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Comparative virulence of *Nosema ceranae* and *Nosema apis* in individual European honey bees

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ABSTRACT

Nosema apis and *Nosema ceranae* are intracellular microsporidian parasites infecting the midgut epithelial cells of adult honey bees. *N. ceranae* was considered to be restricted to the Asian honey bee, *Apis cerana*, but is nowadays a parasite found also in the European honey bee (*Apis mellifera*) across most of the world. Recent surveys and experimental work suggest that *N. ceranae* is a serious threat to the global beekeeping industry. It has been suggested that *N. ceranae* induces significantly higher mortality in honey bees than *N. apis*, but little is known about their comparative virulence. In this study, we used *in vivo* infection experiments to study the two parasites' different virulence (i.e. multiplication rate and infectivity). A qPCR was developed to elucidate within host competition between the two parasites using mixed infections. The outcome of the experiments indicates minor differences in infectious dose and multiplication rate between the two species. Moreover, the mortality caused by *N. ceranae* was not significantly higher than for *N. apis* and *N. ceranae* appeared to have no competitive advantage within host.

1. Introduction

Two microsporidian parasites are described from honey bees, *Nosema apis and Nosema ceranae*. *N. apis* was isolated in the European honey bee (*Apis mellifera*) (Zander, 1909) and *N. ceranae* was isolated from the Asian honey bee (*Apis cerana*) in China (Fries et al., 1996). Infection experiments have demonstrated that both parasites are cross-infective across host species, but that *N. apis* develops less well in *A. cerana* compared to *N. ceranae* in *A. mellifera* (Fries and Feng, 1995; Fries, 1997).

Recently, natural infections of *N. ceranae* in *A. mellifera* were detected in Taiwan and in Spain (Higes et al., 2006; Huang et al., 2007). Samples from across the world now demonstrate that the infection of *N. ceranae* in *A. mellifera* is a world-wide phenomenon (Klee et al., 2007; Giersch et al., 2009) and investigations of historic samples of bees infected with microsporidian spores suggest a replacement process, where *N. ceranae* appears to gradually replace *N. apis* (Paxton et al., 2007). This implies that *N. ceranae* may be a more virulent parasite. Indeed, in one study, experimental infections of *N. ceranae* in the laboratory resulted in a total mortality of 94.1% 1 week post-infection in three infected replicate cages, and by the 8th day post-infection, all infected bees had died (Higes et al., 2007). These survival results of infected bees are in sharp contrast to earlier findings in cage experiments using *N. apis* (Fries, 1988) but also to a limited comparative analysis of the two parasites (Paxton et al., 2007). Other processes such as transmission rates or parasite replication may also influence the relative prevalence of the two parasites over time. In general, there is a positive relationship among parasite reproduction, virulence and increased transmission (Ebert, 1998; de Roode et al., 2009). However, increased parasite virulence may reduce parasite fitness if host mortality results in decreased transmission opportunities. Thus, there should be a trade-off between transmission and virulence. Although the relation

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between transmission and virulence may be complicated (e.g. Ganusov and Antia, 2003), experimental evidence, also using microsporidia infections, do demonstrate this trade-off *in vivo* (Berenos et al., 2009).

Natural infections where hosts are infected by a single parasite alone are rare (Read and Taylor, 2001), and multiple infections seem to be the rule rather than the exception. Within host interactions between parasites can influence both disease severity and epidemiology and mixed infections with *N. apis* and *N. ceranae* are common (Paxton et al., 2007; Fries and Forsgren, 2008; Chen et al., 2009). The effects from multiple species microsporidia infections are unpredictable. One species may out-compete the other in certain tissues or suppress spore formation in competing species (Solter et al., 2002). One species may significantly influence transmission rates of another or have no measurable effect on transmission (Pilarska et al., 2006). Further knowledge about the mechanisms of pathogen competition and the virulence consequences in mixed infections of *Nosema* spp. in honey bees is crucial for predicting future impacts of *N. ceranae* infections on apiculture. In this study we present data on within host competition between *N. apis* and *N. ceranae* and also compare their respective multiplication rate and infectivity using individual feeding of infectious spores to adult bees.

2. Materials and methods

2.1. Infection experiments

Adult worker bees were collected from combs being drawn out from comb foundation in an *A. mellifera* colony in the apiary at the Swedish University of Agricultural Sciences in Uppsala, Sweden and used in the infection experiments. The average age of such bees is approximately 2 weeks (Winston and Bennet, 1982). Prior to experiments, 100 bees from the colony were confirmed to be free from microsporidian spores using light microscopy (400×) on preparations of their ventriculi. The bees were kept in cages in an incubator at +30 °C and after treatment fed *ad libitum* with a 50% (w/v) sucrose solution throughout experiments. Fresh spore suspensions for infection were prepared by mass feeding of *N. apis* spores originating from Sweden and *N. ceranae* spores originating from Italy. Spore suspensions of each species were prepared, counted and diluted to required concentrations in 50% (w/v) sucrose solutions. The *Nosema* species identification was confirmed using a species specific polymerase chain reaction (PCR) method published by Chen et al. (2008).

2.2. Infectious dose and mortality

Initial experiments were made to estimate the dose of the respective microsporidian species needed to infect 50% (ID_{50}) and 100% (ID_{100}) of the bees. For each dose group, 30 adult bees were individually fed 10 μl sugar solution including 10, 10^2, 10^3 and 10^4 spores of *N. apis* and *N. ceranae*, respectively. A total of 90 bees were inoculated for each parasite species. Control bees were individually fed with 10 μl of a 50% (w/v) sucrose solution without any *Nosema* spores. The mortality was monitored daily from day 3 to day 14 post-infection and dead bees were removed from the cages. After 14 days, all bees were killed, each ventriculus dissected, homogenized and checked under a light microscope for infection. The experiment was repeated three times.

2.3. Course of infection

Adult bees ($n = 20$) were individually fed 10 μl sugar solution including high doses (10^4 spores = ID_{100}) of *N. apis* and another group of bees ($n = 20$) were fed equally high dose (10^4 spores = ID_{100}) of *N. ceranae*. Four bees from each group were sampled after 4, 6, 8, 10 and 12 days post-infection. Each ventriculus was dissected and homogenized in 1 ml H_2O and the average number of spores was estimated using a Bürker hemocytometer (Cantwell, 1970). The experiment was repeated twice.

2.4. Within host-competition

Three groups of bees ($n = 30$) were individually fed 10 μl sugar solution including a mix of *N. apis* and *N. ceranae* spores. One group ($n = 10$, group 1) was fed a mixture of *N. apis* and *N. ceranae* spores in the proportion 9–1 (9×10^3 spores of *N. apis* and 1×10^3 spores of *N. ceranae*). Another group ($n = 10$, group 2) was fed equal amounts of both species (i.e. 5×10^3 spores of *N. apis* and 5×10^3 spores of *N. ceranae*). A third group ($n = 10$, group 3) was fed both species in reverse spore concentration compared to group 1 (1×10^3 spores of *N. apis* and 9×10^3 spores of *N. ceranae*). After 14 days, the bees were killed, the ventriculi dissected and stored in −20 °C until analyzed with qPCR for quantification of the two *Nosema* species

2.5. Genomic DNA extraction

The ventriculi were individually grounded in liquid nitrogen and extraction of the genomic DNA was made using a DNeasy® Plant Mini Kit (Qiagen) according to manufacturer's instructions for plant tissue starting from the dissected and homogenized ventriculus. For automated purifications, a QIAcube (Qiagen) instrument was used.

2.6. Primer design and qPCR

Forward and reverse primers were designed from sequence data of the16S rRNA gene available in the GeneBank database. Forward and reverse sequences were selected over regions of specificity for *Nosema* spp. Three primer sites were selected; one *Nosema* universal primer, UnivRev (5′-GTC GCT ATG ATC GCT TGC C-3′), and two species specific reverse primers, NaFor (5′-CTA GTA TAT TTG AAT ATT GTT TAC AAT GG-3′) and NcFor (5′-TAT TGT AGA GAG GTG GGA GAT T-3′).The specificity of the primers was confirmed in PCR reactions using DNA isolated from *N. ceranae*, *N. apis* and *Nosema bombi* as templates. These isolates were earlier identified in PCR reactions using primers described by Chen et al. (2008) and an RFLP (Restriction Fragment Length Polymorphism) method described by Klee et al. (2007).

The quantitative real-time PCR reaction was performed in separate uniplex reactions using 10 μl of iQ™ SYBR® Green Super mix (Bio-Rad), 0.4 μM of each primer (UnivRev and NaFor or NcFor, respectively), 2 μl of template and the final reaction volume adjusted to 20 μl with nuclease-free water. A reaction containing water instead of DNA template was set up as a negative control. All real-time reactions (standards, unknown samples and controls) were performed in duplicate in neighboring wells on the sample plate. Results reported are an average of the duplicates. The amplification and data acquisition were carried out using a MiniOpticon® (Bio-Rad) real-time PCR machine under the following cycling condition: initial activation step, 95 °C for 5 min, PCR cycling (40 cycles of 94 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s) including data collection The amplified product was confirmed using melting curve analysis whereby the reaction was incubated by raising the incubation temperature from 55 to 95 °C in 0.5 °C increments with a hold of 1 second at each increment. Since SYBR® Green I dye binds with any double stranded DNA product, specificity and the absence of non-specific amplification was determined based on the melting temperature (T_m) of the amplified products. Each set of PCR assays comprised serial dilutions of *N. apis* and *N. ceranae* amplicons as external standards. Standard curves was prepared by using serial dilutions of target DNA fragments (i.e. purified PCR products) ranging from 10^{-2} to 10^{-8} as quantification standards in every run.

3. Results

3.1. Infectious dose and mortality

When combining the results from the three infection dose experiments, the mean infectious dose needed to infect 50% (ID_{50}) of the bees was lower for *N. ceranae* (approximately 85 spores) than for *N. apis* (approximately 390 spores, Fig. 1). However, this overall difference in ID_{50} value can be traced to one individual experiment where the ID_{50} for *N. apis* was 670 spores compared to 50 spores for *N. ceranae*. In the other two experiments the ID_{50} value for the parasites was comparable, but variable between experiments. When fed 10^4 spores all bees became infected

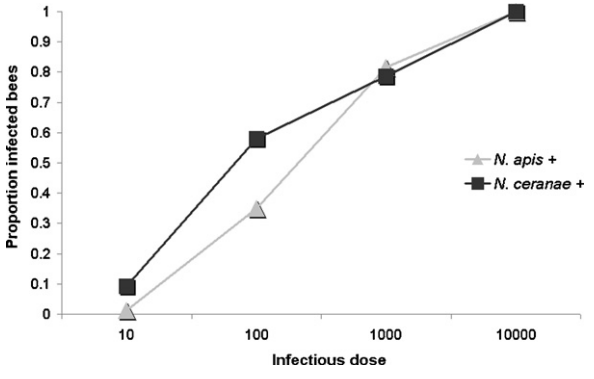

Fig. 1. The proportion of infected bees 14 days post-infection. Bees fed 10, 10^2, 10^3 and 10^4 spores of *N. apis* or *N. ceranae*. Results presented as a mean based on three cages with 30 bees ($n = 90$) for each treatment.

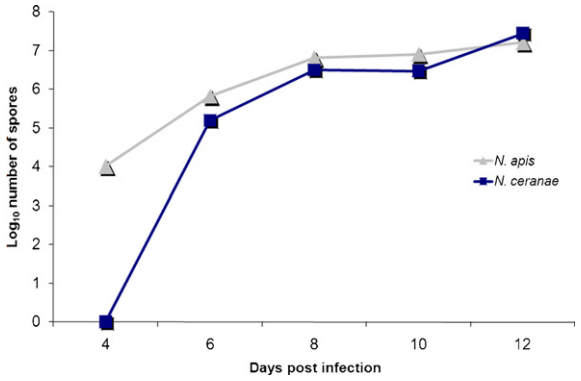

Fig. 2. The course of infection for *N. apis* and *N. ceranae*. Bees were individually infected with 10 000 spores of the respective parasite. At day 12 post-infection, the infection is probably fully developed with a similar number of spores produced by both species.

irrespective of species (Fig. 1). Thus, the ID_{100} value proved to be approximately 10^4 spores for both parasites. There was no significant difference between the cumulative mortality of bees infected with *N. ceranae* compared to those infected with *N. apis* (Chi-square, $p > 0.05$, Mann–Whitney, $p > 0.05$). The mean cumulative mortality in all three experiments after 14 days varied between 0 and 22%, with the highest registered mortality in the group infected with 10^2 *N. apis* spores. The highest registered mortality within one single cage exposed to 10^4 spores of *N. ceranae* was 23% (7 out of 30 inoculated bees).

3.2. Course of infection

The initiation of *N. ceranae* spore production *in vivo* was slower compared to *N. apis* (Fig. 2). With the spore doses given, mature spores of *N. apis* were seen four days post-infection whereas no mature spores of *N. ceranae* could be detected at the same time. However, a fully developed infection in the ventriculus appeared to be reached at about 10-12 days post-infection for both parasites, with a similar number of spores present (approximately $20 \pm 4 \times 10^6$ spores). Possibly, the quantitative spore increase may have continued beyond the course of the experiment for *N. ceranae* (Fig. 2).

3.3. Competition

The mean relative amount of *N. ceranae* DNA in the group fed equal amounts of the two parasites (group 2), was almost unchanged after 14 days of infection (Fig. 3). The mean proportion of *N. ceranae* DNA decreased from 0.50 to 0.47, however the individual bee ratios varied from 0.0 to 0.9 (Fig. 3). In the group fed the low dose *N. ceranae* (group 1), there was a minor, non-significant increase ($p = 0.2793$, one-sample t-test on arcsin transformed proportions) in the mean proportion of *N. ceranae* DNA from the initial 0.10–0.23. However, considering the small sample number ($i = 10$), a difference in proportions of at least 0.22 would have been considered significant given the sample size used. Also in this group (group 1) the inter-individual variation was extensive with individual values ranging from 0.0

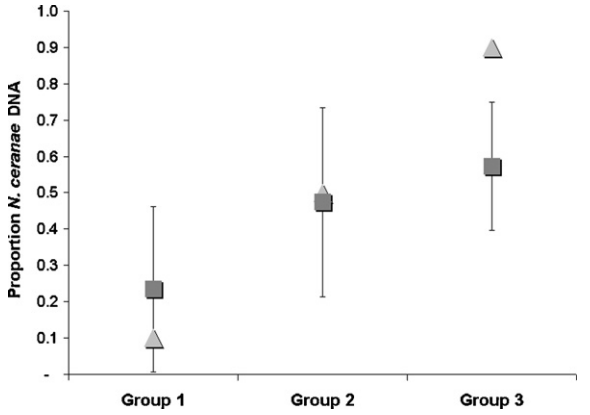

Fig. 3. The mean relative amount of *N. ceranae* DNA 14 days post-infection. Group 1 was infected with 10% *N. ceranae* and 90% *N. apis*, group 2 was infected with equal amounts of both species and group 3 was infected with 90% *N. ceranae* and 10% *N. apis*. The triangles indicate the proportion of *N. ceranae* DNA at the time of infection whereas the squares show the same proportion 14 days post-infection. Error bars show standard deviation.

to 0.69 (Fig. 3). In the group fed the 90% *N. ceranae* and 10% *N. apis* (group 3), there was a decrease in the proportion *N. ceranae* DNA in all but one individual. The mean ratio had decreased from 0.90 to 0.57 ($p = 0.006$, one-sample t-test on arcsin transformed proportions) and individual ratios varied from 0.39 to 1.0.

3.4. qPCR

The presented qPCR method uses external DNA standards (i.e. serial dilutions of PCR amplicons) to calculate the amount of target DNA in a sample. Standard curves were generated by duplicate amplifications of serial dilutions of DNA template, plotting the C(q) values versus nanogram target DNA. The resulting calibration curves indicated a PCR efficiency of between 96.7 and 98.3% and a linear detection range between 10^{-1} to 10^{-9} dilutions of the DNA standard. The level of detection in the quantitative PCR assay was estimated by assaying ten replicates of samples containing 10^{-10}, 10^{-9} and 10^{-8} dilutions of the DNA standard per reaction. Target DNA was successfully amplified from all ten replicates of standard DNA diluted 10^{-9}. However, the efficiencies in an actual sample may in practice be lower due to inhibition of the sample matrix.

4. Discussion

Our results do not indicate a higher virulence of *N. ceranae* compared to *N. apis* in individual bees. Although the (ID_{50}) value for *N. ceranae* may be somewhat lower, the cumulative mortality caused by *N. ceranae* was not significantly higher than the mortality induced by *N. apis*. This is contradictory to earlier results from Higes et al. (2007) who infected bees with *N. ceranae* in cage experiments using an infectious dose of 10^5 spores (10 times the ID_{100} value in our experiments) of the parasites to infect the bees. In their experiment all infected bees died within eight days post-infection. However, this high and rapid mortality has not been confirmed in other reports using cage experiments and individual feeding (Paxton et al., 2007; Martin-Hernandez et al., 2009). The results of Paxton et al. (2007) did suggest a higher virulence in *N. ceranae* compared to *N. apis* but the authors urged caution in interpreting the results as definitive since they were based on a single cage per treatment. Furthermore, by day 14 post-infection 14 out of 25 bees infected with *N. ceranae* were still alive in that experiment.

The virulence of a pathogen is related to its transmission or fecundity (Frank and Schmid-Hempel, 2008; Perlman, 2009), with a trade-off between virulence and transmission. Since *Nosema* spread between hosts as spores, a faster or more effective spore production could imply a higher virulence both at individual and colony levels. In our experiments, the spore load in the individual bee initially built up more slowly with *N. ceranae* compared to *N. apis*, and 12 days after infection the amount of spores was almost the same for both parasites. This is consistent with earlier results from Paxton et al. (2007) where both parasites reached about the same spore levels in the midgut after 10–12 days. This does not imply a faster or more effective spore production in *N. ceranae*. Martin-Hernandez et al. (2009) reported different growth curves at +33 °C with spores produced much faster in *N. ceranae* than in *N. apis*. However, they also infected with a high dose (10^5 spores per bee) and investigated the whole abdomen and not just the ventriculus. This discrepancy in growth pattern may be due to different methods used, but also to different temperatures at which infected bees were exposed.

Infections where hosts are infected with two or more parasite genotypes or species are ubiquitous (Read and Taylor, 2001) and mixed infections with *N. apis* and *N. ceranae* are commonly found (Paxton et al., 2007; Fries and Forsgren, 2008; Chen et al., 2009). Earlier experimental studies have shown that initial conditions such as relative inoculation frequency can be important for which parasite strain or species that outcompetes the other (Read and Taylor, 2001; Mideo, 2009). In our experiments, we inoculated bees with the two *Nosema* species in three relative frequencies but could not see any clear competitive advantage for any of the two parasites. The data (Fig. 3) actually suggest a tendency for the least common variant to be favored, irrespective if this is *N. apis* or *N. ceranae*. To our knowledge, this is the first reported result on within host competition from mixed infections with *N. apis* and *N. ceranae*. The results could reflect a scenario of negative frequency-dependent selection within the host (Weeks and Hoffmann, 2008). Besides frequency dependent selection within host, there may also be intraspecific competitive interactions present where the most abundant parasite inhibits germination and/or growth of conspecifics but not of heterospecifics. We cannot distinguish between these different alternatives, but none of these scenarios would suggest a within host competitive advantage of one parasite species compared to the other. The spores of both species were administered at the same time in this experiment. Different timing of spore administration may give a different result as has been demonstrated for competition between *Nosema lymantriae* and *Vairimorpha dispars* in the gypsy moth, *Lymantria dispars* (Pilarska et al., 2006). In that case the first inoculated species showed a higher rate

of successful establishment irrespective of which species that was first inoculated.

Even though several studies from Spain suggest that infections with *N. ceranae* lead to colony collapse (Martin-Hernandez et al., 2007; Higes et al., 2008), the parasite appears to have no competitive advantage within the individual bee in cage experiments. However, there may be no straightforward relationship between the within-host competitive ability and transmission rates. Differences and variation in severity of the *Nosema* infections may be due to a number of other factors than within-host competition. Furthermore, individual level virulence in honey bee pathogens does not necessarily correlate with colony level virulence (Fries and Camazine, 2001).

In conclusion, our results do not suggest substantial differences in individual level virulence between *N. apis* and *N. ceranae*. The high colony level virulence in *N. ceranae* infected colonies reported from Spain (Higes et al., 2008) may be a regional phenomenon as high mortality in infected colonies are not always present (Cox-Foster et al., 2007; Invernizzi et al., 2009; Pajuelo et al., 2008; Siede et al., 2008). Thus, other factors than *N. ceranae* alone, such as associated virus infections, may be complementary to explain high colony level virulence in this parasite. For example, some viruses, e.g. black queen cell virus (BQCV) and bee virus Y, are known to add to the pathogenic effect of *N. apis* (Bailey and Ball, 1991). The suggested replacement process of one parasite by the other, at least in some parts of the world (Paxton et al., 2007), indicates differences in virulence and/or in transmission rates. Differences in parasite virulence is not supported by studies of individual bees, which makes the observed change in parasite prevalence over time (Paxton et al., 2007) enigmatic. It should be pointed out that the data presented are based on single isolates of both parasites on honey bees from a single colony. Genotype x genotype interactions between hosts and parasites are known to occur, including microsporidian infections (e.g. Schmid-Hempel et al., 1999). Thus, it remains an open question if the results of this case study have more general implications. Obviously more research is needed to elucidate the full effect of *N. ceranae* infection in *A. mellifera* colonies in different geographical areas and to understand if individual level virulence and colony level virulence differ between the two parasites.

5. Conclusions

In this study, the differences in infectious dose and multiplication rate between *N. ceranae* and *N. apis* are minor and the individual bee mortality in cage experiments caused by *N. ceranae* infections is not significantly higher than the mortality caused by *N. apis*. Furthermore, *N. ceranae* seems to have no competitive advantage within host in mixed infections when spores are administered at the same time.

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