Facteurs de variation des effets de *Nosema ceranae* chez l'abeille une approche de la virulence

Présentation

De nombreuses recherches scientifiques se sont intéressées au rôle de *N. ceranae* dans la mortalité des colonies d'abeilles dans différents pays, notamment parce que les effets du parasite ne sont pas consistants dans tous les cas. L'hypothèse des souches de *N. ceranae* portants différents degrés de virulence a été proposée par la communauté scientifique pour expliquer les différences de virulence rencontrées en Espagne par rapport à la France et aux pays du nord de l'Europe, mais n'a jamais été testée. Ainsi l'**hypothèse spécifique** de ce chapitre est que différents degrés de virulence des isolats de *N. ceranae,* provenant du centre de l'Espagne et du sud de la France, sont à la base de la diversité des effets observés dans ces deux régions.

Pour tester cette hypothèse nous avons comparé la virulence des deux isolats de *N. ceranae* de différentes origines géographiques chez *A. mellifera iberiensis*. Cette comparaison a été basée sur le taux de mortalité induit par les deux isolats chez des abeilles infectées au laboratoire et la charge de spores développée au cours de l'infection. Nous avons également conduit une étude permettant d'estimer la proximité génétique des deux isolats.

Article n°4:

Comparative study of *Nosema ceranae* (Microsporidia) isolates from two different geographic origins

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Résumé

Le parasite intestinal de l'abeille *Nosema ceranae* (Microsporidia) est à la base de la perte de colonies dans quelques régions, tandis que dans d'autres sa présence ne cause pas directement de la mortalité. C'est le cas de l'Espagne et de la France respectivement. L'hypothèse que nous formulons vise à expliquer les différences entre les réponses de l'abeille à *N. ceranae* par une différence de degré de virulence de souches de *N. ceranae* provenant de différentes origines géographiques. Afin de tester cette hypothèse nous avons d'abord comparé la variabilité génétique d'un fragment de l'ADNr qui pouvait mettre en évidence des différences entre les deux isolats de *N. ceranae*, l'un provenant d'Espagne et l'autre provenant de la France. En parallèle, nous avons comparé la capacité d'infection des deux isolats de *N. ceranae* chez *Apis mellifera iberiensis*, basé sur les lésions anatomopathologiques conséquentes du développement de *N. ceranae* dans l'intestin. Nous avons comparé également la charge de spores dans l'intestin et le taux de survie des abeilles. Nos résultats suggèrent que les deux isolats de *N. ceranae* utilisés dans cette étude n'ont pas une origine génétique spécifique. Ces résultats sont en accord avec le développement de l'infection, la survie des abeilles et la charge de spores qui n'ont pas présenté de différences entre les abeilles infectées avec les deux isolats de *N. ceranae*. Il se peut que les variations dans les réponses des abeilles à l'infection dans les deux régions, ne soient pas dues aux différences entre les isolats de *N. ceranae*, mais soient plutôt liées au degré de tolérance des sous espèces ou hybrides locaux d'abeilles, ou aux conditions expérimentales (cas des essais conduits en laboratoire). Des recherches plus approfondies pourront aider à estimer la contribution de chaque facteur dans la réponse des abeilles à l'infection.

Abstract

The intestinal honey bee parasite *Nosema ceranae* (Microsporidia) is at the root of colony losses in some regions while in others its presence causes no direct mortality. This is the case for Spain and France, respectively. It is hypothesized that differences in honey bee responses to *N. ceranae* infection could be due to the degree of virulence of *Nosema* strains from different geographic origins. To test this hypothesis, we first performed a study to compare the genetic variability of an rDNA fragment that could reveal differences between two *N. ceranae* isolates, one from Spain and one from France. Then we compared the infection capacity of both isolates in *Apis mellifera iberiensis*, based on the anatomopathological lesions due to *Nosema* development in the honey bee midgut, *Nosema* spore-load in the midgut and the honey bee survival rate. Our results suggest that there is no specific genetic background of the two *N. ceranae* isolates, from Spain or France, used in this study. These results agree with the infection development, honey bee survival and spore-loads that were similar between honey bees infected with both *N. ceranae* isolates. Probably, differences in honey bee response to infection are more related to the degree of tolerance of honey bee subspecies or local hybrids to *N. ceranae*, or experimental conditions in the case of laboratory trials, than to differences between *N. ceranae* isolates. Further studies should be done to estimate the contribution of each of these factors on the response of the honey bees to infection.

1. Introduction

Nosema spp. are obligate unicellular parasites that belong to the phylum Microspora. They are characterized by the production of a resistant spore that contains a polar filament which serves to transmit the genetic material to the host cell (Wittner and Weiss, 1999). Microsporidiosis of adult honey bees caused by *Nosema apis* and *Nosema ceranae* is a common worldwide disease with both, a direct negative impact on colony strength and productivity (Fries, 1988; Higes *et al*., 2010a; Mayack and Naug, 2009) and an indirect effect by interacting with other environmental stressors weakening colony health (Alaux *et al*., 2010b; Pettis *et al* 2012; vanEgelsdorp *et al*., 2009). While it has been known for a century that *N. apis* infects the European honey bee, *N. ceranae* was first isolated in 1996 in the Asian honey bee *Apis ceranae* (Fries *et al*., 1996) and recently detected in the European honey bee *Apis mellifera* in 2005 (Higes *et al*., 2006; Huang *et al*., 2007). Among the factors related to *Nosema* pathology, the existence of different *N. ceranae* isolates from distant geographic areas (Chen *et al*., 2009) that exhibit different degrees of virulence may explain the differences in the response of the honey bee to infection (Genersch, 2010). Contradictory results on *N. ceranae* virulence have been

obtained in a large number of laboratory experiments studying *N. ceranae* effects (Forsgren and Fries, 2010; Higes *et al*., 2007; Martín-Hernández *et al*., 2009; Suwannapong *et al*., 2010) as well as in field surveys of commercial apiaries (vanEgelsdorp *et al*., 2009; Higes *et al*., 2010b).

More precisely, there are apparent differences in honey bee mortality due to *Nosema* spp. between Spain and France. A large study carried out between 2002 and 2006 showed the presence of *Nosema* spp. in France in 50 to 79% of the surveyed apiaries from different regions (Chauzat *et al*., 2010a, 2010b). *N. ceranae* was found to be predominant over *N. apis* (Chauzat *et al*., 2007). In all surveyed years, the presence of the parasite was not significantly related to any acute mortality (Chauzat *et al*., 2010a, 2010b), with the exception of apiaries from a region in France not considered in the previous study where *N. ceranae* appeared to have a central role in massive losses during 2005 – 2006 (Borneck *et al*., 2010). In contrast, a study of the epidemiological factors involved in colony losses in different regions of Spain suggested that *N. ceranae* is a key factor in colony depopulation (Higes *et al*., 2010b). *N. ceranae* was much more common than *N. apis* and it was present in 95% of the samples from depopulated colonies, compared to 5% of asymptomatic beehives (Higes *et al*., 2010b). Laboratory experiments also showed a similar tendency. In France, experimental infections resulted in 10% and 50% bee mortality, 10 and 20 days post-infection respectively (Alaux *et al*., 2010b; Vidau *et al*., 2011); whereas in Spain, higher mortality has been observed over shorter study periods, the most dramatic being 100% of bee mortality 8 days post-infection (Higes *et al*., 2007).

In consequence, we tested the hypothesis that different degrees of virulence of *N. ceranae* isolates from each country could explain this differential effect of *N. ceranae*. We compared the effects on the honey bee of two *N. ceranae* isolates, originating from the National Institute of Agricultural Research (INRA) of Avignon, South of France, and the Regional Apicultural Centre (CAR) in Central Spain. In order to avoid the confounding effects of methodology we used one honey bee subspecies (*Apis mellifera iberiensis*) and a single laboratory protocol. We carried out a genetic study of a variable fragment of rDNA, looking for similar sequences or characteristic fragments of both *N. ceranae* isolates that could define strains from two different geographic origins; at the same time, we performed cage experiments to compare the development of infection of both *N. ceranae* isolates in the honey bee through the observation of histopathological lesions in the midgut and the measurement of daily mortality and spore counts.

2. Materials and methods

In October 2010, naturally-infected forager honey bees from two colonies were collected from Central Spain (Regional Apicultural Center, CAR, at Marchamalo), and the South of France (National Institute of Agronomic Research, INRA, from Avignon), to obtain fresh *N. ceranae* spores to be used in experimental infections. *Nosema ceranae* from Spain are known to cause rapid mortality of honey bees (Higes *et al*., 2008, 2010b), while *N. ceranae* from France have not been related to mass colony depopulation (Chauzat *et al*., 2010a, 2010b).

Both sets of bee samples were collected from the entrance of the hive on the same day at the two different geographic locations and maintained at room temperature, whether in Spain, in France or during the transport of the French samples to the CAR laboratory in Spain where the infections were carried out.

2.1. Obtaining *N. ceranae* spores for experimental infection

Bee samples from both geographic locations obtained as described above, were processed in parallel, in exactly the same way, to obtain fresh mature spores as described in Botías *et al*. (2011). Briefly, the abdomens of all bees were homogenized in 25 ml H_2O PCR grade for 2 min at high speed in a Stomacher® 80 Biomaster (Seward, West Sussex, UK) using strainer bags (BA6040/STR, Seward). The homogenate was recovered in a tube and 15 ml of H_2O PCR grade was again added to the strainer bag to repeat the homogenization under the same conditions. Honey bee homogenates were centrifuged (6 min at 800g), the supernatant was discarded as the spores remained in the sediment. The pellet was resuspended in 1 ml of distilled water. To confirm the *Nosema* species of the spores, an aliquot of each homogenate was analyzed by PCR as previously described (Martín-Hernández *et al*., 2011b) using 218MITOC FOR/218MITOC REV and 321APIS FOR/321APIS REV primers specific for *N. ceranae* or *N. apis* respectively and COI-F/COI-R primers for *A. mellifera* COI, as internal control of each reaction. All the PCRs reactions were carried out in a Mastercycler® ep gradient S (Eppendorf®, Hamburg, Germany). Each PCR product was analyzed in a QIAxcel System (Qiagen, Hilden, Germany), using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002) to detect positive and negative reactions. Negative controls were analyzed in parallel to detect possible contaminations in all phases of this technique. Once the *N. ceranae* species were confirmed, the spores were purified with Percoll® to obtain fresh pure spores suspension for artificial infection. During extraction and before infection, spores were kept at room temperature. The spore number was counted using a hemocytometer chamber and a phase contrast microscope.

2.2. Genetic variability of an rDNA fragment from two *N. ceranae* isolates

Up until now no reliable genetic markers have been found to be suitable to distinguish between strains of *N. ceranae*. In fact, two genetic markers can produce different phylogenetic trees of the same sample, leading to incongruous results (Ironside, 2007). Because of this, we performed a study on the variability of an rDNA fragment between samples as in Sagastume *et al*. (2011), through the amplification by PCR and cloning of the product, to look for a decrease in variability that consequently occurs because of geographic isolation.

Total DNA was previously extracted from honey bee samples naturally infected by *N. ceranae* from Central Spain and South of France (see above). PCR was performed with the pair of primers named NOS3-UPPER (5´ACTGGCTTAACTTCGGAGAG 3´) and NOS3-LOWER (5´AAGTAATACCGTTACCCGTCA 3´) which amplify a 890 bp fragment that contains the intergenic spacer (IGS) and part of the small subunit (SSU) of rDNA. In order to ensure the reliability of our results, PCR was performed using a low initial DNA concentration and a high fidelity polymerase with proof-reading capability that works with an elongation time of one minute, enough to synthesize 890 bp in each cycle of PCR (Meyerhans *et al*., 1990; Bradley and Hillis, 1997). PCR reactions started with 5 µL per tube of 1/10 diluted DNA, 0.4 µM of each nucleotide, 0.4 µM of each primer, 1.5 units of Expand High Fidelity Plus PCR System (Roche, cat no. 3300226) and its 5X buffer, 12 µg of BSA (Roche Diagnostic, cat no. 10711454001) per tube and sterilized distilled water for a final volume of 25 µl. PCR was performed in a Eppendorf Mastercycler EpGradient Pro S thermocycler with the following program: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 57.2°C for 30 sec, and 68°C for 1 min, plus a final step at 68°C for 7 min. The PCR products were kept at 4ºC, and 5 µL of each one were resolved on standard 2% agarose gels (Invitrogen E-GEL 2% Agarose GP, cat no. G8008-02) and visualized by ethidium bromide staining. 4 µL of each PCR product were cloned in *Escherichia coli* plasmid pCR2.1-TOPO® with TOPO TA Cloning® Kit (Invitrogen, cat no. K4500-01) following the manufacturer's instructions. From 4 to 6 clones were obtained per PCR product and the plasmid DNA was extracted and purified using the QIAprep Spin Miniprep Kit (Qiagen, cat no. 27106). A sample of each plasmid was digested with EcoRI (New England biolabs, R0101S) and separated in 1% agarose gel electrophoresis in order to check the correct size of the insert. The inserts from the different clones were sequenced, using the commercial primers M13, at the Alcalá de Henares University, Unidad de Biología Molecular of the Faculty of Environmental Sciences, Spain.

Fourteen sequences from GenBank (http://www.ncbi.nlm.nih.gov/nucleotide) were used to compare our results (Table 1). All the sequences were aligned following the CLUSTAL W algorithm

(Thompson *et al*., 1994) with the program BIOEDIT 7.0.5.2 (Hall, 1999), and the analysis of polymorphic sites and haplotypes generation were carried out with the program DNASP (Rozas *et al*., 2003).

Table 1. *Nosema ceranae* DNA sequences from GenBank (GB). The GB sequences shown here are haplotypes that include clones from samples with different origins (Sagastume *et al*., 2011).

GB accession	Haplotype characteristics
GU131055	Central Spain
GU131067	Central Spain
GU131109	Slovenia
GU131117	France
GU131110	Slovenia
GU131114	North Spain
GU131082	North Spain; Germany
GU131086	Germany
GU131090	Slovakia
GU131111	North Spain
GU131112	North Spain
GU131113	North Spain
GU131115	North Spain
GU131116	France

2.3. Experimental infection

Newly emerged honey bees of the local subspecies *A. m. iberiensis* were obtained as described in Higes *et al*. (2007) and Martín-Hernández *et al*. (2009) from a pool of three *N. ceranae* and *N. apis*-free honey bee colonies with queens naturally mated. Briefly, frames of sealed brood were kept in an incubator at 34ºC ± 1ºC, and when new worker bees started to emerge, they were carefully removed and confined to cages in an incubator for five days at 33ºC ± 1ºC and fed ad libitum with sugar syrup composed of 50% sucrose solution and 2% Promotor L (Calier Lab) through an individual feeder attached to the cage. Three experimental groups of honey bees were assigned as follow: honey bees infected with *N. ceranae* from Avignon (South of France), honey bees infected with *N. ceranae* from Marchamalo (Central Spain) and a group of non-infected honey bees as control. Each group was composed of three replicates of 40 honey bees, 30 of them destined to follow mortality and the other 10 selected at random for optic and electronic microscopy preparations. The five-day-old bees were starved for 2 hours and then anesthetized with CO_2 to be fed individually using a micropipette with 1 µl of a spore solution containing 40 000 spores per bee. After infection honey bees were fed ad libitum

with the same sugar syrup from the beginning. Two similar incubators (Memmert® Mod. IPP500, ± 0.1ºC) were maintained at 33º C, one contained the *N. ceranae* infected honey bees and the other the uninfected honey bees.

2.4. Histopathological study

Two honey bees per cage were collected at random both at days 12 and 16 post-infection to be used for histology preparations as described in Higes *et al*. (2007) and Martín-Hernández *et al*. (2009). After dissecting out the alimentary canal, the ventriculus and attached Malpighian tubes were divided into sections and fixed in 10% buffered formalin for 24 hours. The tissues obtained were then embedded in paraffin, and 4 µm thick sections were stained with haematoxylin-eosin to perform a complete histopathological study as described in Higes *et al*. (2007). For transmission electron microscopy (TEM) we utilized the method described in Higes *et al*. (2007) and Martín-Hernández *et al*. (2009). From each replicate cage the ventriculus and attached Malpighian tubes of two honey bees were processed. Tissue was prefixed in a 2% glutaraldehyde - 2.5% paraformaldehyde solution for a maximum of one hour, before it was washed three times in phosphate buffer (PBS, pH 7.4) and post-fixed in 1% osmium tetroxide at room temperature. After again washing in PBS and dehydrating with an ascending series of acetone, the ventriculus was embedded in Epon-Araldite resin. Semi-thin (0.5 µm) sections were cut with a Reichert-Joung ultracut E microtome, stained with 1% methylene blue in 4 % sodium borate water, and observed with an Olympus Vanox AHB53 photomicroscope. After light microscopy selection of representative tissue areas, the Epon block was trimmed before ultrathin (60 nm) sections were obtained. For TEM studies, grids were double contrasted with 2% uranyl-acetate in water and lead citrate Reynolds solution for 10 min each, then examined and photographed with a Jeol 1010 electron microscope at an accelerating voltage of 80-100 kV.

2.5. Mortality and spore counts

Dead honey bees were counted and removed daily. To compare honey bee survival between groups we used a logrank test. Spores were counted in all dead bees of the infected cages as described in Martín-Hernández *et al*. (2009) and compared through time using ANCOVA.

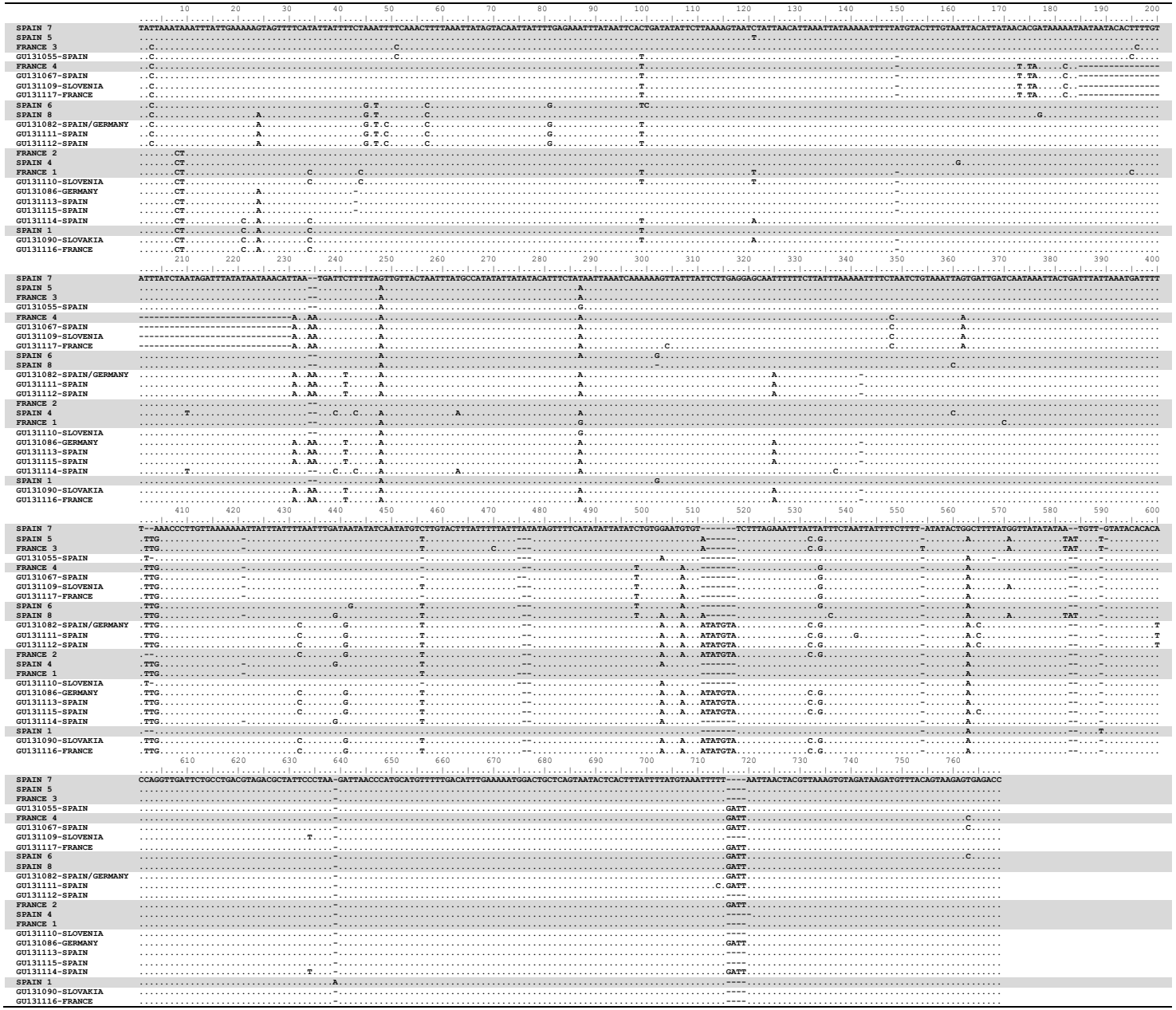
3. Results

3.1 Genetic variability of an rDNA fragment from two *N. ceranae* isolates

A total of 10 different sequences were obtained after cloning PCR products of the two *N. ceranae* isolates rDNA, 4 clones from INRA of Avignon, South of France, named “France 1, 2, 3, 4”, and 6 clones from CAR, Central Spain, named “Spain 1, 4, 5, 6, 7, 8” (GenBank accessions JQ595451 to

JQ595460). We compared them with 14 partial sequences from GenBank (Table 1). All the aligned sequences are shown in Table 2, in which we observed sequences with many polymorphic sites that appeared with different frequencies. Singletons were not discarded. Different sequences and variable sites were analyzed by haplotype generations. We did not find matching sequences, nor characteristic areas that define each sample. One haplotype per sequence was obtained and no evidence of common SNPs (Single Nucleotide Polymorphism) between clones from the same sample (France or Spain) was observed.

Table 2. Sequences of two *N. ceranae* isolates obtained cloning the PCR products of an rDNA fragment. Four and 6 clones from the French and Spanish isolates respectively (in grey) were compared with 14 sequences from the GenBank (in white). From position 1 to 594 corresponds to IGS, and position 595 to 769 corresponds to SSU gene. Polymorphic sites are shown. Dots indicate a nucleotide identical to first sequence (SPAIN 7), and dashes depict gaps.



3.2. Histophatological study

3.2.1. Light microscopy

Epithelial cell morphology in control bees showed no alterations due to methodology. There were no histological differences in the lesions produced by both *N. ceranae* groups, CAR and INRA of Avignon, in the honey bee midgut epithelium in samples taken at days 12 and 16 post-infection. At day 12 almost all the midgut was already infected and the degree of infection increased slightly until day 16. Either at the tips or at the bottom of epithelium folds there were cells that contained *N. ceranae* intracellular stages. As described previously by Higes *et al*. (2007), few unaltered epithelial cells were observed, while parasitized cells showed evidence of degeneration, such as extensive lyses and a perithrophic membrane that appeared broken and fragmented (Fig. 1).

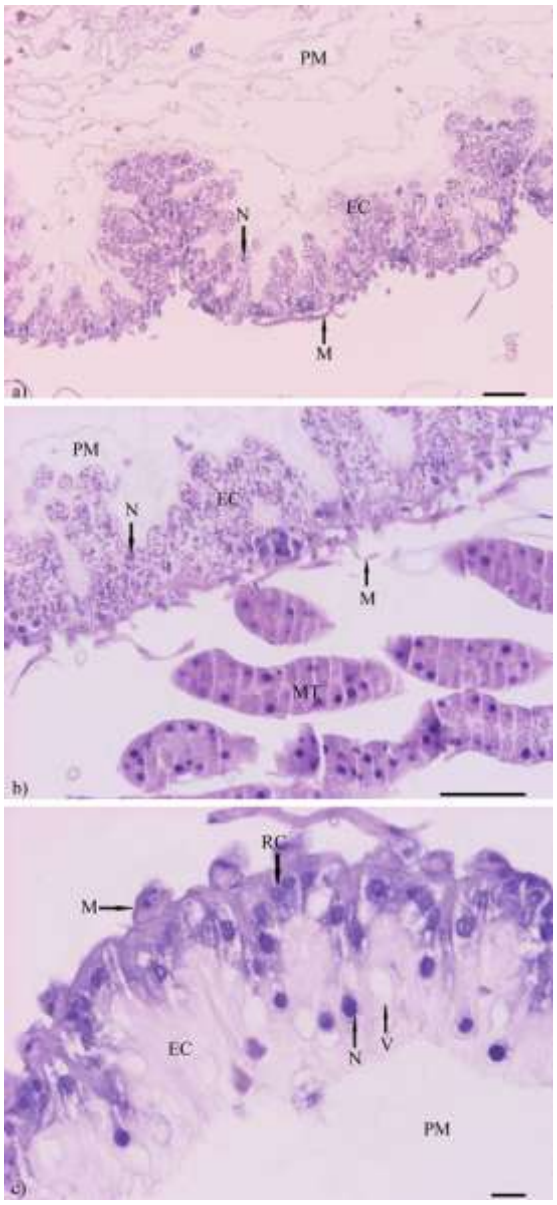


Figure 1. Midgut sections under light microscopy. At day 12th post-infection both *N. ceranae* infected groups showed similar lesions, a) *N. ceranae* from CAR (Central Spain) and b) from INRA of Avignon (South of France): infected midgut epithelial cells (EC) contained different *N. ceranae* stages and presented evidence of extensive lyses, the perithrophic membrane (PM) appeared broken and fragmented, the circular and longitudinal musculature (M) of the midgut was unchanged, no spores were found in the epithelium of Malpighian tubules (MT), small intestine or rectum (not shown in the picture); c) non-infected midgut epithelial cells with non-altered perithrophic membrane and intact cell morphology nucleus (N), vacuoles (V) and regenerative cells (RC). Bar scale: a) and b) 50 µm, c) 10 µm.

3.2.2. Electron microscopy

No differences were observed between spores or other *N. ceranae* stages developed from both isolates. At day 16 post-infection the majority of the epithelial cells were parasitized with the exception of regenerative cells in which it was not possible to distinguish any *N. ceranae* stage, however they were visible in the more developed neighboring cells. Sporoblasts and mature spores were observed in the cytoplasm; the mature spores showed the same number of coils of the polar filament, between 21 and 29, most frequently with coil numbers between 21 and 24 (Fig. 2). These observations are consistent with those of Higes *et al*. (2007, 2008) and García-Palencia *et al*. (2010), as they previously described infected cells as appearing enlarged with the nucleus apically displaced, the cytoplasm containing a larger number of mitochondria and showing evidence of degeneration, such as the presence of vacuoles and lysosomes, most of them secondary and irregularly shaped with heterogeneous electrodense areas. All stages of *N. ceranae* showed diplokaryotic nuclei that were in direct contact with the host cell cytoplasm and no evidence of grouping or enclosure within a vacuolar membrane was evident. Immature and mature stages in invaginations of the nuclear membrane were also visible, and both empty spores and germinating spores were observed inside infected cells. A predominance of basophilic mature spores in the epithelial cells at the tips of the folds was observed, while, in contrast, cells at the bottom of the folds contained less basophilic but larger stages, indicating more cells parasitized by the vegetative stage.

3.3. Mortality and spores count

Survival curves (Fig. 3) were not significantly different between infected bees (X^2 = 3.346 x 10^4, *p*-value = 0.9854, *N*=90), but they were significantly higher compared to controls (X^2 = 132.289, *p*-value < 0.0001). There were no differences between cages within all groups with the exception of one cage from CAR (Central Spain) which showed a higher mortality than others. The difference in mortality started when infected honey bees achieved a mean spore count greater than 9 x 10^6 per bee, and from that point, spore counts in dead bees increased with time from days 12 and 13 post-infection until all infected groups were dead by day 19 post-infection (Fig. 4). Spore count significantly increased with time (*F*-test = 81.792, *p*-value < 0.0001) but was not different between the two groups of *N. ceranae* (*F*-test = 0.001, *p*-value = 0.9771) with mean spore counts from CAR and INRA-Avignon of 17.92 x 10^6 (± 7.78 x 10^6) spores/bee and 16.12 x 10^6 (± 9.62 x 10^6) spores/bee, respectively. As expected throughout the study control bees were *Nosema*-negative.

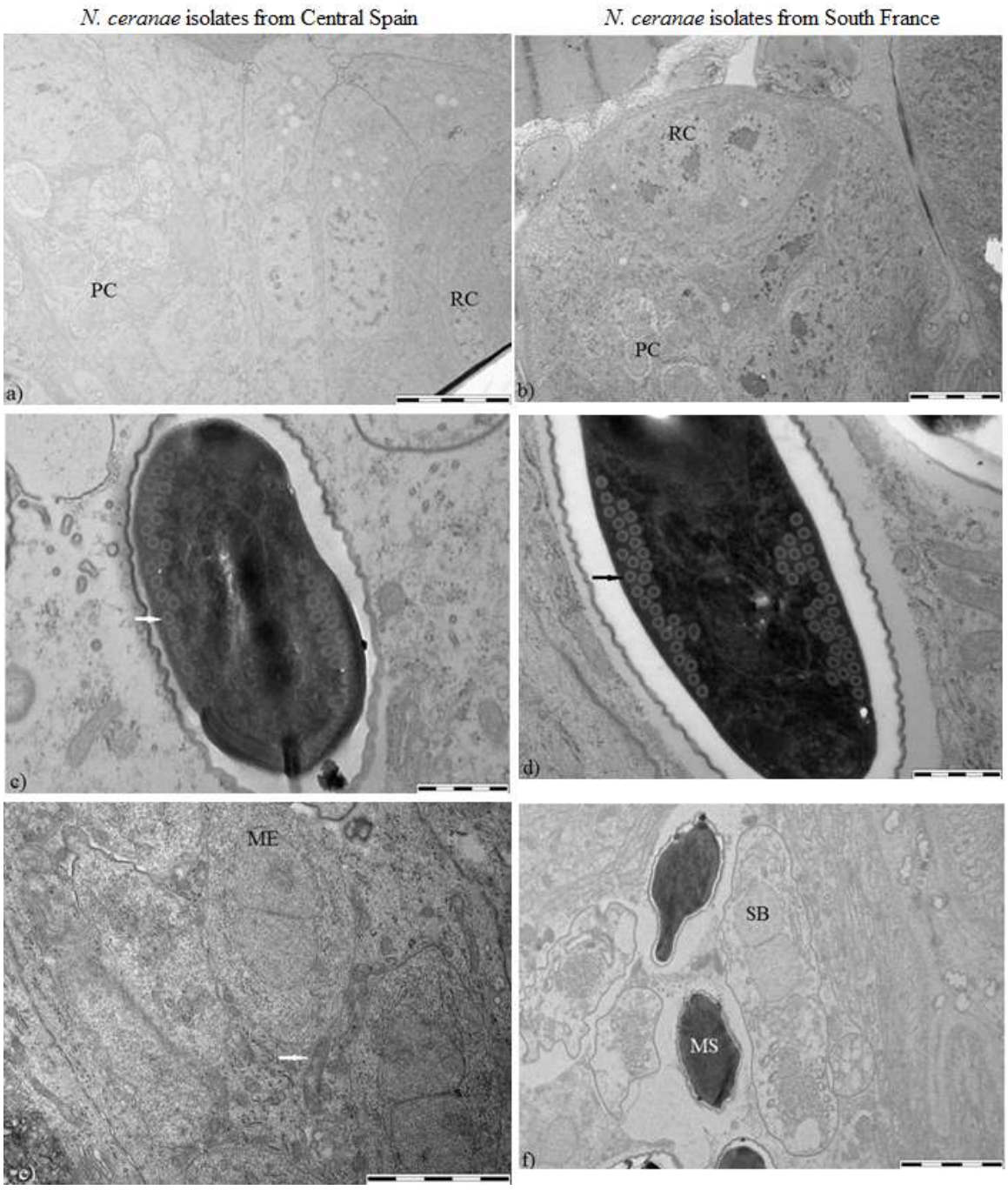


Figure 2. Midgut sections under electron microscopy show similar effects in *N. ceranae* from CAR (Central Spain) and *N. ceranae* from INRA of Avignon (South of France) infected-bees: a) and b) not infected regenerative cells (RC) surrounded by other parasitized cells (PC); c) and d) mature spores showed same number of coils (arrow) of the polar filament, between 21 and 29, been more frequent to observe spores with coil number between 21 and 24, intracellular germination was observed in both cases; e) meronts (ME) surrounded by mitochondria (white arrow), f) sporoblasts (SB) and mature spores (MS) of a *N. ceranae* in the cytoplasm. Bar scale: a) and b) 5 µm, c) and d) 0.5 µm, e) and f) 2 µm.

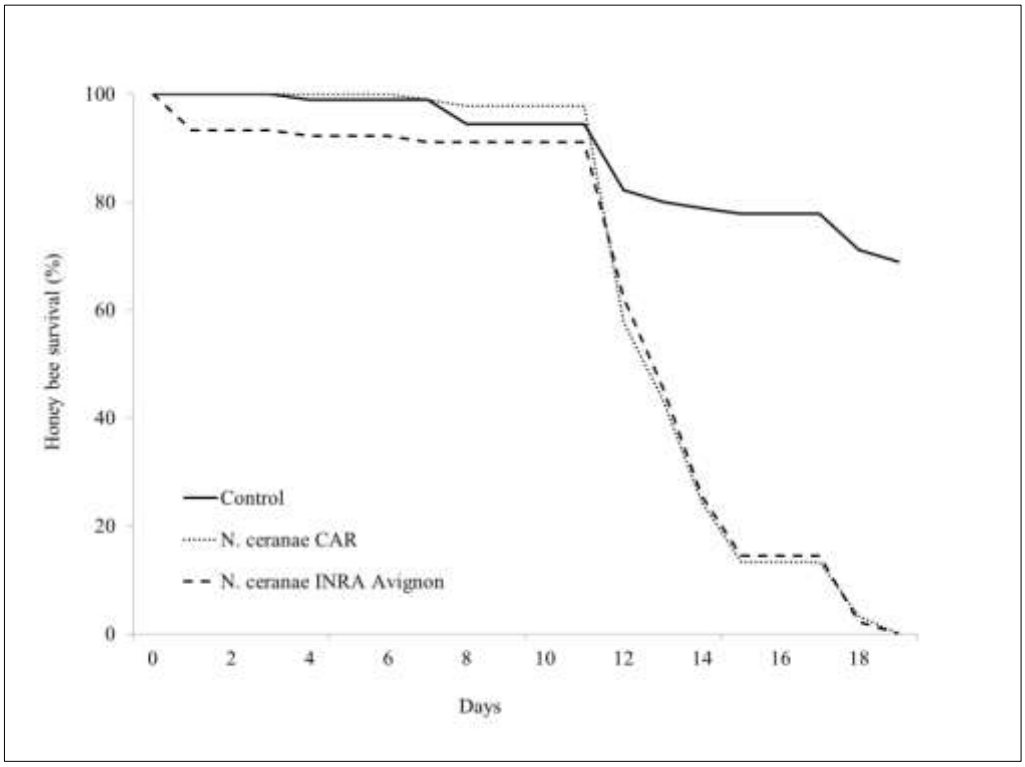


Figure 3. Survival of honey bees infected with *N. ceranae* from CAR (Central Spain) and *N. ceranae* from INRA of Avignon (South France) compared to control bees. Each curve represents a pool of 3 cages with 30 bees each (N = 90). When combining the results within the two infected groups and controls, survival curves were not significantly different between infected bees (X^2 = 3.346 x 104, p-value = 0.9854), but they were significantly higher compared to control (X^2 = 132.289, p-value < 0.0001).

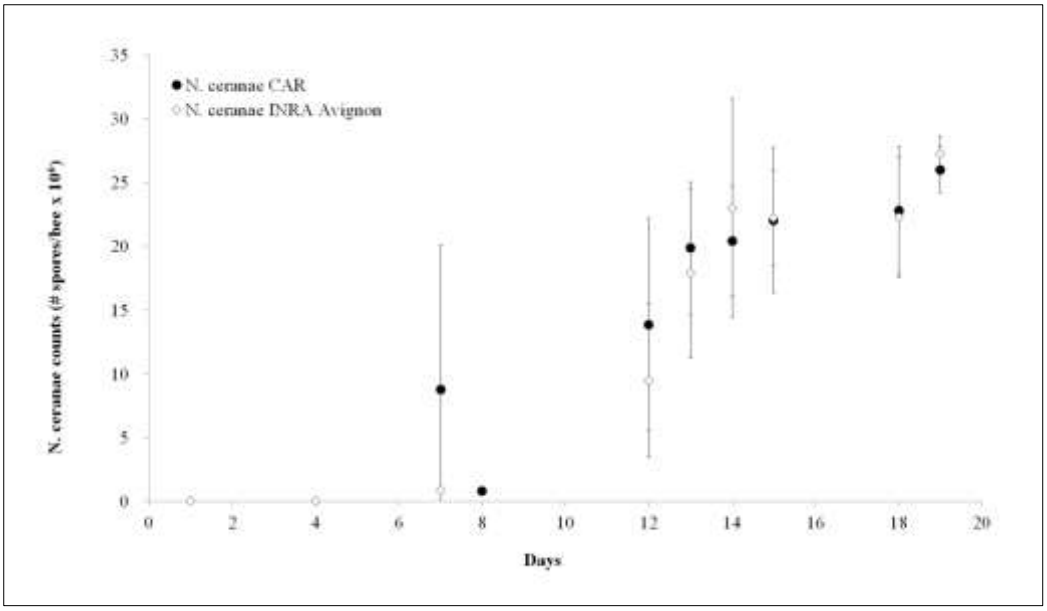


Figure 4. Spore count per bee and day (mean ± SD). Dead honey bees were removed daily from each cage and spores counted. Spore count significantly increased with time but was not different between bees infected with *N. ceranae* from CAR (Central Spain) and *N. ceranae* from INRA of Avignon (South France).

4. Discussion

In our study, we hypothesized that two different *N. ceranae* strains, each one from a different geographic origin, would differ in their virulence. We designed a genetic study amplifying a high variable region of ribosomal DNA. This fragment corresponded to an intergenic spacer (IGS) and to the first part of the small subunit gene (SSU). By now, many sequences of this fragment are available at the GenBank and were used to analyze our results. The IGS is a non-coding region of the DNA that allows the accumulation of mutations. Generally, this kind of variability can be typical of a geographic origin. In addition, a small but conserved degree of variability characterizes the beginning of the SSU gene; i.e. the insertion-deletion (INDEL) GATT at around 100 bp of distance from the beginning of the gene. In 2011 Sagastume *et al*. showed that this region (IGS+SSU) is not only highly variable, in and of itself, but also undergoes genetic recombination that contributes to increase this variability. In addition, they showed that some of these SNPs (single-nucleotide polymorphism) and INDELs are transmitted as whole blocks, generating haplotypes that can be common to multiple sequences. Thus, if the *N. ceranae* spores from South of France or from Spain are a case of genetic differentiation within the same species, we should expect: (i) a decrease in the variability of this fragment, particularly in the SSU gene; (ii) a higher probability of finding similar sequences within the same sample; (iii) groups of SNPs and common haplotypes in function of the geographic origin, that is supposed to contribute to a specialization of *N. ceranae*. We did not find those characteristic zones in our results and the variability of the fragment remained very high. This result suggests that there is no specific genetic background of the *N. ceranae* isolates from Spain or France used in this study.

Results from the genetic characterization were consistent with the anatomopathological lesions observed in the bee's midgut epithelium and the survival rate that were similar in both *N. ceranae* infected-groups but different from control. Indeed *N. ceranae* produced the same pathological lesions at cellular and tissue level in both infected-groups as a consequence of the spore germination in the midgut and subsequent multiplication in the epithelium. Our observations agree with precedent studies carried out by Fries *et al*. (1996) in *A. ceranae*, Higes *et al*. (2007) in *A. m. iberiensis* and Suwannapong *et al*. (2010) in *A. florea*. As well, García-Palencia *et al*. (2010) observed in *A. m. iberiensis* the same lesions in natural infections verifying the previous histopathological studies carried out in laboratory conditions. Spores were not detected in the Malphigean tubes or in the intestinal muscles using either optic or electronic microscopy, which suggests tissue-specificity. However, other studies have found *N. ceranae* in organs other than the honey bee midgut based on PCR signal (Copley and Jabaji 2012). More histological data would be needed to understand the presence of *N. ceranae* in other tissues and the relevance for the host-parasite relationship.

In our experiment all infected honey bees died within 19 days. Similar rates of mortality in the same or shorter periods were also observed using comparable laboratory conditions in *A. m. iberiensis* (Higes *et al*., 2007 and Martín-Hernández *et al*., 2011a for spore loads per bee over 50 000). In contrast other authors obtained lower mortality rates using a variety of honey bee types such as Buckfast (Vidau *et al*., 2011), *A. mellifera* hybrid *ligustica* x *mellifera* (Alaux *et al*., 2010b), *A. mellifera* (Forsgren and Fries, 2010), however it is difficult to compare results since these trials were ended before achieving 100% of mortality and using different rearing methods. The genetic origin of the honey bees could be a key in the understanding of differential mortality of *N. ceranae* infected bees. Indeed, selection of honey bees for low levels of *Nosema* spp. infection seems to be successfully achieved in Denmark as a result of years of selection (Huang *et al*., 2012) suggesting that different degrees of host susceptibility exist.

In our experiment spore count in dead bees showed no differences between infected-groups. The consistency of spore count suggests a similar pattern of infection development, which in turn supports the idea that the *N. ceranae* isolates are the same strain. Huang *et al*. (2012) also used *N. ceranae* isolates from apiaries at INRA Avignon in France, but tested them on two different strains of bees. As mentioned before, one bee strain was selected for tolerance to *Nosema* spp. in Denmark, and the other one was the subspecies *A. mellifera carnica* from Germany. Interestingly, tolerant bees, drones in that case, could resist higher spore-loads and present lower mortality than non-tolerant drones. Again, genetic background of the honey bee seems to be a key factor when studying response to *N. ceranae* infection.

Differences in experimental procedures between laboratories might also explain different results when performing *N. ceranae* infections. First, fresh, purified spores are preferable for experiments, given that refrigeration and freezing may reduce spore viability and virulence (Fenoy *et al*., 2009). Incubation temperature may contribute to differences in honey bee response to infection since less spores are produced at extreme incubation temperatures, like 25°C and 37°C, compared to 33°C; in field conditions sensitivity to low temperature may also explain a higher virulence of *N. ceranae* in warmer regions (Martín-Hernández *et al*., 2009). The age of the honey bees at infection may also influence the infection development (Malone, Giacon and Newton, 1996), however in our experiment we used 5-day-old bees and the rate of mortality was higher than some experiments using younger bees (Alaux *et al*., 2010b) as well as same-age honey bees (Vidau *et al*., 2011) or older honey bees (Forsgren and Fries, 2010). In addition, nutritional sources during artificial rearing are not standardized and they can be more or less favorable to honey bee health (Alaux *et al*., 2010a) and so the development of infection could differ.

5. Conclusion

We tested two *N. ceranae* isolates from different geographical origins, France and Spain, where the effects of this parasite on the honey bee are different, giving rise to the hypothesis that they should differ in virulence. The study of the genetic variability of an rDNA fragment of both *N. ceranae* isolates in addition to similarities observed in histopathological lesions of infected honey bee midgut and honey bee survival rate, suggest that differences in the honey bee response to infection could be more related to honey bee genetics (degree of susceptibility to *N. ceranae*) or experimental conditions than to differences between *N. ceranae* isolates. Future studies should be done to estimate the contribution of each of these factors on the effects of *N. ceranae* on honey bees.

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Synthèse

Les résultats de cette recherche montrent que la variabilité des effets de *N. ceranae* dans différentes régions, notamment les isolats du sud de la France et du centre de l'Espagne, ne s'expliquent pas par l'existence des souches plus ou moins virulentes de *N. ceranae* comme c'est proposé dans notre hypothèse spécifique. Ces différences sont donc dues plus probablement à une base génétique des abeilles liée aux degrés de tolérance à l'infection qui s'exprime lorsque les conditions environnementales sont favorables et que les pratiques apicoles sont appropriées. Ces dernières ont un rôle dans le développement et la transmission des maladies infectieuses chez les abeilles, comme par exemple, l'utilisation des antibiotiques contre *Nosema* spp. (« fumagiline » provenant du *Penicillium fumigatus*) qui empêche la sélection naturelle, ainsi que les grandes densités de populations des ruchers commerciaux qui favorisent la transmission des pathogènes entre colonies.

Une synthèse de l'ensemble des résultats des chapitres 2, 3 et 4 sera présentée dans la discussion générale, structurée de façon à répondre à l'hypothèse globale proposée dans l'introduction de cette thèse.