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ORIGINAL RESEARCH ARTICLE

Nosema ceranae development in *Apis mellifera*: influence of diet and infective inoculum

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Summary

To investigate the effect of nutritional condition of the honey bee *Apis mellifera* on the development of the microsporidian parasite *Nosema ceranae* under laboratory conditions, newly emerged bees were confined and fed on three *ad libitum* diets: high fructose corn syrup (HFCS) + fresh bee bread; HFCS + a commercial mixture of amino acid and vitamin, and HFCS. On day 7 post-emergence, bees from each diet treatment were individually infected with 4.60×10^4, 2.30×10^5 or 1.15×10^6 spores of *N. ceranae*, keeping later on the same diet. On days 3, 6, 9, and 12, post-infection bee midguts were removed to individually quantify the spores developed. The results indicate that this parasite multiplies successfully regardless of the inoculum given or the nutritional status of its host. When bees are fed on pollen, however, the parasite develops quickly, exhibiting significantly higher intensities than under other treatments. The longevity of infected bees fed on the same diet was not affected by the degree of parasitism, but by the quality of the *ad libitum* diet administered. The data demonstrate a parasite development that depends on host-condition. This should be considered when designing experiments to evaluate the development and virulence of this pathogen.

Desarrollo de *Nosema ceranae* en *Apis mellifera*: influencia de la dieta y el inoculado infectivo

Resumen

Para estudiar el efecto de la condición nutricional de obreras de *Apis mellifera* sobre el desarrollo de *Nosema ceranae*, se confinaron abejas recién emergidas y se sometieron a alimentación *ad libitum* con tres dietas diferentes: jarabe de alta fructosa (HFCS) + polen fresco ensilado; HFCS + mezcla comercial de aminoácidos y vitaminas, y una tercera de HFCS. Siete días luego de la emergencia, cada tratamiento nutricio fue subdividido en tres grupos y sus individuos fueron individualmente infectados con 4.60×10^4, 2.30×10^5 o 1.15×10^6 esporos de *N. ceranae*, manteniéndose luego la dieta previa a la infección. A los 3, 6, 9 y 12 días posteriores a la infección se sacrificaron abejas para obtener el ventrículo y cuantificar individualmente el número de esporos presentes. Los resultados demuestran que este parásito se multiplica exitosamente, independientemente del inóculo administrado y del estado nutricional de su hospedador. De todos modos, cuando las obreras son alimentadas con polen fresco, la infección se desarrolla rápidamente, mostrando intensidades significativamente mayores a las desarrolladas bajo otros tratamientos. La longevidad de los individuos infectados y alimentados con la misma dieta no fue afectada del grado de parasitismo desarrollado, sino por la calidad de la dieta administrada. Los datos nos muestran un desarrollo del parásito que es dependiente de la condición del hospedador, y por lo tanto, deberían ser considerados cuando se diseñan experimentos para evaluar el desarrollo y la virulencia de este patógeno.

Keywords: *Nosema ceranae, Apis mellifera,* diet, infective inoculum, experimental infection, survival

Introduction

The microsporidian parasite *Nosema ceranae* affects the gut of adult honey bees causing severe disease. It was first detected on the Asian honey bee *Apis cerana* (Fries *et al.*, 1996), and more recently reported as a parasite of the western honey bee *A. mellifera* (Higes *et al.*, 2006; Huang *et al.*, 2007). *Nosema ceranae* has become a hot

topic due to high mortality shown during some experimental infections and its possible connection with sudden colony losses. Many studies have been carried out to improve molecular diagnosis and control measures, also seeking a better understanding of the virulence, epidemiology, and comparison with *Nosema apis*, long associated with disease in *A. mellifera*. Nevertheless, very little has been discussed about the relation between nutritional resources of honey bees and *N. ceranae* development.

A common practice of beekeepers, mainly in areas with restricted pollen availability, is feeding amino acid and vitamin mixtures in syrup, not only to supply the protein requirements of the bee, but also to strengthen physiological resistance to diseases. Some studies support these practices, showing that nutritional status of bees and other insects is linked to the development of natural barriers against pathogenic microorganisms affecting the digestive tract, such as ventricular epithelial regeneration and development of the peritrophic membrane (Lehane, 1997; Zymaś, 2007; Beckage, 2008). Anyway, the effect of diet composition on the development of *Nosema* has only been studied for *N. apis* (Gontarski and Mebs, 1964; Rinderer and Elliott, 1970).

We therefore carried out the present work to test under laboratory conditions, whether the parasite's reproduction (spore production) is influenced by the quality of resources available to its host (food treatment). It is known that the infection development depends also on the infective inoculum administered, so we studied the development of *N. ceranae* on its relatively new host *A. mellifera,* under different nutritional status and spore doses.

Materials and methods

Experimental design

In April 2008, newly emerged bees were obtained from a healthy colony located in the experimental apiary of the Arthropods Laboratory at Mar del Plata, Argentina (38º10'06" S; 57º38'10" W). Over a month, five consecutive samples of 100 bees from this source were each confirmed to be negative for microsporidian spores using light microscopy squash preparations of their ventriculi. Combs were carried to the laboratory in insulated containers and workers were removed from the combs after emergence (within 6h). Bees were randomly confined to three wooden cages (150 per cage), and supplied with tap water and one of the following *ad libitum* diets: Diet S: High fructose corn syrup (HFCS), commercially named Invertose 026550®; Diet S+C: HFCS supplemented with 0.35 ml/l of a commercial mixture of amino acid and vitamin (**Apipromotor® , Apifey S.A.**); and Diet S+P: HFCS and freshly collected bee bread. The HFCS was composed of: 54.4% levulose; 40.3% dextrose; 3.0% maltose; 0.4% maltotriose; and 0.9% superior sugars. Apipromotor is a commercial product based on soy protein hydrolyzate and vitamins, containing 4g/l of total protein. Polyfloral stored pollen (fresh beebread) was collected from combs using a small palette knife and identified to the plant family taxonomic level (Myrtaceae, Brassicaceae, Fabaceae, Labiatae and Asteraceae). Bees were fed with a small container placed on the floor of the cage and diets were available to bees for six days.

Nosema spores used for inoculation were purified from a laboratory strain developed in confined worker bees. Newly emerged bees were inoculated by means feeding with 66% sucrose syrup containing spores. The bees were then sacrificed to retrieve fresh spores from their midgut. Spores were purified by the triangulation method according to Cole (1970), and were used immediately, without storage. Spores were molecularly characterized according to Martin-Hernández *et al.* (2007). The PCR fragments were purified and sequenced in the Pro-papa Laboratory EEA, INTA, Balcarce, Argentina. Afterwards, they were sequenced and matched with those published in the GenBank (NCBI).

On day 7 post-emergence, bees were starved for two hours and randomly removed from each principal plot box to be individually inoculated. We used one week old worker bees because they have the highest protein consumption both in the colony (Winston, 1987) and in the laboratory (Haydak, 1970).

Since the spore concentrations ingested by the bees in an active colony are unknown, three doses of increasing concentrations were administered, according to the range used in other experimental infections (Higes *et al*., 2007; Paxton *et al*., 2007). Concentrations of 4.60×10^4, 2.30×10^5, and 1.15×10^6 *N. ceranae* spores (inocula 1, 2 and 3 respectively) were individually fed in 10ml of 60% syrup. Manual administration was performed by placing a micropipette tip closer to the mouth parts until to complete consumption.

Each spore concentration was randomly supplied to 45 workers from each of the three principal plot cages (three replicates of 15 bees for each dose and diet treatment). Infected bees (405 workers) continued receiving the same *ad libitum* diet as before inoculation. In order to control unwanted infection due to food contamination, manipulation, or incidental ingestion of spores when the operculum is cut during bee emergence, on day 7 post emergence, 15 bees from each diet treatment (principal plot cages) were confined to three separate cages. They were individually inoculated with 60% (w/v) sucrose syrup without spores (contamination control) and sacrificed at the end of the assay to quantify presence of spores on their midgut. The experimental wooden cages, with transparent mesh walls, (0.11 x 0.14 x 0.05m) were kept in an incubator during all experiments (33 ± 2ºC and 60 ± 4% RH).

Nine randomly selected individual bees per treatment were then removed from replicate cages at 3, 6, 9, and 12 days post-inoculation (observation time 1, 2, 3, and 4, respectively). Bees were anesthetized with cold, their midguts were removed, and then fixed in 4% formaldehyde and individual spore counts were made using a

haemocytometer (Cantwell, 1970). Spore loads are also referred in the text as intensity values, according to Margolis *et al.* (1982). Any dead bees from each replicate cage were recorded daily and removed.

Statistical analysis

Data were analyzed according to a split-split design. Diets were considered as main effect plots; rates of inoculum as sub-plots, and post-infection periods prior to spore counts as sub-sub plots. This design permits the evaluation of interactions amongst all variables.

Data analyses were conducted using PROC MIXED and PROC GLM procedures (SAS Institute, 1999, SAS OnlineDoc®, Version 8, Cary, NC: SAS Institute Inc.). The main effects: diet, inoculum, time, and their interactions, were evaluated. Data were normalized using a Log 10 transformation. One-way ANOVA and Tukey's test were utilized to analyze mortality rate with a 5% level of significance, unless otherwise stated.

Results

After PCR multiplex, the DNA fragments obtained from the spores used for inoculation only exhibited a band corresponding to 218 bp, in agreement with Martín-Hernández *et al.* (2007). Sequencing results were entered in the GenBank BLASTn, which yielded 98% homology with *N. ceranae* (accession N° FJ425736).

Split-split plot analysis

The Fixed Effects Test revealed that diet, inocula and post-infection (p.i.) time were the variables with a significant effect on the number of spores developed in the midgut ($p< 0.0001$). Similarly, the response variable changed significantly under the combined effects of p.i. time with the inocula administered ($p< 0.0001$), and diet supplied ($p< 0.0001$). The interactions of diet and inocula, and the interaction of the three variables had non significant effects ($p = 0.17$ and 0.36, respectively), so we present the results separately for the interactions between p.i. time and inoculum, or p.i. time and diet.

Fig. 1 shows the mean values for the intensity of *Nosema* (untransformed data) as well as the standard error for all treatments; 90% of the sacrificed bees on day 3 p.i. did not develop a detectable degree of infection. From day 6 p.i. onwards, spores were detected in all bee samples except in "contamination control", and "S" and "S+P" diets groups for the lowest inoculum on day 9 p.i. (0.86% and 0.82% of prevalence, respectively).

Diet treatment analysis

Within times

At observation time 1 (o.t. 1), no significant differences were detected among treatments ($p = 0.9985$). At o.t. 2 and 3, significant differences arose: greater spore intensity was found in treatments including pollen than other treatments ($p < 0.0001$). The mean values for "S" and "S+C" diets did not differ during the experiment.

Between times

Spore intensity registered for bees fed with diet "S+P" increased significantly among the four observation times ($p < 0.0001$). Between o.t. 1 and 2, there was no significant increase for "S" and "S+C" diets. Such diets showed an average intensity increment at o.t. 3 ($p < 0.0001$).

Inoculum treatment analysis

Within times

Infective treatments did not show differences at o.t. 1 ($p = 0.9985$). At o.t. 2, "inoculum 1 and 2" values were not significantly different ($p > 0.05$), though the average intensity in "inoculum 3" treatment increased significantly in relation to them. At o.t. 3, "inoculum 1" yielded an average lower than the other treatments ($p < 0.0001$), there being no differences among such other treatments ($p > 0.05$). The intensity values were statistically higher at o.t. 4 for "inoculum 3" treatment ($p < 0.0001$).

Between times

The average intensity for "inoculum 2 and 3" treatments increased significantly ($p < 0.0001$) at each o.t. With regard to "inoculum 1", a significant increase was registered only at o.t. 3 and 4 (both of them $p < 0.0001$).

Descriptive analysis of growth slopes

Growth slopes for each treatment were obtained from the linear function: $m = Yf - Yi / Xf - Xi$ where m indicates the slope value; Y, the mean value of spores' counts without transformation; and X, the time between sacrifices (Table 1).

Mortality

When mortality rates within diets were compared, no significant differences were observed ($p>0.05$) among inoculation treatments (Fig. 2). Diets S and S+C showed a high mortality rate on day 10 p.i., so these treatments were discontinued. This rate was, however, significantly different from that of S+P treatment from day 5 p.i. onwards ($p = 0,005$). The mortality values in the control treatments for each diet were not included in the general statistical analyses because there were no replicates.

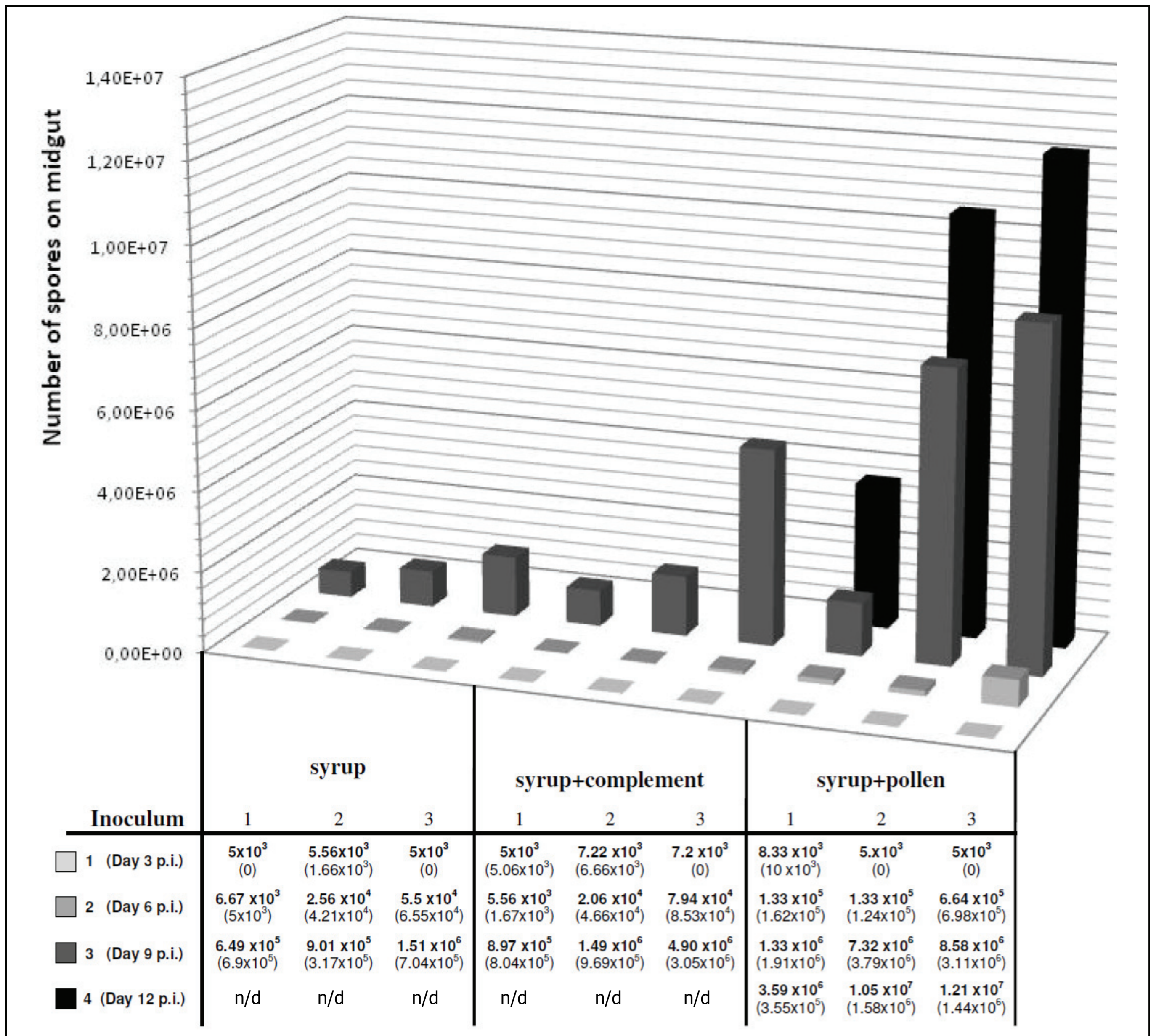

Fig. 1. Mean values ± s.e. (n = 9) of spore load for each treatment at each sacrifice time p.i. (O.T.). Inocula of *N. ceranae* spores: 1, 2 and 3 (4.60×10^4, 2.30×10^5 and 1.15×10^6 respectively). n/d: no data due to treatment discontinuation.

Table 1. Growth slopes values transformed (Log 10 (*m*)) between sacrifice times for all treatments.

		m			
DIET	**INOC.**	**DAYS 0-3**	**DAYS 3-6**	**DAYS 6-9**	**DAYS 9-12**
S+P	1	3.31	3.91	5.26	6.00
	2	3.21	4.38	6.32	6.13
	3	3.22	4.98	6.41	6.14
S+C	1	3.22	2.07	5.33	
	2	3.29	2.80	5.57	
	3	3.31	4.05	5.88	
S	1	3.23	2.55	4.84	
	2	3.25	3.33	5.46	
	3	3.23	3.61	5.43	

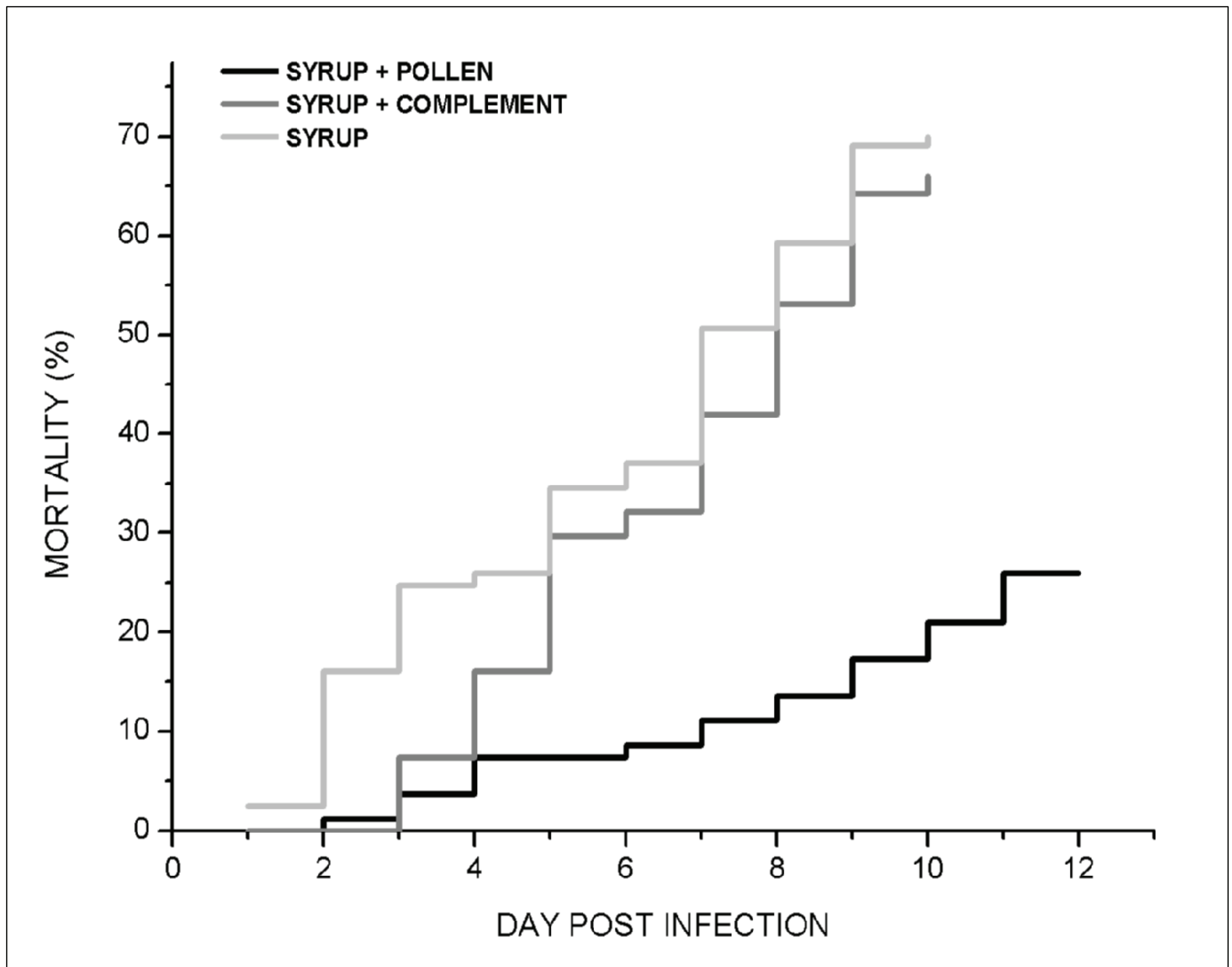

Fig. 2. Daily percentages of cumulative post-infection (p.i.) mortality grouped according to each diet treatment. Syrup+pollen (S+P) treatment showed significant differences compared to other treatments from day 5 p.i. onwards (p = 0.005).

Discussion

Our study shows that the parasite develops according to a host condition-dependent system, where spore production is influenced by food quality. The administration of a highly nutritious diet such as polyfloral bee bread, has promoted in a short period of time, the development of higher counts of *N. ceranae* spores than those for lower quality diets. Despite methodological differences, these results are in agreement with those obtained with *N. apis* spores by Rinderer and Elliott (1977), who infected newly emerged bees and counted their parasite loads in dead individuals. Knowing that the parasite meets its nutritional requirements by taking resources directly from the host cytoplasm (Vávra and Maddox, 1976; Liu, 1984), and that pollen is the natural source of protein, it can then be inferred that an individual bee fed on a highly nutritious diet promotes mechanisms that allow a higher proliferation of the parasite once it has become established. Other factors affecting parasite development cannot be disregarded, for example an expansion of honey bee midgut, and consequently an increased availability of infection sites. As discussed by Rinderer and Elliot (1977), however, this variable by itself does not seem to be able to explain the results obtained

Several authors have studied the link between diet composition and immunity in honey bees (Szymaś, 1976, 1994, 2007; Evans and Lopez, 2004; Alaux *et al.*, 2010; De Grandi-Hoffman, 2010). Our results demonstrate that despite of feeding with fresh bee bread for a period of seven days prior to infection, the multiplication of *N. ceranae* was not negatively affected. This result suggests that immunological barriers dependent on diet (epithelial regeneration of ventricular tissue, peritrophic membrane production, among others) were not useful enough to prevent parasite invasion regardless of the infective dose administered.

The longevity of infected bees fed on the same diet was not affected by spore intensity developed, but by the quality of the *ad libitum* diet administered, which was the decisive factor affecting bee survival during the experimental period. No relation between bee longevity and spore dose was also found for *N. apis* infections by

Malone and Stefanovic (1999). Similar results were registered in the 24 h analysis carried out by Mayack and Naug (2009), who demonstrated that *N. ceranae* infected foragers survive just as well as uninfected ones when fed *ad libitum*. Their results may not be comparable to long term experiments, but support the hypothesis that food availability could mask the mortality caused by energetic stress imposed by the parasite (Naug and Gibbs, 2009). Under our experimental conditions, we can conclude that mortality is a response variable that cannot be related to the pathogenicity caused by the parasite when infected adults are feed *ad libitum*. In future experiments, a greater number of bees per replicate and a longer experimental period could provide more complete results.

Although the spore load was not great for diet treatments without pollen, it is possible that mortality found with these diets has occurred in response to the presence of the parasite in combination with poor nutrition, but the lack of uninfected controls did not allow us to draw conclusions. It should also be noted that the bee mortality that we found was not sudden, as reported previously with an European isolate of the parasite (Higes *et al.*, 2007). Since different *N. ceranae* haplotypes have been described (Huang *et al.*, 2008, Williams *et al.*, 2008), differences in virulence have also been suggested. Given that regional or climatic particularities may be important for the impact of the disease, it is interesting to know specific sequences of the parasite.

Feeding proteinaceous dietary complements such as mixtures of amino acid and vitamins in syrup is a common practice in beekeeping. Malone and Gatehouse (1998) suggested that these compounds may reduce the effects of *Nosema* infections allowing bee nutrition without proteolytic digestion. They also postulated that this diet could accelerate parasite development to the detriment of the bee. In contrast, in our experiment the protein addition to the syrup did not cause significant differences in the spore counts or bee survival when it compared to syrup alone. Field trials in Argentina showed, however, that individually infected bees, introduced into colonies fed with the amino-vitamin compound, significantly increased *N. ceranae* development. At the same time, bees under this treatment were recaptured in greater numbers than workers from non supplemented colonies (Garrido, 2010), confirming the conclusions of Malone and Gatehouse (1998).

The lowest dose of *Nosema* spores administered (4,600 spores) was enough to infect almost all bees on day 9 p.i. This was in accordance with the nearly four times lower ID_{100} found by Forsgren and Fries (2010) 14 days post infection. The expected positive relationship between the infective inoculum concentration and the number of spores developed in the midgut was also confirmed, and clearly shown by the slopes found between days 6 and 9 p.i. for all treatments. After this, the speed of growth seemed to reach its peak before 12 days p.i. ($10\text{-}12 \times 10^6$ spores in the midgut) for the pollen diet, the only treatment alive. Long-term experiments may show an early plateau of parasite proliferation such as obtained by Forsgren and Fries (2010) for intensities around $20 \pm 4 \times 10^6$ spores in midgut.

The fact that bees optimally fed have shown a high rate of parasite multiplication should be considered when designing experiments to evaluate development and virulence of this pathogen. Due to the stress of bees during confinement in the laboratory, field experiments are needed for a better understanding of the interactions between the infective inoculum under hive conditions, nutritional resources, and other predisposing factors affecting parasitism. It is also necessary to investigate disease behaviour in practice when *A. mellifera* colonies parasitized by *N. ceranae* are complemented by a protein-rich diet.

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