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

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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).

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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

a random effect. Model selection was performed based on the comparison of likelihood, AIC and a Chi-squared test of these models.

2.3.2. Competition

To determine the relative production rate of spores, we performed quantitative PCR on bees from each of six different treatments (Treatments 1–6) sampled on days 10, 15, and 20 ($N = 156$ total). We chose bees subjected to dosages with higher ratios of *N. ceranae* because preliminary work indicated a competitive advantage of *N. apis* (supplementary material, Fig. S1). These bees were sampled live, flash frozen in liquid nitrogen for 24 h, and stored at −80 °C until qPCR was performed. Spore samples were purified for PCR using the method described above and DNA was extracted using the Chelex® method (Huang and Solter, 2013; Walsh et al., 1991). Quantitative PCR was performed using the SYBR green method and with the primers described in Huang and Solter (2013) [Na65f: CGT ACT ATG TAC TGA AAG ATG GAC TGC/Na181r: AGG TCT CAC TCT TAC TGT ACA TAT GTT AGC for *N. apis*, and Nc841f: GAG AGA ACG GTT TTT TGT TTG AGA/Nc980r: ATC CTT TCC TTC CTA CAC TGA TTG for *N. ceranae*. ABI SYBR green PCR master mix and 7900HT were used for qPCR with a standard 2-step method and an annealing temperature of 64.5 °C. Quantity was determined by the absolute quantification method using SDS 2.2.2 software (ABI). Standard curves were constructed using serially diluted DNA samples with counted spores that were prepared at the same time. All DNA samples, positive controls, pure *N. apis* and pure *N. ceranae* DNA were tested in both *N. apis* and *N. ceranae* quantifications to identify cross reactions.

To model the single species infections data, we employed a generalized linear model with a log link function with y_i representing the spore count, and the **X** matrix containing p columns for spore type, colony, and treatment and day with the software R (R Development Core Team, 2010).

$$(y_i|x_i) \sim \text{Poission } (\mu_i),\ 1 \leqslant i \leqslant n$$

$$\log(\mu_i) = XB,\ \text{where } B = (\beta_1 \ldots \beta_p)$$

To evaluate the effects of time and treatment on the trend in the proportion of *N. apis* to *N. ceranae* in mixed infections, we used the model below. The outcome variable assumes values in the standard unit interval (0,1). The independent variables of interest were all between-factor measures, and beta regression analysis is appropriate for modeling this issue because the distribution of the criterion variable is frequently heteroskedastic and asymmetric. Because in this data set the outcome variable occasionally accepts extreme values such as 0 or 1 we applied the transformation $(y * (n-1) + 0.5/n)$ where n is the sample size and y is the outcome variable (Smithson and Verkuilen, 2006). The model for the mixed infections is defined as

$$g(\mu_i) = x_i'\beta = \eta_i;\ y_i \sim B(\mu, \phi),\ i \in l, \ldots, n$$

$$Var(y_i) = \frac{\mu_i(l-\mu_i)}{1+\phi}$$

where $\beta = \beta_1, \ldots, \beta_k)'$ and $x_i = (x_{i1}, \ldots, x_{ik})'$. The parameter ϕ is known as the precision parameter.

3. Results

3.1. Survival analysis

When bees from all colonies were examined together, those in the control group survived significantly longer than bees infected with *Nosema*, regardless of species or ratios of the two species in the dosage (Fig. 1, Table 1); almost half (143/297 = 48%) of the bees

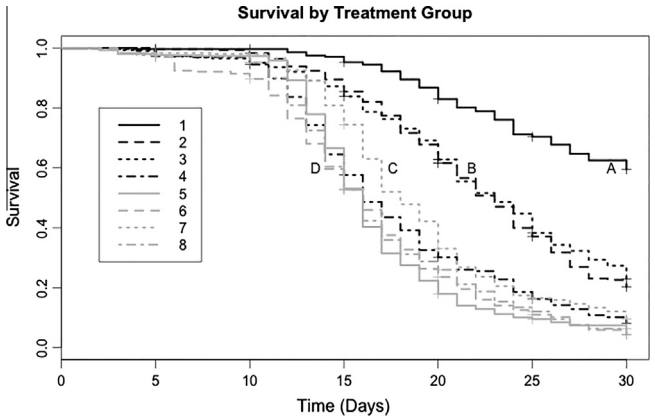

Fig. 1. Estimated Kaplan–Meier cumulative survival function of bees by treatment group, pooled from three trials. Letters denote categories of bees that are significantly different at $p < 0.05$. Group 1 (A) is the control group, Groups 2 and 3 (B) are single-species infections, and Groups 4–8 (C and D) are mixed infections (see Table 1 for details in *Nosema* species and dosages).

in the control group were alive by the end of the experiment (30 days). Bees with single species infections (Treatments 2 and 3) survived significantly longer than bees with mixed infections (Treatments 4–8; Tables 1 and 2). A species-related effect on mortality was not evident when all trials were pooled and colonies (trials) were not included in the model; there were no survival differences between bees with pure *N. ceranae* infections and bees with pure *N. apis* infections ($\chi^2 = 0.3$, $p = 0.61$), and both treatment groups had an estimated median survival of 23 days. All mixed-infection treatments produced similar survival curves and estimated median survival times of 16 days, except for treatment 7 (10^4 *N. ceranae* spores/2×10^4 *N. apis* spores), for which survival was slightly but significantly higher than the other mixed groups (estimated 18 day median survival; Table 2).

The probability of death by day 20 for the control group was 0.24, and the relative risk for the single infections was 1.7 for both *N. ceranae* and *N. apis*. The relative risk for the mixed infections was even higher, ranging from 2.6 for Treatment 7 to 3.4 for Treatment 5. By day 30, the relative risk was 1.6 for both single infection treatments and 1.8–1.9 for all mixed infections.

3.2. Variability in survival among colonies

Survival was similar for all three trials with *N. apis* infected bees (Treatment 2) ($\chi^2 = 2.6$, $df = 2$, $p = 0.28$), but survival in *N. ceranae* infected bees (Treatment 3) was significantly lower for one trial than the other two ($\chi^2 = 24$, $df = 2$, $p < 0.01$). When this trial was excluded, *N. ceranae* infected bees survived slightly, but significantly, longer (median 25 days) than *N. apis* infected bees (Treatment 2, median 23 days) ($\chi^2 = 6.7$, $df = 1$, $p < 0.01$), but still significantly shorter than those in the control group (Treatment 1, median >30 days) ($\chi^2 = 36.9$, $df = 1$, $p < 0.01$).

There were no significant differences among colonies for Treatments 1–6 (except Treatment 3, mentioned above) at $p < 0.05$) (Fig. 2). We did identify significant inter-colony variability in survival for treatments with a higher initial proportion of *N. apis* spores (Treatment 7: $\chi^2 = 28.6$, $df = 2$, $p < 0.01$; and Treatment 8: $\chi^2 = 16.6$, $df = 2$, $p < 0.01$), indicating potential colony related variability in infection responses. When the effect of colony was included as a random variable using a Cox proportional hazard (ph) model with frailty, the Akaike Information Criteria (AIC) was slightly lower than our original model (22842.08 and 22863.13, respectively), indicating that a model including colony differences was a slightly better fit (Table 3). Pairwise comparisons among

Table 2
Pairwise comparisons of Kaplan–Meier survival curves (top right) and Cox proportional hazard model (bottom left), using the log-rank test among the eight treatments. Top number as X^2 value followed by its associated p value, with bold text indicates significance at 5% level ($p < 0.05$).

	1	2	3	4	5	6	7	8
1		**84.2**	**72.8**	**221.0**	**286.0**	**279.0**	**197.0**	**274.0**
		0	0	0	0	0	0	0
2	**90.2**		0.3	**55.7**	**111**	**99.4**	**38.3**	**91**
	0		0.609	8.3×10^{-14}	0	0	5.9×10^{-10}	0
3	**120.0**	**33.1**		**56.9**	**106**	**97.4**	**40.1**	**89.5**
	0	3.6×10^{-06}		0	0	0	2.4×10^{-10}	0
4	**224.0**	**58.3**	**76.6**		**4.7**	**4.9**	**4.2**	**3.2**
	0	2.7×10^{-11}	4.3×10^{-15}		0.0306	0.026	0.041	0.075
5	**289.0**	**114.0**	**126.0**	5.6		0	**23.8**	0.2
	0	0	0	0.34		0.96	1.1×10^{-06}	0.67
6	**298.0**	**114.0**	**128.0**	**15.0**	10.9		**19.2**	0.2
	0	0	0	0.01	0.05		1.2×10^{-05}	0.62
7	**249.0**	**79.4**	**99.3**	**45.8**	**23.8**	**51.2**		**16.1**
	0	1.1×10^{-11}	2.4×10^{-10}	9.9×10^{-09}	1.1×10^{-06}	7.7×10^{-10}		6.1×10^{-05}
8	**307**	**125**	**138**	**20.1**	**18.1**	**25.7**	**62.6**	
	0	0	0	0.00119	0.0029	0.000103	3.6×10^{-12}	

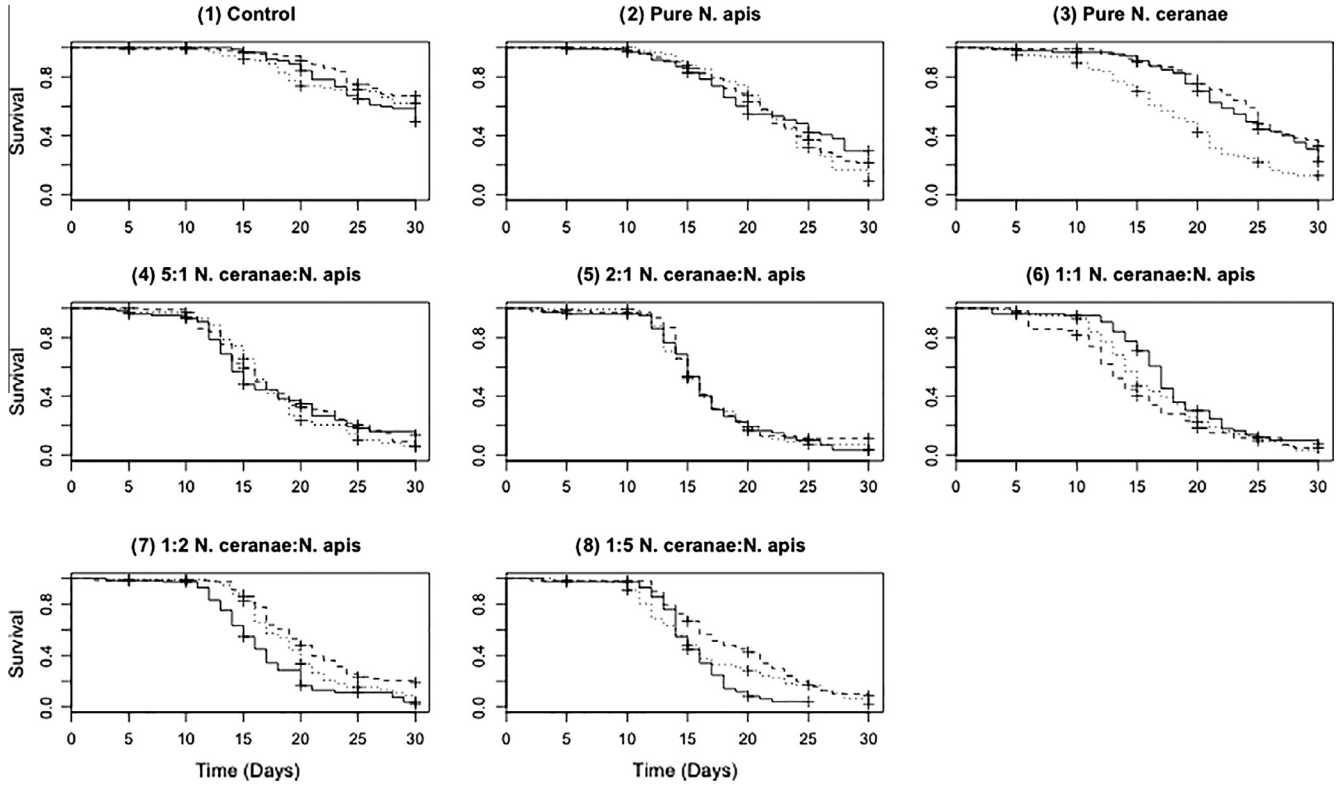

Fig. 2. Inter-colony differences in survival (in days) for each treatment. Differences were observed in Treatments 3, 7, and 8 ($p > 0.05$).

Table 3
Comparison of Cox-proportional hazard models without (Model 1) and with (Model 2) colony included as a random effect.

Model	Colony effects	Log-likelihood	X^2	AIC
Model 1	Not included	−11,431	362.15 ($<2.2 \times 10^{-16}$)	22863.13
Model 2	Included	−11,418	24.987 (1.55×10^{-05})	22842.09

treatments were similar to those in the initial model (Table 2) with the following exceptions: (1) in the Cox ph model there were significant differences between the groups with single species infections (Treatments 2 and 3, $p < 0.01$); (2) Treatment 8 was significantly different than Treatments 4 and 5; and (3) Treatments 4 and 5 were statistically similar ($p = 0.34$).

3.3. Spore counts

PCR efficiencies were in similar ranges to those in Huang and Solter (2013), and no cross-reaction between positive controls was noted. Overall, spore counts ranged from 0 to over 180 million/bee (max = 1.83×10^8 spores; *N. apis*, Treatment 5, day 20). Of the bees in the control group, 65% were free of infection (17/26). Among the nine infected bees, four were infected with *N. ceranae*, four with *N. apis* spores, and one with both species. All of these infections were relatively low intensity, with over 1 million spores counted in only two bees. Likewise, some cross infection was detected in the single species *Nosema* infections. Of the bees in Treatment 2 (inoculated with only *N. ceranae*), 10 (28%) were also infected with *N. apis*. In Treatment 3 (those inoculated with

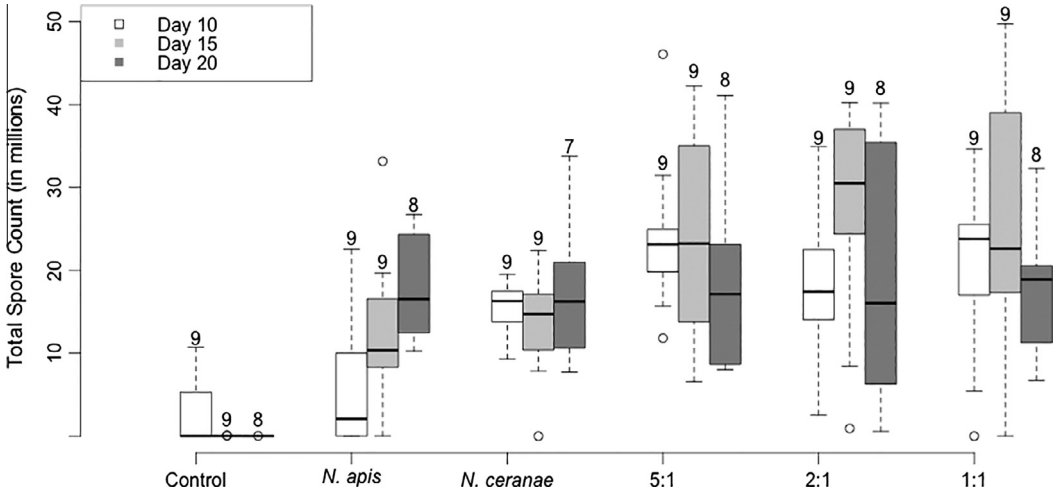

Fig. 3. Total spore production (in millions) by treatment. All groups were initially inoculated with 30,000 spores. Ratios indicate the proportion of *N. ceranae* spores to *N. apis* spores in the initial inoculation for mixed infections (corresponding to Treatments 4, 5 and 6 in Table 1). White boxes indicate bees sacrificed at day 10, light gray at day 15, and dark gray at day 20. Numbers above the boxplot indicate number of bees analyzed. One outlier is not shown in Treatment 5 (2:1 *N. ceranae*: *N. apis*) day 20 at 182,938,787.

only *N. apis*), *N. ceranae* spores were detected in 3 (12%). There were similar rates of infection for both species in the mixed infection groups: 91% were infected with *N. apis*, and 86% with *N. ceranae*.

3.3.1. Spore counts in single species infections

Overall, we observed higher total spore counts in *N. ceranae* infections (Treatment 2) than in *N. apis* infections (Treatment 3), corroborating the results of Huang and Solter (2013). At day 10 the odds of spore counts from *N. ceranae* single infections being higher than those of *N. apis* was 2.5:1 ($p < 0.01$). These two single species infections did not exhibit similar spore production over time (Fig. 3): *N. apis* increased over time from day 10 to day 20, whereas spore counts remained relatively stable for *N. ceranae* over this time period. By day 20, the spore counts in *N. ceranae* infections were still slightly but significantly (alpha = 0.05) higher than those in *N. apis* infections (odds = 2.90:1).

3.3.2. Spore production in mixed infections

Spore production in mixed infections was much more variable, both within and among treatments. Overall, mixed species infections had higher spore counts than single species infections; the odds of a mixed species infection of having a higher spore count than single species infections is 1.8:1 ($p < 0.01$). We conducted a sensitivity analysis to determine if this difference was influence by the one bee with a substantially higher spore count (in Treatment 5). After running the model with and without this individual specimen, it was established that the higher infection intensity of this bee did not exert undue influence on the results, and an assessment of the residuals with the subject case in the analytic model did not produce any alarming statistics. Thus, we only report the comprehensive results.

3.4. Competition in mixed infections

We found no indication of a competitive advantage for growth for *N. ceranae* spores in mixed *Nosema* infections. Of the 78 bees infected with both species of microsporidia, 71% had more *N. apis* spores than *N. ceranae* spores. On days 10 and 15, 67% and 93% had higher counts of *N. apis*, respectively, but on day 20, only half had higher counts of *N. apis*.

The outcome of the treatment with a 1:1 initial dosage (Treatment 6) was different from the other mixed infections with higher initial dosages of *N. ceranae* (Treatments 4 and 5) (Fig. 4). The proportion of *N. apis* spores in Treatment 6 remained consistent over time, compared to Treatments 4 and 5, which had a higher proportion of *N. apis* spores only on day 15. The highest proportion of *N. apis* spores was recorded on day 15 in all three treatment groups. We performed a likelihood ratio test to compare a model of the interaction of day and treatment to one without the interaction terms, and we found that the interaction does not contribute to our understanding of the data generation process in any meaningful way ($p > 0.05$).

4. Discussion

Mixed species *Nosema* infections often occur in the western honey bee, but neither the effects on individual bees nor the implications for colony health are well understood. Here, we demonstrated that mixed *Nosema* infections significantly decreased survival of caged *A. mellifera* workers. This increased mortality is not due to higher dosages (total spores) at infection, as we used same initial dosage as for single species infections. These higher mortality rates may be a consequence of higher spore reproduction – mixed infections resulted in higher numbers of total spores-although the mechanism for this increased reproduction is unknown. Alternatively, the two species of *Nosema* may attack different molecular or physiological systems in honey bees, thus resulting in a synergistic effect of mixed species infections. However, earlier host mortality may not favor overall microsporidian production, or it may potentially favor the fastest reproducing or environmentally tougher species in a given host population. Because co-infections with different combinations of honey bee diseases commonly occur, it is important to understand these interactions and their effects on their host bees.

Our data demonstrated different trajectories over time for single species microsporidia infections. Single species infections of *N. ceranae* had higher spore counts on day 10, which is opposite of what is observed by Forsgren and Fries (2010), who observed an earlier build up of *N. apis*. The mean spore counts in midgut tissues for the single species infections were similar at days 15 and 20, which is consistent with the trend observed at 14 days by several previous studies (Paxton et al., 2007; Forsgren and Fries, 2010). Difference in counts among laboratories may be related to methodology of inoculation, age of bees inoculated, as well counting methods. Huang and Solter (2013) measured total spores in the

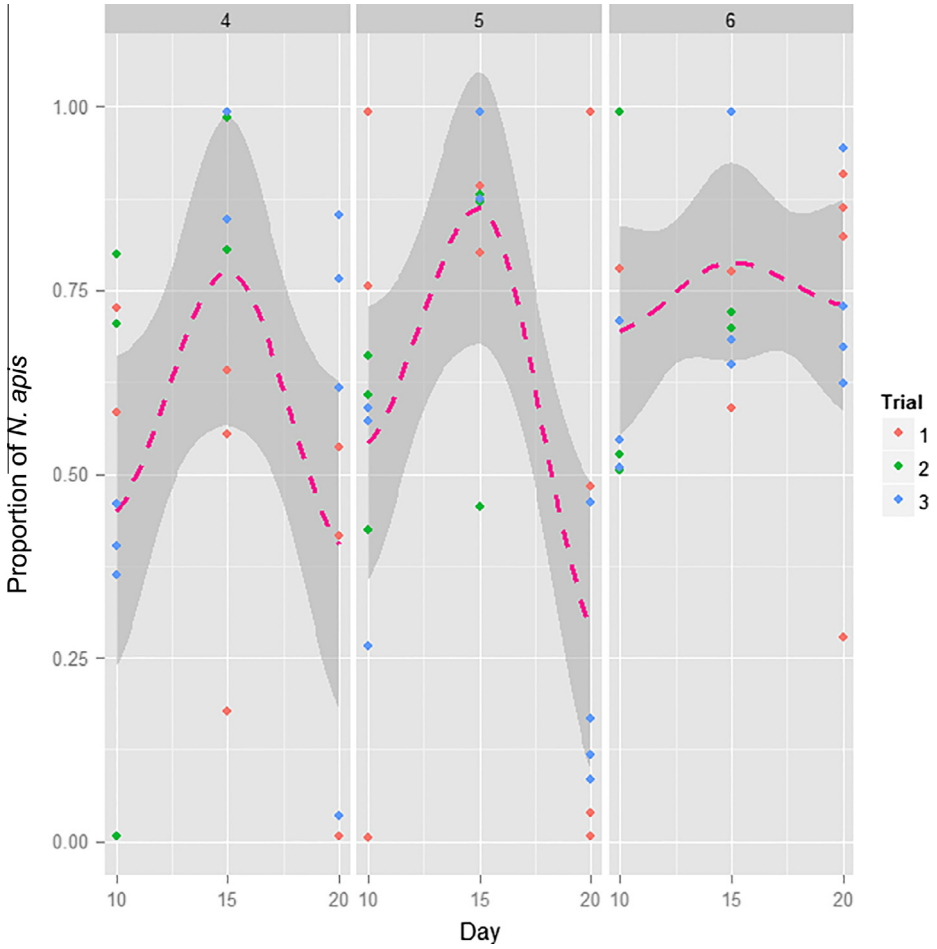

Fig. 4. Nonparametric trajectories of proportion of *N. apis* spores for Treatments 4, 5, and 6 by day, when day is treated as n interval level measure. Trials (indicated by colored dots) indicate different colonies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gut (midgut + hindgut) and found overall greater total spore production for *N. ceranae* from 14 to 20 days post inoculation. Our data here only includes spores in midgut, thus changes in spore counts could be due to differences in rate of movement from midgut to hindgut.

Our data do not support a clear advantage for *N. ceranae* spore growth in mixed infections that would lead to a global replacement of *N. apis* by *N. ceranae* in *A. mellifera*, as suggested by epidemiological evidence (Klee et al., 2007; Paxton et al., 2007; Chen et al., 2008; Fries, 2010; Martín-Hernández et al., 2012). Spore counts were similar for both species in many of the mixed infections, and for some treatments there was a greater number of *N. apis* spores. Differences in infectivity also did not account for potential replacement. We found similar rates of infection for both species in mixed and single infections, with *N. apis* having a slightly higher rate (91% compared to 86% for *N. ceranae* in mixed infections, and 96% compared to 85% for *N. ceranae* in single infections). We experienced minor cross-contamination of both species: 12% of bees inoculated with only *N. apis* had *N. ceranae* spores, and 28% of bees inoculated with only *N. ceranae* had *N. apis* spores, which may dilute some of the observed outcomes.

Other mechanisms besides spore growth and infectivity may be responsible for the apparent global replacement of *N. apis* by *N. ceranae*. For example, *N. ceranae* may respond differently to environmental cues, resulting in different, more efficient infection patterns (Gisder et al., 2010; Chen et al., 2012; Higes et al., 2013). A difference in thermal plasticity has been demonstrated between spores of the two species that may result in different infection rates in more natural and variable settings (Fenoy et al., 2009; Fries, 2010; Higes et al., 2013). It has been suggested that *N. ceranae* may infect tissues differently (Chen et al., 2009b; Bourgeois et al., 2012a), leading to different transmission patterns. Recent work, however, indicates that both species infect only the midgut tissues with possible involvement of proximal Malpighian tubules (Huang and Solter, 2013). Response to treatment may be another factor for this observed replacement: Huang et al. (2013) found that *N. ceranae* is released more quickly than *N. apis* from the effects of fumagillin as drug concentrations decline, and has a higher natural resistance to treatment.

In reporting the results we were occasionally confronted with the unavoidable circumstance of enormous statistical power. With very high power, the *p*-value's utility as a gauge of practical differences in the outcome declines dramatically. One way to convey the importance of a finding when faced with high statistical power is by reporting its effect size (e.g., Cohen, 1992; Murphy and Myors, 2003), a descriptive measure used to communicate the strength of a result. When the outcome variable is categorical, the usual methods for reporting effect size do not function appropriately. Alternative measures have been proposed for models that accommodate these type outcomes (Cox and Snell, 1989; Allen and Le, 2008), including the odds-ratio, which we employ here (Peng et al., 2002).

We noted high variability of spore counts within treatments, which may be due to variation in natural resistance or possibly patrilineal genetic differences (Bourgeois et al., 2012b; Tarpy, unpublished data). This is supported by the differences we noted

in survival among colonies, but our study was too small to formulate conclusions about colony-level resistance or tolerance. There are little published data on resistance of insects to their naturally occurring microsporidia (e.g., Hoch et al., 2008; Bourgeois et al., 2012b), and determination of natural differences among genotypes that may prevent or deter spore reproduction is an area that warrants further research. Breeding for such characteristics could reduce the prevalence and severity of *Nosema* infection.

Our data suggest that the apparent global advantage of *N. ceranae* is not due to a difference in spore production or dosage/infectivity, and that replacement of *N. apis* by *N. ceranae* is occurring by mechanisms other than a competitive advantage for within-host spore production. Our study demonstrates in a controlled setting the decreased survival of honey bees with mixed *Nosema* infections, and these results were robust over 20 days of infection. Further research in controlled field trials in a more natural colony context can elucidate the natural history of these infections, their synergistic effects, and environmental factors affecting transmission that may be the cause of the competition between these two pathogens.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2014.12.006.

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