Journal of Invertebrate Pathology 125 (2015) 9–15

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Comparative virulence and competition between *Nosema apis* and *Nosema ceranae* in honey bees (*Apis mellifera*)

Meghan O. Milbrath [a,1], Toan van Tran [a,b,1], Wei-Fong Huang [c], Leellen F. Solter [c], David R. Tarpy [d], Frank Lawrence [e], Zachary Y. Huang [a,*]

[a] Department of Entomology, Natural Science Building, 288 Farm Lane Room 243, Michigan State University, East Lansing, MI 48824, USA
[b] Bee Research and Development Center, N^0 19 Truc Khe, Lang Ha, Dong Da, Ha Noi, Viet Nam
[c] Illinois Natural History Survey, Prairie Research Institute at the University of Illinois at Urbana-Champaign, 1816 S. Oak St, Champaign, IL 61820, USA
[d] Department of Entomology, North Carolina State University, Raleigh, NC 27695-7613, USA
[e] Center for Statistical Training and Consulting, 178 Giltner Hall, Michigan State University, East Lansing, MI, USA

ARTICLE INFO

Article history:
Received 28 June 2014
Revised 3 November 2014
Accepted 8 December 2014
Available online 16 December 2014

Keywords:
Honey bee, *Apis mellifera*
Nosema apis
Nosema ceranae
Disease transmission, infectivity
Microsporidia
Microsporidiosis
Co-infection, mixed-infections

ABSTRACT

Honey bees (*Apis mellifera)* are infected by two species of microsporidia: *Nosema apis* and *Nosema ceranae.* Epidemiological evidence indicates that *N. ceranae* may be replacing *N. apis* globally in *A. mellifera* populations, suggesting a potential competitive advantage of *N. ceranae*. Mixed infections of the two species occur, and little is known about the interactions among the host and the two pathogens that have allowed *N. ceranae* to become dominant in most geographical areas. We demonstrated that mixed *Nosema* species infections negatively affected honey bee survival (median survival = 15–17 days) more than single species infections (median survival = 21 days and 20 days for *N. apis* and *N. ceranae*, respectively), with median survival of control bees of 27 days. We found similar rates of infection (percentage of bees with active infections after inoculation) for both species in mixed infections, with *N. apis* having a slightly higher rate (91% compared to 86% for *N. ceranae*). We observed slightly higher spore counts in bees infected with *N. ceranae* than in bees infected with *N. apis* in single microsporidia infections, especially at the midpoint of infection (day 10). Bees with mixed infections of both species had higher spore counts than bees with single infections, but spore counts in mixed infections were highly variable. We did not see a competitive advantage for *N. ceranae* in mixed infections; *N. apis* spore counts were either higher or counts were similar for both species and more *N. apis spores* were produced in 62% of bees inoculated with equal dosages of the two microsporidian species. *N. ceranae* does not, therefore, appear to have a strong within-host advantage for either infectivity or spore growth, suggesting that direct competition in these worker bee mid-guts is not responsible for its apparent replacement of *N. apis.*

1. Introduction

Honey bees (genus *Apis*) are parasitized by two species of microsporidia, *Nosema apis* and *Nosema ceranae. N. apis* was first identified in the western honey bee, *Apis mellifera*, over 100 years ago (Zander, 1909), while the recently described *N. ceranae* was thought to be restricted to the Eastern honey bee, *Apis cerana* (Fries et al., 1996). Shortly after *N. ceranae* was described, it was found in colonies of *A. mellifera* worldwide (Klee et al., 2007; Chen et al., 2008); a possible shift from its original host (Huang et al., 2007; Paxton et al., 2007). Currently, the ranges for the two *Nosema* pathogens strongly overlap. Infections with *N. apis* and *N. ceranae* can co-occur, and recent prevalence studies indicate that mixed infections occur in both Europe and North America (Klee et al., 2007; Gisder et al., 2010; Copley et al., 2012).

Within-host competition between two microsporidian species in mixed infections can lead to unequal transmission or replacement. This type of competitive replacement has been reported in mixed microsporidian infections in other insects such as the gypsy moth, *Lymantria dispar* (Solter et al., 2002; Pilarska et al., 2006). A similar interaction may be occurring between the two *Nosema* species in *A. mellifera*, with *N. ceranae* having a competitive advantage. At the population level, it appears that *N. ceranae* may be displacing *N. apis*; prevalence studies found that in many regions *N. apis* infections are becoming rarer, and those of *N. ceranae* more frequent (Klee et al., 2007; Paxton et al., 2007; Fries, 2010; Martín-Hernández et al., 2012).

* Corresponding author. Tel.: +1 517 353 8136.
[1] These two authors contributed equally to this study.

http://dx.doi.org/10.1016/j.jip.2014.12.006
0022-2011/

A competitive advantage for within-host growth of *N. ceranae* is supported in *A. cerana*, where prevalence and pathogen loads of *N. ceranae* are higher than *N. apis* in natural mixed infections (Chen et al., 2009a). In *A. mellifera*, however, no competitive advantage was reported for *N. ceranae* over *N. apis* in bees that had been infected with both species (Forsgren and Fries, 2010), and infectivity was similar for both species. Even in the absence of a competitive advantage in mixed infections, the increasing prevalence of *N. ceranae* could be explained by faster infection dynamics in single-species infections. However, studies comparing single-species *Nosema* infections found lower initial spore production for *N. ceranae* than *N. apis* (Forsgren and Fries, 2010), but similar overall growth rates and spore loads for both parasites, with later mature spore production in *N. ceranae* (Paxton et al., 2007).

In this work we examined the survival effects and within-host competition of mixed *Nosema* infections in *A. mellifera*. We examined individual bees from three colonies to determine if there is an effect of colony (genetic background) in mixed infections; we used a broad range of inoculum ratios to identify effects related to initial dosage; we examined relative spore growth over multiple time points; and we examined the effects on worker survival related to these treatments. These methods enabled us to better identify the competitive abilities and the pathogen population dynamics in mixed infections of *N. ceranae* and *N. apis* in *A. mellifera*, as well as to understand the potential impacts and outcomes of single and mixed infections.

2. Methods

2.1. Experimental design

To understand the effects of initial dosage and examine competition, we infected bees with eight different combinations of the two microsporidian species (Table 1). We conducted three trials, using bees from three different *Nosema*-free colonies to identify potential colony effects. Each treatment consisted of 100 bees from each colony separated into wooden cages (14 × 12 × 16 cm, 50 bees/cage), for a total of 300 bees per treatment ($N = 2400$ bees total).

2.2. Experimental infection

2.2.1. Spore preparation

Fresh spores of each *Nosema* species (harvested within 24 h) were used to inoculate honey bees. *N. apis* spore stock was obtained from Tom Webster (Kentucky State University), and fresh *N. apis* spores were produced in bees artificially infected in the laboratory from this stock. *N. ceranae* spores were obtained from foragers in naturally infected colonies at the Michigan State University apiary (East Lansing, Michigan, GPS position: N42°40′44.97″, W84°28′39.16″). To obtain *Nosema* spores for inoculations, we homogenized the midgut tissues of infected bees in distilled water using a plastic pestle. The spore suspension was centrifuged to pellet spores and the supernatant with insect cells was discarded (Solter et al., 2012). Spores were confirmed to be mono-specific using PCR with previously described primers (Chen et al., 2008). We determined spore counts using a hemocytometer (Hausser Scientific) and resuspended the cleaned spores in appropriate amounts of 50% sucrose solution to provide treatment dosages.

2.2.2. Insect handling

We inoculated newly emerged bees with *Nosema* spores, instead of using 5 day old bees (Higes et al., 2007) because the latter requires the use of carbon dioxide, which results in added mortality (Milbrath et al., 2013). Frames of sealed brood were obtained from three *Nosema*-free colonies of *A. mellifera* and incubated at 34 ± 0.5 °C, 50% RH (Percival 136NL, Percival Scientific, Perry, IA, USA). After emerging, worker bees were starved for 2 h and then fed 2 μl 50% sucrose solution with 30,000 *Nosema* spores in differing proportions of the two species (Table 1). The spore/sucrose solution was vortexed after every third bee to ensure a uniform suspension. After feeding, bees were isolated for 30 min in individual vials in the growth chamber to ensure that the sugar solution was not transferred among bees and the entire dosage was ingested. Control bees (Treatment 1) were treated in an identical manner using a 50% sucrose solution containing no spores. Bees were caged by trial and treatment, then maintained in the same growth chamber set at 30 ± 0.5 °C, 50% RH, and total darkness (24 h dark). Sucrose solution (50%), distilled water, and pollen were provided *ad libitum* and changed every 5 days. Prior to administration, the pollen was subjected to 3 cycles of freezing/heat (−20/60 °C, 12 h minimum each half cycle) to inactivate any *Nosema* spores, which can be potentially present in corbicular pollen (Higes et al., 2008).

2.3. Analyses

2.3.1. Survival

Each cage was checked daily for bee mortality for the duration of the experiment (30 days). Dead bees were recorded and removed for storage at −80 °C. Bees that died within the first 24 h post-inoculation were excluded from analysis to eliminate handling effects, and bees that were sampled for *Nosema* spores were included in the survival analysis as right-censored data. A non-parametric MLE estimate of the survival function for each treatment was determined using the Kaplan–Meier estimate. A post hoc pair-wise comparison was applied to assess differences between treatments using log-rank tests with the software R (R Development Core Team, 2010). The effect of colony was examined using a Cox proportional hazard frailty model with trial included as

Table 1
Initial dosage (number of spores) of each *Nosema* species and summary of survival data (in days) for bees in each treatment.

Dosage				Survival data (in days)			
Treatment	*N. ceranae* spores	*N. apis* spores	*N. ceranae*:*N. apis* ratio	Min	Median	Mean	Max
1	–	–	–	4	27	24.07	30
2	30,000	–	–	5	20	20.5	30
3	–	30,000	–	2	21	20.32	30
4	25,000	5,000	5:1	3	15	16.45	30
5	20,000	10,000	2:1	2	15	15.53	30
6	15,000	15,000	1:1	3	15	15.25	30
7	10,000	20,000	1:2	2	17	17.57	30
8	5,000	25,000	1:5	2	15	15.77	30