Journal of Invertebrate Pathology 124 (2015) 107–113

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Infectivity and virulence of *Nosema ceranae* and *Nosema apis* in commercially available North American honey bees

Wei-Fone Huang [a,*], Leellen Solter [a], Katherine Aronstein [b], Zachary Huang [c]

[a] Illinois Natural History Survey, Prairie Research Institute, University of Illinois, 1816 S. Oak St, Champaign, IL 61820, USA
[b] USDA-ARS, Honey Bee Breeding, Genetics and Physiology Research Unit, 1157 Ben Hur Rd., Baton Rouge, LA 70820, USA
[c] Department of Entomology, Natural Science Building, 288 Farm Lane Room 243, Michigan State University, East Lansing, MI 48824, USA

ARTICLE INFO

Article history:
Received 25 July 2014
Accepted 20 October 2014
Available online 24 October 2014

Keywords:
Nosema ceranae
Nosema apis
Infectivity
Virulence

ABSTRACT

Nosema ceranae infection is ubiquitous in western honey bees, *Apis mellifera,* in the United States and the pathogen has apparently replaced *Nosema apis* in colonies nationwide. Displacement of *N. apis* suggests that *N. ceranae* has competitive advantages but *N. ceranae* was significantly less infective and less virulent than *N. apis* in commercially available lineages of honey bees in studies conducted in Illinois and Texas. At 5 days post eclosion, the most susceptible age of adult bees tested, the mean ID_{50} for *N. apis* was 359 spores compared to 3217 *N. ceranae* spores, a nearly 9-fold difference. Infectivity of *N. ceranae* was also lower than *N. apis* for 24-h and 14-day worker bees. *N. ceranae* was less infective than reported in studies using European strains of honey bees, while *N. apis* infectivity, tested in the same cohort of honey bees, corresponded to results reported globally from 1972 to 2010. Mortality of worker bees was similar for both pathogens at a dosage of 50 spores and was not different from the uninfected controls, but was significantly higher for *N. apis* than *N. ceranae* at dosages $\geqslant$500 spores. Our results provide comparisons for evaluating research using different ages of bees and pathogen dosages and clarify some controversies. In addition, comparisons among studies suggest that the mixed lineages of US honey bees may be less susceptible to *N. ceranae* infections than are European bees or that the US isolates of the pathogen are less infective and less virulent than European isolates.

1. Introduction

From the first identification of a microsporidian pathogen, *Nosema apis,* in honey bees (*Apis mellifera*) in the early 20th Century (Zander, 1909) to the recognition of global distribution of honey bee nosemosis in the 1990s (Matheson, 1996), the disease has been recognized as a cause of significant losses in managed colonies (Bailey and Ball, 1991). The discovery of *Nosema ceranae,* a microsporidian species described from *Apis cerana* in Asia (Fries et al., 1996), in western honey bees (Higes et al., 2006; Huang et al., 2007) and its apparent replacement of *N. apis* as the dominant microsporidian pathogen in *A. mellifera* have elicited interest in nosemosis as a factor contributing to the global decline of honey bee populations (Cox-Foster et al., 2007; Higes et al., 2009; Bromenshenk et al., 2010). Chronic *Nosema* disease reduces productivity of a colony and the life-span of foragers (Fries, 1993; Higes et al., 2007; Paxton et al., 2007; Bailey and Ball, 1991) and induces earlier task shifting of workers (Fries, 1993; Goblirsch et al., 2013). In addition, infected bees consume significantly higher amounts of sugar and have higher energy demands and stress (Mayack and Naug, 2009; Martin-Hernandez et al., 2011).

Metagenomic analyses have shown a high prevalence of *N. ceranae* in honey bee hives that exhibit sudden colony losses known as colony collapse disorder (CCD) (Cox-Foster et al., 2007; Bromenshenk et al., 2010), however, a recent comparison of pathogens in healthy and CCD colonies did not suggest a correlation because *N. ceranae* was ubiquitous in all colonies (Cornman et al., 2012). Additional investigations have shown the establishment of *N. ceranae* in *A. mellifera* colonies much earlier than the reported occurrence of CCD (Chen and Huang, 2010; Teixeira et al., 2013). It is not known when introduction of *N. ceranae* occurred in the US or at what point it apparently outcompeted *N. apis* (Chen and Huang, 2010) but *N. apis* is now rarely recovered from honey bee colonies in the US.

The reasons *N. ceranae* became the dominant microsporidian pathogen of honey bees in the US remain controversial. *N. ceranae* is not necessarily dominant at similar latitudes in Europe nor is there strong evidence that *N. ceranae* is becoming dominant in these areas (Gisder et al., 2010; Forsgren and Fries, 2013).

* Corresponding author.
E-mail address: wfhuang@illinois.edu (W.-F. Huang).

http://dx.doi.org/10.1016/j.jip.2014.10.006
0022-2011/

N. ceranae is apparently less cold-tolerant than *N. apis*; isolated spores lose viability more quickly in cold storage (Gisder et al., 2010; Paxton, 2010). However, while the climatic conditions in the US Midwest and Canada would appear to favor *N. apis*, *N. ceranae* is also the dominant microsporidian in apiaries in these areas (Chen and Huang, 2010). Only *N. ceranae* was found in 3 years of monitoring the University of Illinois apiaries using PCR detection, and *N. apis* was rarely found in Michigan State University apiaries (Huang, unpublished data).

N. apis is a midgut pathogen that is transmitted among adult honey bees, probably via combs contaminated by fecal material (Bailey and Ball, 1991) and trophallaxis (Webster, 1993). Initial suggestions that *N. ceranae* and *N. apis* occurred in cephalic tissues (Chen et al., 2009; Gisder et al., 2010; Copley and Jabaji, 2012) raised speculation that both *Nosema* species are systemic pathogens with variable transmission routes (Copley and Jabaji, 2012); however, Huang and Solter (2013) recently demonstrated that *N. ceranae* does not infect the cephalic tissues, nor were PCR signals for the pathogens detected in the hemolymph. Transmission routes appear to be the same for the two species with no apparent advantage for *N. ceranae*.

Various subspecies of honey bees have responded differently to *N. ceranae* infection (Bourgeois et al., 2012), possibly a result of strain differences of either the pathogen or the host (Antúnez et al., 2013). Researchers in Spain tested *Apis mellifera iberiensis* (Higes et al., 2007), a honey bee strain ancestrally derived from North African and western European bees, while other researchers in Europe most likely studied infection in European strains or mixed strains, such as *Apis mellifera mellifera* and *Apis mellifera ligustica*. The published results from Spain and other European countries on infectivity and mortality differed (reviewed by Fries et al., 2013). In addition, the ages of bees selected for infectivity and virulence testing often differed in reported studies (Fries, 1988; Higes et al., 2007; Forsgren and Fries, 2010), possibly leading to different results since the physiology and behavior of workers vary significantly among stages (Huang et al., 1994). Co-infection of *N. apis* and *N. ceranae* in the same host produced different results in Sweden (Forsgren and Fries, 2010), Canada (Williams et al., 2014) and in the US (Milbrath et al., in press). Forsgren and Fries (2010) reported that the species ratio of spore production at the termination of their experiments was correlated with inoculum ratio, and Williams et al. (2014) found similar results using Buckfast bees. However, Milbrath et al. (in press) showed that *N. apis* tended to produce more spores than *N. ceranae* at similar times post inoculation of hybrid bees in the US regardless of the species ratio in the inoculum, although the advantage appeared to diminish as time post inoculation increased.

One microsporidian spore can theoretically infect a host (Maddox et al., 1981) but the chance that a single spore will fail is high. Identifying appropriate inoculation dosages for *N. ceranae* is important for studying infectivity and pathogenicity, results of which can be variable due to presence of uninfected bees in the experimental group. Although high dosages can produce 100% infection, the initial dosage also affects pathogen development and proliferation (Cuomo et al., 2012; Fries, 1988; Forsgren and Fries, 2010; Fries et al., 2013) and overdosing may result in atypically severe pathogenicity. We inoculated 5-day bees with 10^6 *N. ceranae* spores and observed fewer spores in the midgut tissues than at lower dosages due to early mortality (unpublished data).

Forsgren and Fries (2010) reported that the ID_{100} dosage (dosage producing infection in 100% of inoculated bees) for *N. ceranae* is 10^4 spores per bee, but this dosage did not produce 100% infection in preliminary trials in Illinois, nor in the collaborating laboratories in Michigan and Texas (Z. H. and K. A., unpublished data). Bees in the US may have been exposed to *N. ceranae* earlier than 1995 (Chen et al., 2009), and the earliest record of *N. ceranae* in *A. mellifera* was reported as 1978 in Brazil (Teixeira et al., 2013). Huang et al. (2012) demonstrated that honey bees in Denmark developed tolerance to *Nosema* after continuous selection for decades. Although *Nosema* resistance or tolerance has not been tested in the US, apiculturists usually select the better performing hives for breeding queens, which could lead to the acquisition of some level of resistance to a ubiquitous pathogen such as *N. ceranae.*

We conducted infectivity and mortality bioassays at two different sites in the US, the USDA-ARS Honey Bee Research Unit in Weslaco, Texas and the University of Illinois at Urbana-Champaign, Illinois, using commercially available bees from hybrid queens. In an initial mortality study in Texas, we inoculated bees with different dosages of *N. ceranae* and *N. apis* and found significantly lower mortality for *N. ceranae* infections at lower dosages. The results also suggested that lower dosages generated infection rates that were lower for *N. ceranae* than for *N. apis*. We then conducted bioassays comparing *N. apis* and *N. ceranae* using workers from multiple colonies to address variation in genetic backgrounds in both Texas and Illinois. Our results provide comparisons for evaluating research using different ages of bees and pathogen dosages and clarify some controversies regarding results obtained in different laboratories.

2. Materials and methods

2.1. N. ceranae and N. apis isolates

N. ceranae was isolated from honey bees collected from apiaries at University of Illinois, Urbana-Champaign for trials in Illinois and from a commercial apiary in Louisiana for trials in Texas. *N. apis* spores were provided by Dr. Thomas Webster, Kentucky State University. Both microsporidian species were produced in caged bees in the laboratory using group inoculation with spores suspended in 50% sugar water (w:w, approximately 10^4 spores per microliter in final concentration), and we inoculated bees throughout the experimental period (May to early September) to maintain fresh spore supplies for the infectivity study. Mature spores were freshly isolated from infected midgut tissues at $\geqslant$12 days post inoculation (dpi) and counted under a microscope using an Improved Neubauer hemocytometer (Huang and Solter, 2013). Briefly, the infected midgut tissues were dissected and homogenized in sterile water using a glass tissue grinder, then centrifuged at low speed (<3000*g*) to pellet the spores. Supernatant and tissue debris were removed and the spores were re-suspended in sterile ddH_2O. The process was repeated twice to remove most tissue debris and virus particles (Carter and Saunders, 2007). Freshly isolated spores were used immediately for each experimental treatment. *Nosema* isolates were confirmed to be pure species using PCR diagnoses (Huang and Solter, 2013; Chen et al., 2009).

2.2. Cage rearing

2.2.1. Texas

100 bees per treatment were tested in wooden cages ($14 \times 12 \times 16$ cm) (Milbrath et al., in press) for mortality studies. Honey bee colonies were comprised of a majority of the Italian (*A. mellifera ligustica*) phenotype and included commercially produced queens (Koehnen and Sons Inc., USA). Colonies with no obvious virus disease symptoms and low *Varroa* mite infestations were selected. Each cage was provided with a 50% sugar water solution in a 20 ml gravity feeder placed on top of the cage. Growth chamber conditions were 33 °C, 50% RH, 24 h dark.

2.2.2. Illinois

Bees were harvested from brood frames and held in a 34.5 °C growth chamber, 60% RH, 24 h dark. The University of Illinois

apiary used commercially produced multiple-mated queens purchased from Florida, as well as queens reared on site. The majority of bees were the *A. mellifera ligustica* phenotype and we tested bees produced by a different queen for each trial. Colonies were not treated with fumagillin to avoid interference of the drug with infection (Huang et al., 2013). To accommodate more dosages, smaller cages were used, each consisting of a 480-ml HDPE cup with a 0.64 cm hardware cloth screen stapled over a cut-out on the lid (Webster et al., 2004). Two 15.2 cm wooden applicator sticks were placed in each cup for perching, and pollen patties (15% radiated pollen, MegaBee) as a protein supplement and a 35-ml gravity feeder containing 50% sugar water were placed on the screen window and fed *ad libitum*. Inoculated bees were held in a growth chamber, 30 °C, 60% RH, 24 h dark.

2.3. Infectivity tests (Illinois only)

Experimental honey bees were held in cages as described above, approximately 100 bees per cage, in a growth chamber (34.5 °C, 60% relative humidity, 24 h dark) until inoculation. Bees at three selected ages post eclosion, <24 h, 5 days, and 14 days, were individually inoculated with a series of dosages of freshly isolated *N. ceranae* and *N. apis* spores, from 50 to 10^6 spores (Table 1). Bees were starved for 2 h and anesthetized on ice before inoculation. Anesthetized bees were secured to a foam board with two crossed insect pins between the thorax and abdomen (Huang and Solter, 2013) and were inoculated individually by feeding 2 µl 50% sugar water/spore suspensions in a 10-µl pipette tip. Bees were held on the foam boards for 30 min to assure ingestion of the inoculate. Multiple infectivity trials were conducted, each using bees from a different hive, and the dosages were adjusted based on results of the first trial (Table 1).

Inoculated bees were caged, 30 bees per cage, fed *ad libitum* as previously described and incubated at 30 °C for 10 days before examination. Each treatment consisted of one cage for each trial; treatments are listed in Table 1. A minimum of three successful trials, and up to six trials, were conducted for each treatment for 24-h and 5-day bees. Insufficient 14-day bees were available for a third trial. To avoid counting transmitted infections that were expected to occur in treatment cages after the latent period of infection (Goertz et al., 2007), we used only microscopic examination to identify infections. For each determination, the posterior portion of the midgut of a surviving bee was smeared on a slide and observed under 400× magnification. Only bees with mature infections (mature spores within the host cells) were counted as infected to avoid including infections transmitted during the experimental period (Solter et al., 2010). Cages with fewer than 20 surviving bees after incubation were excluded from the analyses to avoid counting handling deaths as "uninfected".

We conducted parallel trials for *N. ceranae* and *N. apis* using the same cohort of bees, and bees for each trial were offspring of a different queen. The trials were conducted concurrently with our previous studies (Huang and Solter, 2013; Huang et al., 2013) and we used bees that emerged from the same brood frames.

2.4. Mortality tests

2.4.1. Multiple dosages (Texas)

Bees at 48 h post eclosion were inoculated by feeding either *N. apis* or *N. ceranae* spores mixed in a pollen substitute diet (19% water, 43% sugar, 38% MegaBee powder). 100× dosages (Table 1) were mixed in 1 g pollen substitute and placed at the bottom of the cages. On average, all diet was consumed within the first 24 h by the 100 caged bees. Control bees were provided pollen substitute without spores. The experiment was repeated three times using bees from different hives for a total of 27 cages. Dead bees were removed from cages daily, counted and stored at −20 °C for later analysis of infection status. Five live bees were randomly selected each week from each treatment to evaluate development of infection by counting spores. The trials were conducted from late spring through the summer season.

2.4.2. Single ID_{100} dosage (Illinois)

Individual 5-day-old bees were inoculated with 10^5 spores of either *N. apis* or *N. ceranae*, a dosage that results in 100% infection by both species based on results from preliminary infectivity tests. Bees were starved for 2 h and anesthetized on ice before inoculation. Bees were inoculated as described for the infectivity experiments. Each experiment consisted of one cage of 30 bees for each

Table 1
Dosages and bees used in mortality and infectivity trials.

Trial	Location	Dosages		*Nosema* species	Bees[a]
Mortality	Texas	5.0×10^1	5.0×10^4	*N. apis* and *N. ceranae*	3 colonies
		5.0×10^2			
		5.0×10^3			
	Illinois	1.0×10^5		*N. apis* and *N. ceranae*	3 colonies
Infectivity	Illinois	1.0×10^2	1.0×10^4	*N. ceranae*	Newly emerged bees (<24 h), 5 colonies
		5.0×10^2	5.0×10^4		
		1.0×10^3	1.0×10^5		
		5.0×10^3	1.0×10^6		
		5.0×10^2	1.0×10^4	*N. apis*	Newly emerged bees (<24 h), 3 colonies
		1.0×10^3			
		5.0×10^3			
		1.0×10^2	5.0×10^3	*N. ceranae*	5-day-old bees, 6 colonies
		5.0×10^2	1.0×10^4		
		1.0×10^3	5.0×10^4		
		5.0×10^1	1.0×10^3	*N. apis*	5-day-old bees, 6 colonies
		1.0×10^2	5.0×10^3		
		5.0×10^2	1.0×10^4		
		5.0×10^2	1.0×10^4	*N. ceranae*	14-day-old bees, 2 colonies
		1.0×10^3	5.0×10^4		
		5.0×10^3	1.0×10^5		
		1.0×10^2	5.0×10^3	*N. apis*	14-day-old bees, 2 colonies
		5.0×10^2	1.0×10^4		
		1.0×10^3			

[a] One colony was used for each trial; the same colony was used for different ages of bees.

microsporidian species and a control cage (fed sugar water without spores). Three trials were conducted.

2.5. Statistical analysis

Kaplan–Meier survival analysis was used to compare mortality results. Significance of the difference between different experimental sets was calculated using the Log-rank (Mantel–Cox) test. Linear regression between log phase of dosages and infection rate was used to estimate the ID_{50} (dosage infecting 50% of inoculated bees) and ID_{100} for each trial (Forsgren and Fries, 2010). Two-way ANOVA was used to determine the effect of *Nosema* species (*N. ceranae* or *N. apis*) and ages of workers on ID_{50} and ID_{100}. Survival analyses were done using Prism 6.0 (GraphPad software) and using SPSS 16.0 (IBM) for two-way ANOVA with Tukey's Post-Hoc test and additional one-way ANOVA to determine the significance of single variants (one pathogen or specific age of bees).

3. Results

3.1. Infectivity

Newly emerged bees were evaluated in three trials for *N. apis* infectivity and five trials for *N. ceranae* infectivity. A fourth trial for *N. apis* (five cages) was excluded due to unusually high mortality in all treatments. We excluded another six cages over all the trials, four from newly emerged bees (two *N. apis* and two *N. ceranae*) and two from 5-day bees (one *N. apis* and one *N. ceranae*). Six trials for both *Nosema* pathogens were completed for 5-day bees, and two trials were completed for both pathogens for 14-day bees (Table 2). There were not sufficient 14-day bees for additional trials, so only the results for newly emerged bees and 5-day bees were statistically analyzed. However, with 60 bees per treatment for 14-day bees, the results are included in Fig. 1 and Table 2 for comparison.

Differences in ID_{50} and ID_{100} for different experimental treatments are shown in Fig. 1. The mean ID_{50} of *N. ceranae* was 10,053 (±4147) spores for 24-h old bees, 3217 (±1268) spores for 5-day-old bees, and 5009 spores for 14 day-old bees (Table 2). The mean ID_{50} of *N. apis* was 3192 (±1105) spores for 24-h bees, 359 (±92) spores for 5-day bees, and 263 spores for 14-day bees. Analysis using two-way ANOVA showed significant interaction between *Nosema* species and age of tested bees on ID_{50} ($P = 0.051$), but not on ID_{100} ($P = 0.852$). The ID_{50} was significantly higher for *N. ceranae* than *N. apis* for all tested ages ($P < 0.001$). Newly emerged bees were significantly less susceptible than 5-day bees for both *Nosema* species. Overall, *N. ceranae* ID_{50} was significantly affected by host age differences but *N. apis* infectivity was not.

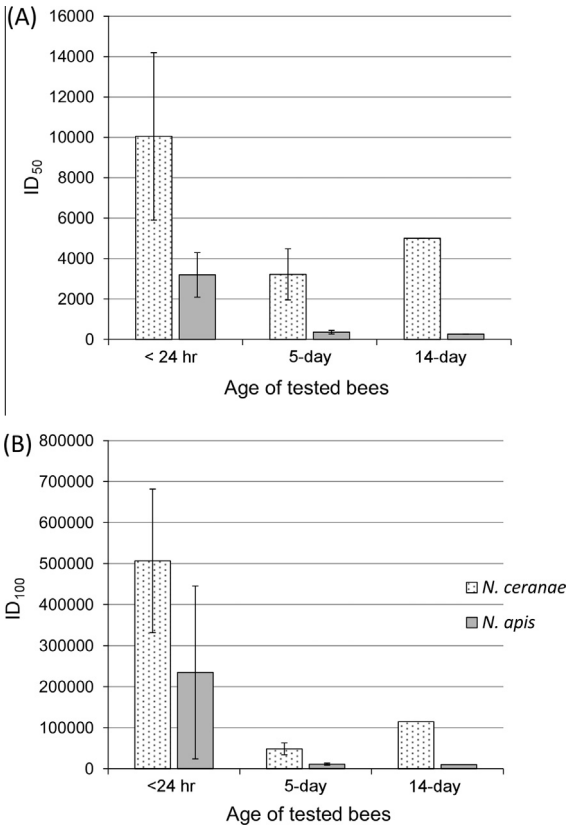

Fig. 1. Infectivity results for *Nosema apis* and *Nosema ceranae* infection in honey bees. (A) ID_{50} of *N. apis* and *N. ceranae* at 24 h, 5-day, and 14-day post eclosion; (B) ID_{100} at 24 h, 5-day, and 14-day post eclosion. The error bars show standard errors for the trials. Two trials conducted for 14-day bees were not statistically analyzed and are shown only for comparison with the newly emerged and 5-day bees. The difference between infectivity of *N. ceranae* and *N. apis* was smaller for the ID_{100} test than for the ID_{50} test.

N. ceranae and *N. apis* ID_{100} results (Fig. 1B and Table 2) differed somewhat from the ID_{50} results. Although the tested dosages (Table 1) for 24-h bees did not cover the calculated ID_{100} (Table 2) for *N. apis*, the projected ID_{100} dosages across tested ages was significantly higher for *N. ceranae* than for *N. apis* ($P = 0.001$). ID_{100} for both *Nosema* species were significantly affected by age ($P = 0.02$). The 95% confidence levels for treatments are listed in Table 2.

The standard errors (SE) associated with infectivity for newly emerged bees were higher than for 5-day old bees for both *N. ceranae* and *N. apis*.

Table 2
Infectivity of *Nosema apis* and *Nosema ceranae* in honey bees and the infectivity reported in studies conducted in Europe, New Zealand and the US.

Nosema species	Age of bees post eclosion	ID_{50}	ID_{100}	Previously published results
Nosema apis	<24 h	3192.7 ± 1105.3	234620.1 ± 210564.8 (1,140,600[a])	2×10^5 (ID_{100}) spores (Malone and Gatehouse, 1998); ID_{50} = approx. 5000 spores (Malone et al., 2001)
	5 days	359.7 ± 92.2	10918.7 ± 2630.7 (17681.2[a])	ID_{50} = 22 spores (Bailey, 1972); ID_{50} = 88 spores (Fries, 1988)
	14 days	263.4	9792.3	$ID_{100} = 10^4$ spores (Forsgren and Fries, 2010)
Nosema ceranae	<24 h	10053.1 ± 4147.0	506778.2 ± 175030.2 (992739.9[a])	40–60% infection using $1–2 \times 10^5$ spores, group feeding (Pettis et al., 2013)
	5 days	3217.2 ± 1267.5	48441.2 ± 14427.6 (85528.5[a])	1.25×10^5, >ID_{100} spores (Higes et al., 2007)
	14 days	5009.8	114861.1	$ID_{100} = 10^4$ spores (Forsgren and Fries, 2010)

[a] Upper end of 95% confidence.

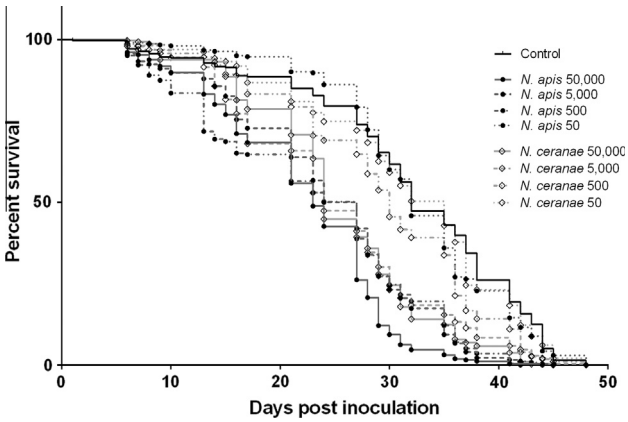

Fig. 2. Survival curves for serially increasing dosages of *Nosema apis* and *Nosema ceranae*. Median survival times for *N. apis* infections: 23 d for 50,000 spores, 25.5 d for 5000 spores, 27 d for 500 spores, and 32 d for 50 spores. Median survival times for *N. ceranae*: 24 d for 50,000 and 5000 spores, 30 d for 500 spore, and 35 d for 50 spores. Median survival time for uninfected control bees: 32 d.

3.2. Mortality – multiple dosages

The survival curves for multiple spore dosages are shown in Fig. 2. All but the lowest dosage of both pathogens significantly reduced the life span of the caged bees ($Ps < 0.001$ in log-rank tests between inoculated bees and control bees for all treatments $\geqslant 500$ spores per bee). The median survival time (LT_{50}) was 32 days for the control, and 23–35 days for inoculated bees (Fig. 2). We did not find significant dosage dependent mortality rate changes for the same pathogen. The survival curve for *N. apis* was significantly lower than for *N. ceranae* at dosages of 500–50,000 spores. Survival of bees fed 500 *N. apis* spores was significantly lower ($P = 0.0442$ in Log-rank test) than that of the bees fed 5000 *N. ceranae* spores. Spore production was dosage dependent for both *Nosema* species (data not shown). However, we note that the infection rate was not 100% and the *N. apis* infection rate was higher than *N. ceranae* at the same dosage.

3.3. Mortality – single ID_{100} dosage

Survival curves for the treatments 10^5 *N. apis* spores/bee, 10^5 *N. ceranae* spores/bee and an uninfected control are shown in Fig. 3. Bees in plastic cups in the Illinois study did not survive as long as bees in the wooden cages used in Texas; mean survival time

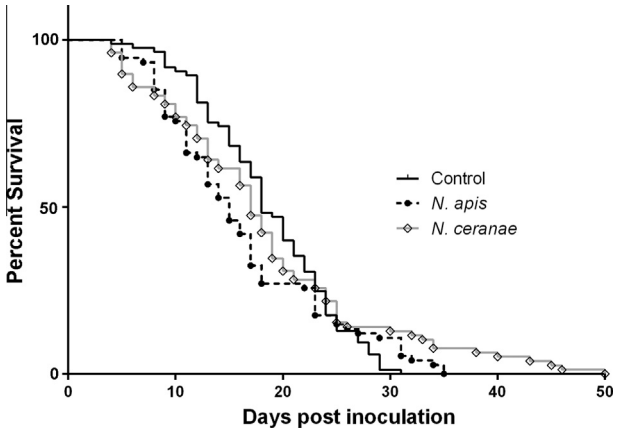

Fig. 3. Survival curves for the ID_{100} dosage of 10^5 *Nosema apis* or *Nosema ceranae* spores/bee, inoculated 5 days after honey bee eclosion.

of control bees in IL was 18 days. Survival of the *N. ceranae* treatment group ($LT_{50} = 17$ days) was similar to the control ($P = 0.587$ in Log-rank test) and slightly higher than *N. apis* ($LT_{50} = 15$ days), but the results are not statistically significant ($P = 0.097$ in Log-rank test). Variation among trials (bees from different colonies) was high. Mortality was similar for control bees and bees infected with both *Nosema* species in the first trial ($LT_{50} = 22$–24 days). In the second trial, *N. ceranae* infection resulted in a lower LT_{50} (7 d), whereas the LT_{50} for *N. apis* infection was 11 d and the control was approximately 18 d. The LT_{50} of *N. ceranae* infection in the third trial was 19 d, *N. apis* was 10 d, and the control was 18 d.

4. Discussion

Experimental results for mortality and infectivity studies conducted in Illinois and Texas using honey bees of different origin, *N. ceranae* of local origin in each region, and different methods of caging bees were consistent between laboratories and suggest that *N. ceranae* infections in US honey bees are not as infective nor possibly as virulent as reported in European studies. Our results corresponded with other US studies in Michigan (Milbrath et al., in press) and Maryland (Chaimanee et al., 2012; Pettis et al., 2013) that suggested *N. ceranae* requires more spores to infect bees than previous studies showed for *N. apis*. These results do not appear to explain the dominant status of *N. ceranae* in the US (Chen and Huang, 2010), but it is possible that honey bees have developed some tolerance to *N. ceranae* after decades of exposure.

The infectivity of *N. apis* in newly emerged and 5-day bees (Table 2) did not differ from previous studies. Newly emerged bees tended to be less susceptible to infection than older bees, similar to results reported by Malone et al. (2001), and the ID_{50} for 5-day bees was slightly higher in our study but within the 95% confidence range reported by Fries (1988). Results for 14-day bees also corroborated those of Forsgren and Fries (2010). *N. apis* infectivity was, therefore, consistently similar among years (1972–2013), pathogen isolates, and strains of bees in New Zealand, Europe and the US.

Infectivity levels of *N. ceranae* were consistent with those reported in other studies in the US for newly emerged bees (Pettis et al., 2013; Chaimanee et al., 2012), including high variation among trials. These variations could possibly be caused by a difference in susceptibility among bees inoculated at eclosion vs. increasing age to 24 h post-eclosion, as well as sensitivity of newly emerged bees to handling. Susceptibility to *N. ceranae* differed significantly among ages of experimental workers. Previous studies evaluated newly emerged, 5-day, and 14-day old bees (Higes et al., 2007; Forsgren and Fries, 2010; Pettis et al., 2013; Huang and Solter, 2013), but typically only one age group was tested per experiment. Our comparative study found that newly emerged bees are the least susceptible to both *Nosema* pathogens but standard errors were large among trials. Bees at 5-days post eclosion were the most susceptible to *N. ceranae*. Differences in susceptibility among age groups may indicate that *N. ceranae* relies on a transmission cycle between foragers and housekeeping bees. Bees at the age of approximately 5-days perform housekeeping tasks such as hygienic behaviors and feeding nestmates (Seeley and Kolmes, 1991) and can acquire *N. ceranae* spores (Huang and Solter, 2013; Smith, 2012) from the environment and from foragers. Bees fed spores at 5 days post eclosion usually have fully developed infections in the midgut at 10–14 days dpi (Forsgren and Fries, 2010; Huang and Solter, 2013) at which time they have become foragers (Seeley and Kolmes, 1991) and are potentially the inoculum source for the next group of younger housekeeping bees.

Previous *N. apis* studies hypothesized that contaminated comb ingested when emerging bees chew the wax cover of the cell is

an important transmission route (Bailey and Ball, 1991). However, our results suggest that newly eclosed bees are the least susceptible adult stage to both *Nosema* species. Higher spore concentrations were used in past studies (2×10^5 spores) for newly emerged bees (Malone and Gatehouse, 1998) to achieve consistent ID_{100} results. Honey bee larvae may not be susceptible to *N. ceranae* as newly emerged adults appear to be free of infection (Smith, 2012; Huang and Solter, 2013). Higher susceptibility at 5 or more days post eclosion may allow the infection to reach highest levels that do not result in high early mortality in foraging bees, allowing spores to be spread in the environment as well as directly to the natal colony and to other colonies by drifting bees (Fries and Camazine, 2001).

Mortality experiments suggested that *N. ceranae* is less virulent than *N. apis* when tested in the same conditions, a result that was consistent for two different experimental methods. These results differed from those reported by Paxton et al. (2007) and Higes et al. (2007) in Europe; Williams et al. (2014) in Nova Scotia, Canada also suggested that *N. ceranae* is more virulent than *N. apis* in Buckfast bees originating from the United Kingdom. In the Illinois experiments, we used similar methods to those of Higes et al. (2007) and Paxton et al. (2007) for the high dosage treatments and, although *N. ceranae* produced higher mortality in one trial, similar to studies in Spain (Higes et al., 2007); mortality was similar or lower than *N. apis* mortality in two trials and was statistically insignificant over the three trials in our study, each using different colonies. Regression analysis for each trial (accumulated mortality/days post inoculation) showed that differences among the three trials for multiple dosages and a single ID_{100} dosage were greater than the differences between the *Nosema* pathogens. Although we selected apparently healthy bees for the trials, we did not comprehensively screen for other pathogens, including viruses, in our studies. In addition to the genetic differences of the bees among trials, background or latent virus infections may have contributed to the differences we noted. Nevertheless, our overall results and those of previous studies (Pettis et al., 2013; Chaimanee et al., 2012) suggest that in the US more *N. ceranae* spores are required to produce infection in 100% of inoculated hosts and virulence of *N. ceranae* is lower than that of *N. apis.*

Honey bees in the US have been exposed to *N. ceranae* for an unknown amount of time prior to 1995 (Chen and Huang, 2010) and it is possible that the US honey bee population has had sufficient time and generations to adapt somewhat to *N. ceranae* infection. There is little difference in genetic markers for *N. ceranae* isolates globally (Huang et al., 2008; Chen et al., 2009) and infectivity of different isolates appear to be similar under similar conditions (Dussaubat et al., 2013). Different strains and patrilines of bees may differ in susceptibility to *N. ceranae* (Bourgeois et al., 2012) and *N. ceranae* tolerance through selection has been observed (Huang et al., 2012). It is possible that differences in strains of bees could explain results that differed from European studies and the recent study in Canada (Williams et al., 2014).

Results of the infectivity and mortality tests do not explain why *N. ceranae* has become the dominant microsporidian pathogen in US honey bees. *N. ceranae* was suggested to be at an advantage in warm climates (Chen et al., 2012; Martin-Hernandez et al., 2009); it has slightly higher spore production (Forsgren and Fries, 2010; Paxton et al., 2007; Huang and Solter, 2013), and apparently has some natural resistance to fumagillin, a commonly used antibiotic treatment in the US (Huang et al., 2013). In addition, the less virulent and age-specific infections may be beneficial for *N. ceranae*, providing more opportunities to proliferate and spread via infected hosts. Conversely, *N. ceranae* may be less tolerant of cold environments and cold storage (Paxton et al., 2007) and this pathogen was also less competitive than *N. apis* in co-infections (Milbrath et al., in press). Overall infectivity and mortality in the US suggest, however, that US bees and *N. ceranae* are more co-adapted than the pathogen and European honey bees, possibly explaining the difference between our results and those in Europe (Forsgren and Fries, 2010; Paxton et al., 2007). Intriguingly, *N. apis* was more virulent and infective than *N. ceranae* in our studies; perhaps virulence is related to lack of exposure to *N. apis* in recent decades (Chen and Huang, 2010). We did not identify markers to determine if bees have adapted to *N. ceranae* infection. Such research efforts and new management or treatment methods for *N. ceranae* are urgent needs in the US.

Acknowledgments

The authors thank G. Robinson for use of the University of Illinois research bee colonies, C. Nye, E. Swenson, and A. Nelson for laboratory assistance, and Bob Cox of the USDA/ARS for laboratory and field assistance. We also thank anonymous reviewers for helpful comments. This research was supported by USDA National Institute of Food and Agriculture, Agriculture and Food Research Initiative Managed Pollinator Coordinated Agriculture Project (CAP) no. 2009-85118-05718, subaward to KAA and subaward no. RC293-365/4692038 to LFS, and USDA-CSREES Project no. ILLU-875302-0250249 S-1024.

References

Antúnez, K., Mendoza, Y., Santos, E., Invernizzi, C., 2013. Differential expression of vitellogenin in honey bees (*Apis mellifera*) with different degress of *Nosema ceranae* infection. J. Apicult. Res. 52, 227–234.

Bailey, L., 1972. The preservation of infective microsporidian spores. J. Invertebr. Pathol. 20, 252–254.

Bailey, L., Ball, B.V., 1991. Honey Bee Pathology. Academic Press, London; New York.

Bourgeois, A.L., Rinderer, T.E., Sylvester, H.A., Holloway, B., Oldroyd, B.P., 2012. Patterns of *Apis mellifera* infestation by *Nosema ceranae* support the parasite hypothesis for the evolution of extreme polyandry in eusocial insects. Apidologie 43, 539–548.

Bromenshenk, J.J., Henderson, C.B., Wick, C.H., Stanford, M.F., Zulich, A.W., Jabbour, R.E., Deshpande, S.V., McCubbin, P.E., Seccomb, R.A., Welch, P.M., Williams, T., Firth, D.R., Skowronski, E., Lehmann, M.M., Bilimoria, S.L., Gress, J., Wanner, K.W., Cramer Jr., R.A., 2010. Iridovirus and microsporidian linked to honey bee colony decline. PLoS One 5 (10), e13181.

Carter, J., Saunders, V., 2007. Methods used in virology. In: Carter, J., Saunders, V. (Eds.), Virology: Principles and Applications. Wiley & Sons Ltd., West Sussex, UK, pp. 9–28.

Chaimanee, V., Chantawannakul, P., Chen, Y.P., Evans, J.D., Pettis, J.S., 2012. Differential expression of immune genes of adult honey bee (*Apis mellifera*) after inoculated by *Nosema ceranae*. J. Insect Physiol. 58, 1090–1095.

Chen, Y.P., Huang, Z.Y., 2010. *Nosema ceranae*, a newly identified pathogen of *Apis mellifera* in the USA and Asia. Apidologie 41, 364–374.

Chen, Y., Evans, J.D., Zhou, L., Boncristiani, H., Kimura, K., Xiao, T., Litkowski, A.M., Pettis, J.S., 2009. Asymmetrical coexistence of *Nosema ceranae* and *Nosema apis* in honey bees. J. Invertebr. Pathol. 101, 204–209.

Chen, Y.W., Chung, W.P., Wang, C.H., Solter, L.F., Huang, W.F., 2012. *Nosema ceranae* infection intensity highly correlates with temperature. J. Invertebr. Pathol. 111, 264–267.

Copley, T.R., Jabaji, S.H., 2012. Honeybee glands as possible infection reservoirs of *Nosema ceranae* and *Nosema apis* in naturally infected forager bees. J. Appl. Microbiol. 112, 15–24.

Cornman, R.S., Tarpy, D.R., Chen, Y.P., Jeffreys, L., Lopez, D., Pettis, J.S., vanEngelsdorp, D., Evans, D., 2012. Pathogen webs in collapsing honey bee colonies. PLoS One 7 (8), e43562.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V., vanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S.K., Simons, J.F., Egholm, M., Pettis, J.S., Lipkin, W.I., 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318 (5848), 283–287.

Cuomo, C.A., Desjardins, C.A., Bakowski, M.A., Goldberg, J., Ma, A.T., Becnel, J.J., Didier, E.S., Fan, L., Heiman, D.I., Levin, J.Z., Young, S., Zeng, Q., Troemel, E.R., 2012. Microsporidian genome analysis reveals evolutionary strategies for obligate intracellular growth. Genome Res. 22, 2478–2488.

Dussaubat, C., Sagastume, S., Gomez-Moracho, T., Botias, C., Garcia-Palencia, P., Martin-Hernandez, R., Le Conte, Y., Higes, M., 2013. Comparative study of *Nosema ceranae* (Microsporidia) isolates from two different geographic origins. Vet. Microbiol. 162, 670–678.

Forsgren, E., Fries, I., 2010. Comparative virulence of *Nosema ceranae* and *Nosema apis* in individual European honey bees. Vet Parasitol. 170, 212–217.

Forsgren, E., Fries, I., 2013. Temporal study of *Nosema* spp. in a cold climate. Environ. Microbiol. Rep. 5, 78–82.

Fries, I., 1988. Infectivity and multiplication of *Nosema apis* Z. in the ventriculus of the honey bee. Apidologie 19, 319–328.
Fries, I., 1993. *Nosema apis* – a parasite in the honey bee colony. Bee World 74, 5–19.
Fries, I., Camazine, S., 2001. Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. Apidologie 32, 199–214.
Fries, I., Feng, F., daSilva, A., Slemenda, S.B., Pieniazek, N.J., 1996. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). Eur. J. Protistol. 32, 356–365.
Fries, I., Chauzat, M.P., Chen, Y.P., Doublet, V., Genersch, E., Gisder, S., Higes, M., McMahon, D.P., Martin-Hernandez, R., Natsopoulou, M., Paxton, R.J., Tanner, G., Webster, T.C., Williams, G.R., 2013. Standard methods for *Nosema* research. J. Apicult. Res. 52 (1), http://dx.doi.org/10.3896/IBRA.1.52.1.14.
Gisder, S., Hedtke, K., Mockel, N., Frielitz, M.C., Linde, A., Genersch, E., 2010. Five-year cohort study of *Nosema* spp. in Germany: does climate shape virulence and assertiveness of *Nosema ceranae*? Appl. Environ. Microbiol. 76, 3032–3038.
Goblirsch, M., Huang, Z.Y., Spivak, M., 2013. Physiological and behavioral changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection. PLoS One 8 (3), e58165.
Goertz, D., Solter, L.F., Linde, A., 2007. Horizontal and vertical transmission of a *Nosema* sp. (Microsporidia) from *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae). J. Invertebr. Pathol. 95, 9–16.
Higes, M., Martin, R., Meana, A., 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J. Invertebr. Pathol. 92, 93–95.
Higes, M., Garcia-Palencia, P., Martin-Hernandez, R., Meana, A., 2007. Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). J. Invertebr. Pathol. 94, 211–217.
Higes, M., Martin-Hernandez, R., Garrido-Bailon, E., Gonzalez-Porto, A.V., Garcia-Palencia, P., Meana, A., del Nozal, M.J., Mayo, r., Bernal, J.L., 2009. Honeybee colony collapse due to *Nosema ceranae* in professional apiaries. Environ. Microbiol. Rpt., http://dx.doi.org/10.1111/j.1758-2229.2009.00014.x.
Huang, W.F., Solter, L.F., 2013. Comparative development and tissue tropism of *Nosema apis* and *Nosema ceranae*. J. Invertebr. Pathol. 113, 35–41.
Huang, Z.Y., Robinson, G.E., Borst, D.W., 1994. Physiological correlates of division of labor among similarly aged honey bees. J. Comp. Physiol. A 174, 731–739.
Huang, W.F., Jiang, J.H., Chen, Y.W., Wang, C.H., 2007. A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. Apidologie 38, 30–37.
Huang, W.F., Bocquet, M., Lee, K.C., Sung, I.H., Jiang, J.H., Chen, Y.W., Wang, C.H., 2008. The comparison of rDNA spacer regions of *Nosema ceranae* isolates from different hosts and locations. J. Invertebr. Pathol. 97, 9–13.
Huang, Q., Kryger, P., Le Conte, Y., Moritz, R.F., 2012. Survival and immune response of drones of a Nosemosis tolerant honey bee strain towards *N. ceranae* infections. J. Invertebr. Pathol. 109, 297–302.
Huang, W.F., Solter, L.F., Yau, P.M., Imai, B.S., 2013. *Nosema ceranae* escapes fumagillin control in honey bees. PLoS Pathogens 9 (3), e1003185.
Maddox, J.V., Brooks, W.M., Fuxa, J.R., 1981. *Vairimorpha necatrix*, a pathogen of agricultural pests: potential for pest control. In: Burges, H.D. (Ed.), Microbial Control of Pests and Plant Diseases. Academic Press, London, pp. 587–594.
Malone, L.A., Gatehouse, H.S., 1998. Effects of *Nosema apis* infection on honey bee (*Apis mellifera*) digestive proteolytic enzyme activity. J. Invertebr. Pathol. 71, 169–174.
Malone, L.A., Gatehouse, H.S., Tregidga, E.L., 2001. Effects of time, temperature, and honey on *Nosema apis* (Microsporidia: Nosematidae), a parasite of the honeybee, *Apis mellifera* (Hymenoptera: Apidae). J. Invertebr. Pathol. 77, 258–268.
Martin-Hernandez, R., Meana, A., Garcia-Palencia, P., Marin, P., Botias, C., Garrido-Bailon, E., Barrios, L., Higes, M., 2009. Effect of temperature on the biotic potential of honeybee microsporidia. Appl. Environ. Microbiol. 75, 2554–2557.
Martin-Hernandez, R., Botias, C., Barrios, L., Martinez-Salvador, A., Meana, A., Mayack, C., Higes, M., 2011. Comparison of the energetic stress associated with experimental *Nosema ceranae* and *Nosema apis* infection of honeybees (*Apis mellifera*). Parasitol. Res. 109, 605–612.
Matheson, A., 1996. World bee health update 1996. Bee World 77, 45–51.
Mayack, C., Naug, D., 2009. Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. J. Invertebr. Pathol. 100, 185–188.
Milbrath, M., Tran, V.T., Huang, W.F., Solter, L., Tarpy, D., Lawrence, F., Huang, Z.Y., in press. Comparative virulence and competition between *Nosema apis* and *Nosema ceranae* in honey bees (*Apis mellifera*). J. Invert. Pathol. http://dx.doi.org/10.1016/j.jip.2014.12.006.
Paxton, R.J., 2010. Does infection by *Nosema ceranae* cause "Colony Collapse Disorder" in honey bees (*Apis mellifera*)? J. Apicult. Res. 49, 80–84.
Paxton, R.J., Klee, J., Korpela, S., Fries, I., 2007. *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie 38, 558–565.
Pettis, J.S., Lichtenberg, E.M., Andree, M., Stitzinger, J., Rose, R., Vanengelsdorp, D., 2013. Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. PLoS One 8 (7), e70182.
Seeley, T.D., Kolmes, S.A., 1991. Age polyethism for hive duties in honey-bees – illusion or reality. Ethology 87, 284–297.
Smith, M.L., 2012. The honey bee parasite *Nosema ceranae*: transmissible via food exchange? PLoS One 7 (8), e43319.
Solter, L.F., Pilarska, D.K., McManus, M.L., Zubrik, M., Patočka, J., Huang, W.F., Novotny, J., 2010. Host specificity of microsporidia pathogenic to the gypsy moth, *Lymantria dispar* (L.): field studies in Slovakia. J. Invertebr. Pathol. 105, 1–10.
Teixeira, E.W., Santos, L.G., Sattler, A., Message, D., Alves, M.L., Martins, M.F., Grassi-Sella, M.L., Francoy, T.M., 2013. *Nosema ceranae* has been present in Brazil for more than three decades infecting Africanized honey bees. J. Invertebr. Pathol. 114, 250–254.
Webster, T.C., 1993. *Nosema apis* spore transmission among honey bees. Am. Bee J. 133, 869–870.
Webster, T.C., Pomper, K.W., Hunt, G., Thacker, E.M., Jones, S.C., 2004. *Nosema apis* infection in worker and queen *Apis mellifera*. Apidologie 35, 49–54.
Williams, G.R., Shutler, D., Burgher-MacLellan, K.L., Rogers, R.E., 2014. Infra-population and community dynamis of parasites *Nosema apis* and *Nosema ceranae* and consequences for honey bee (*Apis mellifera*) hosts. PloS One 9 (7), e99465.
Zander, E., 1909. Tierische parasiten als krankenheitserreger bei der biene. Münchener Bienenzeitung 31, 196–204.