

European isolates of the Microsporidia *Nosema apis* and *Nosema ceranae* have similar virulence in laboratory tests on European worker honey bees

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Abstract – *Nosema apis* and *Nosema ceranae* are gut parasites that infect western honey bees (*Apis mellifera*) worldwide. *N. ceranae* is an exotic infectious disease agent of *A. mellifera*, having been originally described in the Asian honey bee (*Apis cerana*), while *N. apis* is native to the western honey bee. To better understand the dynamics and epidemiology of the two pathogens, we examined the impact of European isolates of both Microsporidia on the longevity of European *A. mellifera* in a controlled laboratory experiment. *N. ceranae* caused slightly higher host mortality compared to *N. apis*, but differences in virulence were subtle and non-significant. Variation across published studies may reflect geographic differences in the coadaptation of hosts and parasites and seasonal differences in host susceptibility.

Apis mellifera / Microsporidia / host / parasite / survival

1. INTRODUCTION

The western honey bee (*Apis mellifera*) is the most important commercially managed pollinator, with its services to agriculture estimated to exceed \$200 billion per year (Klein et al. 2007; Gallai et al. 2009; Potts et al. 2010). Honey bee health is therefore a topic that has received considerable attention, especially given reports of dramatic and unexpected colony losses in the last decade (Carreck and Neumann 2010). Several factors have been suggested to account for the decrease in honey bee health status, especially the introduction and spread of pathogens due to

beekeeping activities (Carreck and Neumann 2010; vanEngelsdorp and Meixner 2010; Williams et al. 2010; González-Varo et al. 2013; Vanbergen and the Insect Pollinators Initiative 2013). Of the wide range of pathogens infecting honey bees, several have been listed as high-risk stressors reducing individual host lifespan, with subsequent detrimental effects on the colony (reviewed by Genersch 2010; Evans and Schwarz 2011).

Many studies have focused on an exotic emerging pathogen, the Microsporidian *Nosema ceranae* (Higes et al. 2006; Chen et al. 2008; Fries 2010). First described in the Asian honey bee (*Apis cerana*, Fries et al. 1996), *N. ceranae* successfully infected the western honey bee, probably prior to 1997 (Paxton et al. 2007), and is nowadays globally distributed (Klee et al. 2007). In some regions, *N. ceranae* has been suggested to be replacing the native *Nosema apis* (Klee et al. 2007; Higes et al. 2013), the

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Microsporidian that was for years considered to be the only disease agent of nosemosis in western honey bees (Zander 1909; Bailey 1955). Our recent experimental data suggest that *N. ceranae* exhibits a strong priority effect and a competitive advantage over *N. apis* when infecting a worker bee before its congener, plausibly explaining its predominance (Natsopoulou et al. 2015).

Given the threat that *Nosema* spp. pose to honey bees, comparative studies investigating differences in host responses to both Microsporidia have been undertaken, often focusing on possible fitness costs to hosts, e.g. death as a measure of pathogen virulence of infection by *N. ceranae* versus *N. apis*. Both Microsporidia are obligate intracellular pathogens that infect the epithelial cells of the ventriculus of adult bees, resulting in gut tissue degeneration (Fries et al. 1996; Higes et al. 2007; Huang and Solter 2013). *N. ceranae* has been additionally associated with nutritional stress (Mayack and Naug 2009; Martín-Hernández et al. 2011) and immune suppression (Dussaubat et al. 2012; Aufauvre et al. 2014). Moreover, a direct link between *N. ceranae* infection and colony depopulation or even total loss has been shown in Spanish honey bee (Martín-Hernández et al. 2007; Higes et al. 2008, 2009, 2010; Botías et al. 2013). However, in other regions of the world, colony loss due to *N. ceranae* infection has not been reported (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009; Gisder et al. 2010); the impact of *N. ceranae* may indeed vary across countries and climatic conditions.

At the level of the individual honey bee, studies are contradictory concerning the virulence of *N. ceranae* over *N. apis*. Paxton et al. (2007), Martín-Hernández et al. (2011) and Williams et al. (2014) found that *N. ceranae* induced higher mortality than *N. apis*, but this was not confirmed by Forsgren and Fries (2010), Huang et al. (2015) or Milbrath et al. (2015), who found the two Microsporidia species to induce similar mortality. The experiments by Forsgren and Fries (2010) and Milbrath et al. (2015) were not designed focusing on host mortality, and authors did not comment in detail on the lack of differences in host mortality induced by the two Microsporidia. Huang et al. (2015), on the other hand, explicitly tested for differences in virulence; they (Huang

et al. 2015) hypothesised that adaptation of *A. mellifera* to *N. ceranae* may have already occurred in North America (but not in Europe), which could explain why North American honey bees exhibit similar mortality to *N. apis* and *N. ceranae*, in contrast to Europe, where *N. ceranae* seems to be more virulent than *N. apis* (Paxton et al. 2007; Martín-Hernández et al. 2011).

Because of these contradictory results and the still open question of the possibly greater virulence of the exotic *N. ceranae* over the native *N. apis*, we test the hypothesis of Huang et al. (2015) that *N. ceranae* is more virulent than *N. apis* for honey bees in Europe. Studies of comparative virulence may also help to explain why *N. ceranae* has become dominant over *N. apis* in warmer regions of the world, and if the variation in virulence reported in the literature is a reflection of differences in geographic coadaptation of hosts and Microsporidia. Specifically, we performed mortality bioassays using honey bee workers originating from Germany in which bees were experimentally infected with European isolates of either *N. apis* or *N. ceranae*, and host fitness (parasite virulence) in terms of host longevity was monitored in carefully controlled cage experiments.

2. MATERIAL AND METHODS

2.1. Host and pathogen preparation

Three queen-right, unrelated colonies of *A. mellifera* (typical local Halle (Germany) beekeeper colonies of the subspecies *A. mellifera carnica*) were used as the source of worker bees. Bees that newly emerged in the laboratory were placed in metal cages, with bees from source colonies equally mixed across cages (18 bees per cage) so as to eliminate any colony genotypic effect, and then kept for 3 days in an incubator at +30 °C with ad libitum 50 % (w/v) sucrose solution following standard procedures (Williams et al. 2013) until inoculation.

Spores of both *Nosema* species were obtained from artificial propagations kept in the lab through mass feeding of caged adult honey bees. *N. ceranae* spores originated from Germany, while *N. apis* spores originated from Sweden. On the day of infection, several (>15) host bees per propagation were checked under the

microscope in order to verify inoculation success; each bee contained millions of spores. Freshly prepared inocula containing spores of either *N. ceranae* or *N. apis* were obtained by crushing the ventriculus of honey bees from the propagations in distilled water and purified by triangulation (Fries et al. 2013). *Nosema* spores were counted using a Fuchs-Rosenthal haemocytometer. Spore solutions were kept at room temperature throughout the whole procedure until inoculation of all experimental bees on the same day (maximum 8 h between extractions from propagation bees to feeding to experimental bees). The same procedure was followed using uninfected caged bees that served as control inoculum.

2.2. Inoculation

Four-day-old caged workers were individually fed with 10 μ l of 50 % w/v sucrose solution containing a control solution, or 10⁵ spores of either *N. ceranae* or *N. apis*: this dosage has been repeatedly shown to result in 100 % *Nosema* infection in honey bees (Forsgren and Fries 2010; Huang et al. 2015). For inoculations, bees were immobilised by hand following the recommendations of Human et al. (2013) without prior anaesthesia; attention was given to avoid any physical harm to hosts. After feeding, each individual was kept isolated in a 1.5-mL Eppendorf tube for ca. 30 min post infection to avoid any pathogen exchange through trophallaxis (Fries et al. 2013). Bees that did not consume the entire volume of inoculum during feeding or were observed to have discharged the inoculum during the first 30 min were discarded. Each treatment was replicated five times (five independent replicate cages) with 18 bees per cage. All replicates and all treatments were set up within the same day. Cages were placed back in the incubator and checked every 24 h starting the day after inoculation. Dead bees were counted and removed from each cage. Observations continued until all bees in all replicates were dead.

Absence of cross-contamination in the inocula used for the infections was confirmed with a multiplex PCR as described in Fries et al. (2013). To verify if the infection had been successful, bees that died at 21 days post infection (p.i.) were individually checked under the microscope for the presence of *Nosema* spp. At that age, both species have already asymptoted in number in the ventriculus (Forsgren and Fries 2010; Huang and Solter 2013). DNA was also extracted from these bees

(pooled per treatment) and, using the same multiplex PCR described above, they were checked for cross-contamination.

2.3. Survival analysis

Survival analysis was performed in R v 3.1.0 using a mixed effects Cox proportional hazard model (R package ‘survival’ (Therneau and Grambsch 2000), and the R package ‘coxme’ (Therneau et al. 2003)), with treatment as a fixed factor and cage (replicate) as a random effect. Including cage as a random effect did not improve the model ($P > 0.05$), indicating that there was no significant variation between replicates. It was nevertheless included in the model as an integral component of the experimental design. Post hoc analysis to test significant differences between treatment means was performed using the R package ‘multcomp’ (Hothorn et al. 2007), applying a Bonferroni correction for multiple testing.

We used two approaches to evaluate the statistical power with which differences in host mortality between Microsporidia species could be detected. Firstly, given our sample sizes and the variation we observed in our dataset, we increased the difference in mean survival between the two treatments by reducing the survival of the *N. ceranae* treatment group while keeping that of the *N. apis* treatment group constant, then tested for significance of differences in survival. Secondly, we estimated the power of our current analysis (β) and the sample size of host individuals necessary to increase statistical power so as to be able to reject the null hypothesis of no difference between *Nosema* spp. treatments using the R package ‘powerSurvEpi’ (Qiu et al. 2012).

3. RESULTS

PCR demonstrated that inocula fed to bees contained only *N. ceranae* or *N. apis*, while control solutions were devoid of Microsporidia DNA and, by visual inspection, they were devoid of spores, too. Microscopic analysis of individual bees that died 21 days p.i. showed that infections were successful as *Nosema* spores were observed across all replicate cages in both Microsporidia treatments ($n=8$ individual bees per treatment). Spores were not observed in control bees ($n=5$)

and PCRs on the same bees (pooled per treatment) verified the absence of cross-contamination.

Nosema treatments resulted in slightly but significantly faster mortality of caged honey bees (Figure 1). During the first 10 days p.i., mortality was slight and below 10 % across all treatments (Figure 1). The median survival time (LT₅₀) was 23 days p.i. (95 % CI, 22–24) for the control, 20 days p.i. (19–22) for *N. ceranae* and 21 days p.i. (20–22) for *N. apis* treatments. Treatment was a significant predictor of survival; when it was included as an explanatory variable, the statistical model was significantly better compared to the null model without treatment (likelihood ratio $\chi^2=16.8$, $df=2$, $P<0.05$). Though infection with *N. ceranae* or *N. apis* significantly increased mortality compared to the controls (hazard ratio:control vs. *N. ceranae*: $z=3.44$, $P<0.05$, control vs. *N. apis*: $z=3.51$, $P<0.05$), survival did not differ significantly between Microsporidian treatments *N. apis* and *N. ceranae* ($P>0.05$).

Our power analysis showed that, given our samples size and the variance detected in our dataset, if differences in mean survival of the two Microsporidia treatments had been >3 days, the difference would have been significant. Differences in virulence that we observed were

clearly less than 3 days. Indeed, the statistical power (β) of our survival analysis given the observed differences in hazard ratio was found to be very low (0.03), indicating that small differences in hazard ratio, as we found, need a great number of replicates in order to detect a difference as statistically significant (estimated sample size, $>15,000$ individuals per treatment). Overall, our power analyses suggest that the observed differences in virulence between Microsporidia species were minimal in our experimental paradigm.

4. DISCUSSION/CONCLUSION

Our experiment confirms that *Nosema* spp. are virulent for their host *A. mellifera*; infection significantly decreased longevity of honey bee workers, albeit only by 2–3 days in our laboratory set-up. Yet, we found only slight and statistically non-significant differences in virulence (mortality) between *N. ceranae* and *N. apis*. We can thus reject the hypothesis of Huang et al. (2015) that honey bees in Europe respond differently to *N. ceranae* infection versus *N. apis* infection; we find that honey bees in Europe respond similarly to *N. ceranae* infection and *N. apis* infection, as they do in USA (Huang et al. 2015).

These results contrast with others from Europe (Paxton et al. 2007, a coauthor of the current study; Martín-Hernández et al. 2011) and Canada (Williams et al. 2014), in which *N. ceranae* was found to be much more virulent than *N. apis* for caged honey bees. Rather, our results correspond more closely with the recently reported results from the USA showing that *N. ceranae* is not more virulent than *N. apis* in terms of inducing host mortality (Huang et al. 2015; Milbrath et al. 2015). Huang et al. (2015) argue that differences between their results and those reported from Europe may reflect an adaption of USA bees to *N. ceranae* through long-term exposure and thus reduced susceptibility, or lower virulence of USA isolates of *N. ceranae*. Our data show that the same host responses can be observed in European bees and thus the similarity in virulence of *N. apis* and *N. ceranae* found by Huang et al. (2015) does not represent a regional effect found only in North American honey bees and North American isolates of Microsporidia.

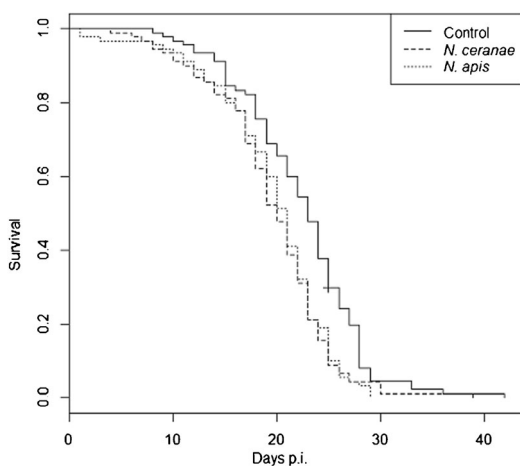


Figure 1. Survival curves for the different *Nosema* treatments. Both species were found to reduce significantly the survival of honey bee workers compared to control ($P<0.05$) but the difference in survival between *Nosema* treatments was not significant ($P>0.05$).

One explanation for the variability across published studies is that differences in virulence between the two Microsporidian species are sensitive to experimental conditions. We consider this unlikely because most reported studies employ standard experimental approaches in which honey bee workers are individually fed freshly harvested spores of *Nosema* (Fries et al. 2013) and are then caged en masse (Williams et al. 2013).

Variation exists across published studies in the age at which bees are inoculated, variation that could potentially explain differences in results across studies. Differences in susceptibility among bees infected at different ages has been demonstrated by Huang et al. (2015), who found newly emerged bees (<24 h after eclosion) to be less susceptible to both *Nosema* spp., particularly to *N. ceranae*. Yet, studies using same-age bees have generated inconsistent results: Williams et al. (2014) and Milbrath et al. (2015) each inoculated bees a few hours after eclosion (<24 h) and reported higher virulence of *N. ceranae* or no difference between the two species, respectively. Martín-Hernández et al. (2011) and Huang et al. (2015) both infected 5-day-old bees in their experiments but also found contradictory results, with *N. ceranae* more virulent or no different in virulence, respectively. Differences in study protocols and age of bees at infection therefore seem unlikely to explain differences in study results. However, extensive studies on the interaction between age-dependent susceptibility to infection and host genotype are lacking. In our study, we followed procedures to minimise handling effects on bees and ensure high spore viability in both inocula by employing standard experimental approaches (Fries et al. 2013; Human et al. 2013; Williams et al. 2013); we acknowledge, though, that differences in spore viability coupled to length of host exposure might also explain our results.

The discrepancy between our results and those of previous studies in Europe may alternatively reflect different responses of the host due to genetic differences of either the host or the pathogen. As host genotype is considered a key factor determining infection outcome (Minchella 1985; de Roode et al. 2004), different host subspecies may vary in their ability to counter infection. At

least 26 subspecies of *A. mellifera* have been described (Ruttner et al. 1978), and variation in response to *Nosema* spp. may lie in inherent host subspecific differences. For example, experiments in Spain are conducted using *A. mellifera iberiensis* (Higes et al. 2007) while *A. mellifera mellifera*, *A. mellifera carnica* or Buckfast (a cross of many subspecies) are usually tested in central and northern Europe (Mulholland et al. 2012; Fontbonne et al. 2013; Francis et al. 2014). However, recent studies suggest that genetic variation among individual bees or colonies within a subspecies—and not between subspecies—is a better predictor of host response to *N. ceranae* (Fontbonne et al. 2013; Francis et al. 2014; Meixner et al. 2014).

Genetic differences among pathogen isolates from different geographic locations may also lead to variation in response of the host. *N. ceranae* in Spain has been associated with colony depopulation and colony losses and a higher prevalence than *N. apis* (Higes et al. 2008; Fernández et al. 2012). In Germany, both *Nosema* species co-exist in populations at a similar prevalence (Gisder et al. 2010), with no impact on colony losses (Genersch 2010; Gisder et al. 2010). This suggests the presence in Europe of *N. ceranae* strains that differ in virulence. However, a study comparing *N. ceranae* strains from France and Spain failed to show any difference, either genetically in the parasite or in terms of virulence, measured as the mortality it induced in host worker honey bees (Dussaubat et al. 2013). A recent genomic study also supports the idea that *N. ceranae* does not differ across the range of *A. mellifera* and exists as a single clonal variant (Pelín et al. 2015).

Though we have argued that genetic differences among host subspecies or pathogen isolates are unlikely to account for differences observed in studies of the virulence of *N. apis* versus *N. ceranae*, specific combinations of host-pathogen genotypes might nevertheless lead to different infection outcome. Interaction effects between host and parasite genotypes (G×G) have been found to play a key role determining infection traits such as virulence in several host-parasite systems (Lambrechts et al. 2006). Moreover, genetic differences may lead to differences in virulence if they are dependent on environment

(e.g. climatic conditions), known as a genotype×environment interaction (Thomas and Blanford 2003; Wolinska and King 2009). This notion may explain why *N. ceranae* in Spain induced 100 % host mortality at 8 days post infection (Higes et al. 2007; Martín-Hernández et al. 2011), whereas in Sweden and Canada, it induced <25 % host mortality in the same time frame (Forsgren and Fries 2010; Williams et al. 2014). Whether the response of hosts to *N. apis* varies similarly is not known.

Worker honey bee physiology varies profoundly across the year in temperate regions of the world (Winston 1987), and this variation may potentially be of greater importance than genotype×environment interactions in explaining differences among published results in virulence of pathogens. For example, Doublet et al. (2015) found mortality induced by *N. ceranae* to exhibit a strong seasonal effect. If the susceptibility of honey bees to *N. apis* and *N. ceranae* varies differentially across the year, this might also explain differences observed between studies in the relative virulence of *N. apis* and *N. ceranae*.

That we did not find a significant difference between the two pathogens in inducing mortality in our experiment may not be surprising as the size of the effect was small; the median survival time of bees infected with *N. ceranae* was only 1 day less than that of *N. apis* (20 vs. 21 days for *N. ceranae* and *N. apis* respectively). As we have shown, small effect sizes require greater experimental replication to detect differences. We note that another recent study with European honey bees also found *N. ceranae* to induce a slightly higher, albeit non-significant, mortality than *N. apis* (Gajda et al. 2014).

Of course, subtle differences in the response of caged honey bees in a benign environment may translate into more profound differences in behaviour or longevity in a harsh field environment (e.g. homing ability, Wolf et al. 2014). We cannot therefore predict what the impact of the small and insignificant difference we observed in our cage experiment might be under natural conditions, where worker honey bees face more stressful conditions. However, the successful spread of *N. ceranae* and its dominance over *N. apis* in some locations is probably more related to the

competitive advantage of *N. ceranae* over *N. apis* in warmer regions of the world (Natsopoulou et al. 2015) rather than to its higher virulence in terms of inducing host mortality. That our results concur with those from the USA (Huang et al. 2015; Milbrath et al. 2015) in showing that *N. ceranae* differs little in virulence from *N. apis* highlights the necessity for future research to focus on exploring other mechanisms responsible for shaping the current patterns of distribution and prevalence of *N. ceranae* and *N. apis*, such as comparative transmission dynamics, parasite inter-species competition and host genotype×parasite genotype effects.

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Des isolats européens des microsporidies *Nosema apis* et *Nosema ceranae* présentent la même virulence vis-à-vis des ouvrières de l'abeille européenne dans des essais en laboratoire

Apis mellifera / relation hôte parasite / survie / Microsporidia

Europäische Isolate der Mikrosporidienarten *Nosema apis* und *Nosema ceranae* zeigen in Labortests ähnliche Virulenz gegenüber Arbeiterinnen der europäischen Honigbiene

Apis mellifera / Microsporidien / Wirt / Parasit / Überlebenszeit

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