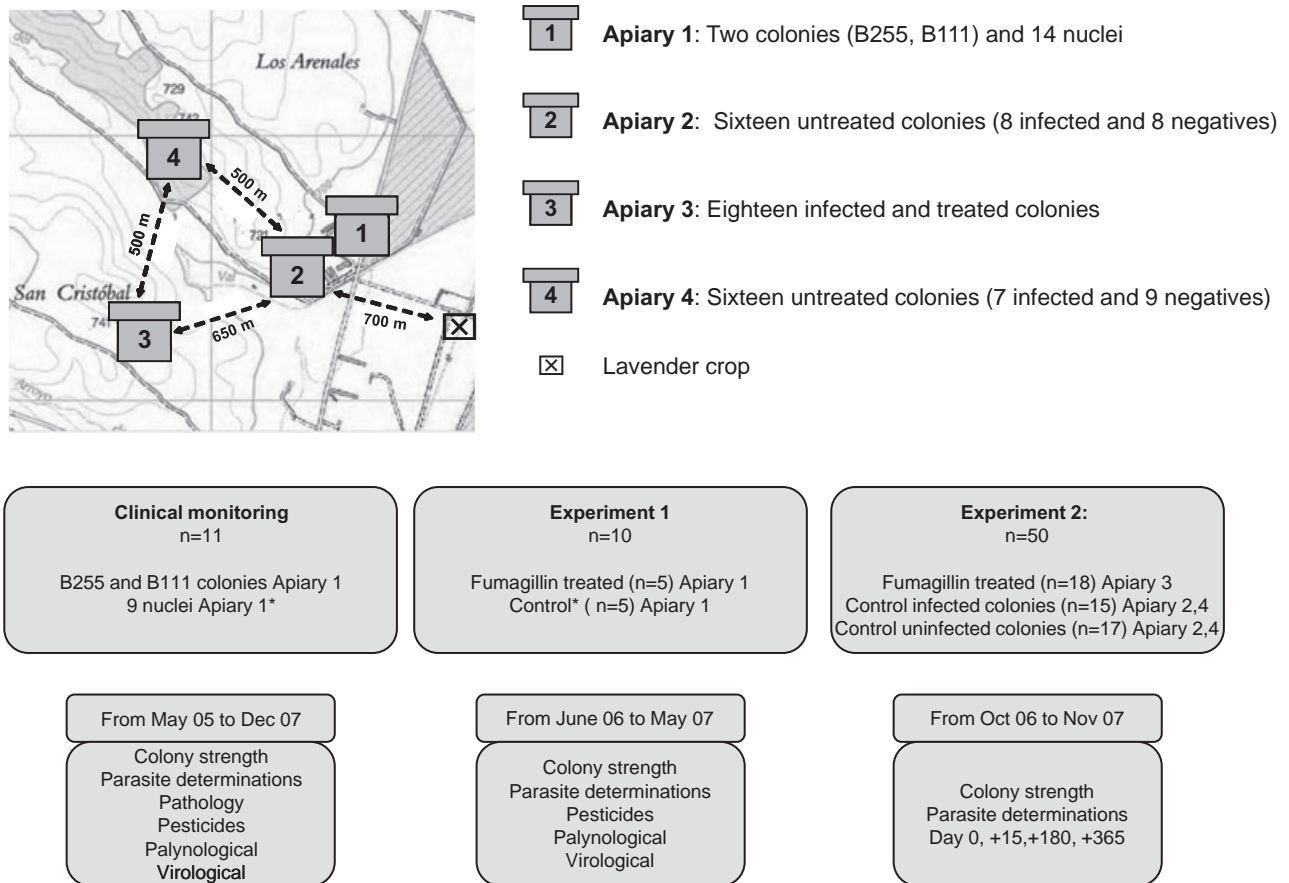
**Experimental procedure**

*Honeybee colonies*

The studies were performed on the experimental apiaries at the 'Centro Apícola de Marchamalo' (CAR, Spain; 40°40′57.47′N, 03°12′3.08′W, Fig. 4). No genetically modified agricultural crops are found in the surrounding areas, nor are insecticides such as fipronil or imidacloprid used.

All the colonies (10 bee combs) and nuclei packages (5 bee combs) were analysed to confirm the absence of any bee pathology prior to their introduction into hives at the CAR apiaries. *Varroa* was controlled during the study by twice yearly treatment with amitraz (Apiva®) and flumetrin (Bayvarol®), alternating the products each year. The absence of any brood pathology was also confirmed.

*Experimental design*

i. Clinical monitoring. The first *N. ceranae*-infected colony detected was a successful and productive colony named B255, previously used as a healthy control in various studies. The presence of the parasite was detected microscopically on the 25 May 2005, and the species was later confirmed by PCR and sequencing of the SSUrRNA gene. This colony was left untreated and from the date of detection to the final death of the colony (11 December 2006), it was monitored monthly (about colony strength, parasitic burdens, pathological studies and virological, palynological and pesticide determinations as described below).

**Apiary 1**: Two colonies (B255, B111) and 14 nuclei

**Apiary 2**: Sixteen untreated colonies (8 infected and 8 negatives)

**Apiary 3**: Eighteen infected and treated colonies

**Apiary 4**: Sixteen untreated colonies (7 infected and 9 negatives)

☒  Lavender crop

| **Clinical monitoring** n=11 | **Experiment 1** n=10 | **Experiment 2:** n=50 |
|---|---|---|
| B255 and B111 colonies Apiary 1 9 nuclei Apiary 1* | Fumagillin treated (n=5) Apiary 1 Control* ( n=5) Apiary 1 | Fumagillin treated (n=18) Apiary 3 Control infected colonies (n=15) Apiary 2,4 Control uninfected colonies (n=17) Apiary 2,4 |

| From May 05 to Dec 07 | From June 06 to May 07 | From Oct 06 to Nov 07 |
|---|---|---|
| Colony strength Parasite determinations Pathology Pesticides Palynological **Virological** | Colony strength Parasite determinations Pesticides Palynological Virological | Colony strength Parasite determinations Day 0, +15,+180, +365 |

*5 of this colonies were used as control in Experiment 1

**Fig. 4.** Diagram summarizing the distribution and location of colonies and nuclei between the different apiaries, the timecourse of sampling and infection status of *N. ceranae*.

One year after *N. ceranae* was first detected in B255 (June 2006), 14 uninfected nuclei (NSN1–NSN4; CN1–CN10) and another uninfected hive (B111) were placed close to the original hive (B255). All but four nuclei (NSN1–NSN4) were checked monthly, analysing samples in a similar manner to those of B255. These colonies (10 nuclei and B111 hive) were used to establish differences in the evolution of the clinical signs of natural infection. The remaining four nuclei were only observed visually to avoid any negative effect of manipulation on bee health and behaviour.

Once infection was confirmed, nuclei were used in an experiment designed to determine the effect of an antibiotic on the clinical evolution of nuclei through a response to treatment trial (see below).

In summer 2006 and 2007, when the bees were foraging intensively, dead bee samples were also collected from the ground from a *L. latifolia* crop situated 700 m from the apiaries 1 and 2 (Fig. 4).

ii. Experiment 1 was carried out in 10 nuclei (CN1–CN10), five of which (CN1–CN5) were treated with Fumidil B (fumagillin a.i., 1.5 g colony$^{-1}$ in a 250 cc syrup of sugar/distilled water 50%, four treatments a week, so a total of 120 mg of fumagillin per hive). Nuclei CN6–CN10 were offered the same syrup without the antibiotic (250 cc × 4 applications a week), and the treatments were applied from October 2006 to November 2006. Syrup, with or without Fumidil B, was placed in a plastic bag over the upper surface of brood combs and the entire content was consumed by the bees in fewer than 48 h. All colonies were checked monthly.

iii. Experiment 2. The same administration regime as in Experiment 1 was used for the entire 50 colonies, located in three experimental apiaries, to reproduce the real situation of beekeeping under field conditions. The distance that separated the three apiaries was ∼500 m (Fig. 4). *Nosema* infection was diagnosed in October 2006 and 18 *N. ceranae*-parasitized colonies were treated with Fumidil B in November 2006, while the other 32 colonies were left untreated as a control (13 *N. ceranae*-positive, 2 *N. apis*- and *N. ceranae*-positive and 17 microsporidia-negative).

*Sample collection and determination of colony strength*

Throughout the study, forager and interior adult bee samples were taken monthly from hives and nuclei at 12:00 at noon. Initially, the hive entrance was closed and forager bees coming from the outside were taken for 30 min. Afterwards, a

sample of interior bees was taken. Stored pollen samples from the combs were also taken monthly from the nuclei and colonies. These samples were used for multi-residue and palynological analysis in order to confirm the absence of pesticides in foraged crops and the availability of pollen reserves. Honey was only sampled monthly from hive B255.

Samples of dead adult foraging honeybees were picked up from the ground in *L. latifolia* crop.

Colony strength was determined monthly by visually counting the number of combs covered with bees or brood.

### Parasite determination

All samples from hives and the nuclei were studied to detect the presence of *Nosema* spores, *Malpinghamoeba* cyst, *V. destructor* and *Acarapis woodi* mites in forager and interior bees according to Office International des Epizooties methods (OIE, 2004). The percentage infection and the mean *N. ceranae* spore burden were calculated in 30 interior bees and 30 foragers analysed individually and checked each month (Doull and Cellier, 1961; Pickard and El-Shemy, 1989). After dissecting the bees, the gut was placed in a sterile Epperdorf and 200 μl of ddH2O was added. After thorough grinding (Eppendorf), a drop was visualized under a microscope to detect the presence of spores (Higes *et al.*, 2007). Afterwards, the 30 samples were mixed, homogenized, centrifuged and the spore count obtained. The remaining homogenate was used for PCR analysis.

Molecular diagnosis was performed monthly to identify the species in all *Nosema*-positive colonies. The analysis was performed in B255 from the 25 May 2005 to collapse on the 11 December 2006, and the nuclei were monitored monthly from June 2006 to April 2007. Determinations in B111 started in June 2006 until collapse day on the 13 December 2007. Infection was confirmed by PCR in all cases (Higes *et al.*, 2006; Martín-Hernández *et al.*, 2007).

The identification of *Nosema* was carried out when a positive spore count was identified, except for the treated nuclei where it was carried out monthly even when spores were not identified. DNA was extracted from 500 μl of the remaining homogenate after microscopy analysis of interior and foragers bees. Samples were shaken with ceramic beads (MagNA Lyser Green Beads, Roche 03 358941 001) in MagNA Lyser (Roche) at 9500 r.p.m. for 95 s and the DNA extracted with MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche 03 730964 001) in a MagNA Pure Compact instrument (Roche). From May 2005 to June 2006, PCR was performed with NOS FOR/NOS REV primers, and the products were subjected to SSUrRNA sequencing (Higes *et al.*, 2006; 2007). More recent samples were analysed by multiplex PCR (Martín-Hernández *et al.*, 2007).

### Pathological studies

Samples for pathological studies were taken from B255 and on dying bees collected from the surrounding area. Two adult interior bees and two foragers were analysed monthly by optical microscopy. After dissecting out the gut, the ventriculus was divided into sections, fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 4 μm and stained with haematoxylin and eosin. A complete histopathological study was performed in each case. The ventriculus from another three interior and forager bees from colony B255 were also analysed monthly in semithin (0.5 μm) and TEM studies (Higes *et al.*, 2007). Representative tissue areas for TEM analysis were selected after examining the semithin sections. For thin and semi-thin sections, an Olympus BX 50 microscope with an Olympus television camera was used connected to a Phillips 50× max Pentium computer with micro Image Version 4.0 for Windows 98 image analysis software. The TEM images were obtained and photographed with a Jeol 1010 electron microscope at an accelerating voltage of 80–100 kV.

### Virological determinations

Virus analysis was performed on interior and forager bees by Dr Ribiere (AFSSA) and Dr Sela (University of Jerusalem) as described previously (Stoltz *et al.*, 1995; Hung, 2000; Benjeddou *et al.*, 2001; Grabensteiner *et al.*, 2001; Bakonyi *et al.*, 2002; Blanchard *et al.*, 2007; Maori *et al.*, 2007). The samples for virological analysis were collected from the B255 colony in July 2006, October 2006 and December 2006 and on the day of colony collapse. Samples from B111 were collected in October 2006, June 2007 and November 2007. Samples from untreated nuclei were collected on the day when each nucleus collapsed. Samples from treated nuclei were also collected in April 2007, on the same day when the last untreated nucleus collapsed. In the unmanaged control nuclei (NS1–NS4), IAPV was only analysed when they collapsed.

### Palynological determinations

To confirm the type of foraging crop and the nutritive availability of pollen in colonies (B255 and nuclei CN1–CN10), 0.5 g of stored pollen was diluted in 10 ml of acidulated $H_2O$ (0.5% $H_2SO_4$) and 1 ml was taken for pollen extraction (Erdtman, 1969). Species were identified using a photographic atlas (Valdés *et al.*, 1987; Faegri and Iversen, 1989) and the reference collection of pollen slides from the CAR.

### Presence of pesticides

Honey, pollen and bee samples from B255, and pollen samples from B111 and nuclei were stored at −20°C in dark recipients until they were analysed. All samples were subjected to a multi-residue analysis (Table S2) at the Chemical Laboratory (University of Valladolid) following slight modifications of previously reported methods. Briefly, pollen and bee samples were extracted with acetonitrile, water was added to the extract and the mixture was subjected to solid-phase extraction on octadecyl silane cartridge (Jiménez *et al.*, 2007). Honey samples were diluted with methanol and solid-phase extraction on a Florisil-packed column was performed (Jiménez *et al.*, 1998). Finally, the analysis of residues in the extracts was carried out by gas chromatography with mass spectrometric detection in combination with a matrix-matched calibration.

### Meteorological data

Meteorological data were obtained from the CREA-SIAR of the 'Consejería de Agricultura de la JCCM' (http://crea.uclm.

es/~siar/datmeteo/resu_hist.php). Monthly rainfall and temperatures were recorded by standard procedures.

*Statistical analysis*

Pearson correlations (two-tailed, $P < 0.01$ level) were obtained to determine the relationship between the percentage infection and the mean spore counts in forager and interior bees, the brood combs, the combs with bees and the meteorological data in B255 colony. A principal components analysis for categorical data, CATPCA, was used to determine the relationship between multiple parameters derived from measurements on B255, and the variables were transformed with an optimal scaling process and subsequently correlated. The relationships between the data are represented in a component loading graph. A multiple linear regression analysis was performed in which the choice of predictive variables was carried out automatically selecting the best-fit model. The relationships were established between a criterion variable (frames full of bees and brood combs) and a set of predictors (percentage infection of interior and forager bees, temperatures and monthly rainfall). Statistical analyses were performed using SPSS 15.0.1 and SAS 9.1 for Windows XP.

To compare colonies from the second response trial, data were grouped as uninfected colonies ($n = 17$), infected and treated ($n = 18$) and infected and untreated ($n = 15$) at the moment the trial commenced. To study the dependence of these groups upon bee health, the statistical analysis was cross-tabulated. Chi-squared tests ($P < 0.01$) and a correspondence analysis were used to calculate the exact probabilities. To perform these analyses, a variable related to bee health at the end of the trial was established with different levels.

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## Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Component loading graph of measurements on the B255 colony. Correlated transformed variables matrix interpretation is: angle 0° related with absolute positive dependency, angle 180° with absolute negative dependency and angle 90° with independence.

**Fig. S2.** Experiment 2. Component analysis of treated and untreated hives of groups of treatment (NEG, free of infection; POS + TREATED, infection and treated; POS + UNTREATED, infection and non-treated) and bee health degrees (Phase 1, Phase 2, Phase 3, Phase 4, Phase 5_d1, colony breakdown in 12.15.06; Phase 5_d2, colony breakdown in 04.26.07; Phase 5_d3, colony breakdown in 09.26.07; Phase 5_d4, colony breakdown in 11.11.07).

**Table S1.** A. Average *N. ceranae* spore counts in treated (CN1–CN5) and untreated (CN6–CN10) nuclei.
B. Percentage of *N. ceranae*-infected bees in treated (CN1–CN5) and untreated (CN6–CN10) nuclei.

**Table S2.** Compounds studied and detection limits (LOD) by multi-residue analysis. None of them were detected in any stored pollen samples from B225 and the nuclei analysed. Adult bee and honey analysed from B255 were also negatives.

**Table S3.** Palynological analysis of stored pollen collected from cells. Percentage of taxa present in stored pollen. The percentage pollen in the nuclei was calculated as the average and standard deviation of the nuclei CN1–CN10 (treated and untreated). Spring stored pollen samples were collected in June 2006. Collapse samples from B255 on the 11 December 2006 and untreated nuclei (CN6–CN10) from February to April 2007 on the collapse date. Pollen samples stored in treated nuclei (CN1–CN5) were collected when the last untreated nuclei was found dead (April 2007).

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