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Original article

Nosema ceranae has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis**

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Abstract – *Nosema ceranae*, a microsporidian formerly regarded as confined to its Asiatic host *Apis cerana*, has recently been shown to parasitise *Apis mellifera* and to have spread throughout most of the world in the past few years. Using a temporal sequence of N = 28 *Nosema* isolates from Finland from 1986–2006, we now find (i) that *N. ceranae* has been present in Europe since at least 1998 and (ii) that it has increased in frequency across this time period relative to *Nosema apis*, possibly leading to higher mean spore loads per bee. We then present results of a single laboratory infection experiment in which we directly compare the virulence of *N. apis* with *N. ceranae*. Though lacking replication, our results suggest (iii) that both parasites build up to equal numbers per bee by day 14 post infection but that (iv) *N. ceranae* induces significantly higher mortality relative to *N. apis*.

Microsporidia / disease / exotic / emergent / PCR-RFLP

1. INTRODUCTION

The Microsporidia are highly specialized fungi (Keeling and McFadden, 1998; Hirt et al., 1999) that are obligatory intracellular parasites found in all major animal phyla (Larsson, 1986). Only two microsporidian species have been described as parasites of honey bees (*Apis*); the western honey bee, *Apis mellifera* L., harbours *Nosema apis* Zander (Zander, 1909) whilst the eastern honey bee, *Apis cerana* Fabricius, harbours *Nosema ceranae* Fries (Fries et al., 1996). Both species infect the ventriculus of adult honey bees and are transmitted horizontally via spore ingestion (*per os*), for example whilst workers clean combs of infected faeces (Fries, 1988a; Fries et al., 1996).

When first discovered near Beijing, China, in 1994, *N. ceranae* was originally considered to be restricted to *A. cerana* in East Asia (Fries et al., 1996; Fries, 1997). More recently, Huang et al. (2007) demonstrated the species to be present in colonies of *A. mellifera* collected in 2005 from Taiwan and Higes et al. (2006) detected it in Spanish *A. mellifera* also sampled in 2005. Our subsequent analyses (Klee et al., 2007) of historical (pre-2003) and recent (2003 onwards) samples have demonstrated that *N. ceranae* is nowadays (2003 onwards) a parasite of *A. mellifera* across much of the world, most likely because of a host jump from *A. cerana* to *A. mellifera* in the past four or more years. Exactly when *N. ceranae* first arrived in European *A. mellifera* is still a matter of conjecture.

For *N. apis*, the lifespan of infected bees is reduced and infected colonies suffer increased winter mortality or poor spring build-up and reduced honey yield (Fries et al., 1984; Anderson and Giacon, 1992). Though *N. apis*

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Figure 1. The seven sampling localities in Southern Finland.

infected colonies may not exhibit overt infection symptoms, this disease organism is nevertheless considered to be a major scourge of beekeeping in temperate climates (Fries, 1993; Fries et al., 2003). For *N. ceranae*, an emergent pathogen of *A. mellifera*, there is less information available. It has been thought to be a factor in the increased mortality of colonies detected across the year in central and southern Europe (Higes et al., 2005, 2006; Imdorf et al., 2006). In support of this view, a recent infection experiment with *A. mellifera* comparing the effects of *N. ceranae* versus uninfected control bees revealed high mortality of infected bees (Higes et al., 2007). We note, however, the lack of comparison in these experiments of the virulence of *N. ceranae* with *N. apis* under identical laboratory conditions.

Our first objective was to use a temporal sequence of *Nosema* infected honey bees from approximately the same location in Finland to determine more precisely the arrival of *N. ceranae* in Europe. Our second objective was to compare the virulence of *N. ceranae* with that of *N. apis* under identical experimental conditions.

2. MATERIALS AND METHODS

2.1. Sample collection

Naturally *Nosema* infected *A. mellifera* were collected from 28 Finnish colonies between 1986 and 2006 (Fig. 1). From each colony, 25 adult bees were homogenized in a mortar with 25 mL of water. The mean spore load per bee was determined by counting the number of spores in 5 blocks of 16 squares per block (0.00025 μL per square) of an improved Neubauer haemocytometer (Cantwell, 1970) and calculating the number of spores per mL (the equivalent of one bee). Afterwards, the spore suspension was filtered to remove coarse bee parts and 1 mL was stored in 99% ethanol.

2.2. *Nosema* species identification

Visual identification of microsporidia to species is often difficult (Larsson, 1986; Weiss and Vossbrinck, 1999) whereas molecular genetic methods can be more successful (e.g. Klee et al., 2006). We therefore used our recently developed and highly accurate (100% accuracy) genetic technique to differentiate *N. apis* from *N. ceranae* based on 16S (or SSU) rRNA restriction fragment length polymorphisms (RFLP) (Klee et al., 2007). DNA extractions follow the methods given in Klee et al. (2007). Extracts were kept at –20 °C until needed as DNA template in PCRs.

As negative controls, DNA was also extracted from (i) ethanol-washed legs of bees, parts of the anatomy that are considered not to be infected with *Nosema* (Fries, 1997) and (ii) from honey bee abdomens visually showing no sign of *Nosema* infection (10 extracts), with the order of infected *A. mellifera* samples and negative controls randomized to test for potential contamination with *Nosema* DNA across extracts. These negative controls were used as template in >40 PCRs, as described below, but never generated an amplicon, indicating that our methods of DNA extraction did not lead to contamination of extracts with DNA from another sample.

The oligonucleotide primers SSU-res-f1 (5'-GCCTGACGTAGACGCTATTC -3') and SSU-res-r1 (5'- GTATTACCGCGGCTGCTGG -3') were used with DNA spore extracts as template in PCRs to amplify ca. 400 bp of the *Nosema* 16S rRNA gene (see Klee et al., 2007, for details). PCR products were digested with the restriction enzymes *Pac* I, *Nde* I and *Msp* I and fragments were resolved on 3% NuSieve agarose gels (Cambrex Bio Science) that were stained with ethidium bromide to reveal specific restriction patterns for the two *Nosema* species: *N. apis* (fragments of 175 bp, 136 bp and 91 bp) and *N. ceranae* (fragments of 177 bp, 116 bp and 104 bp) (see Klee et al., 2007, for details). Reference DNA extracts of *N. apis* and *N. ceranae* were provided by Dr. N. Pieniazek (CDCP-USA) and were used as positive controls in PCRs. They always generated amplicons of the correct size and RFLPs of the correct pattern. Water as template and DNA extracts of negative controls as template never generated a PCR amplicon.

2.3. Infection experiments

Three cages, each containing 50 *A. mellifera* workers from the brood nest of one hive, were established in 1994 in an incubator at 30 °C. Prior to collection of bees for the cages, three consecutive samples of 100 bees each were confirmed to be negative for microsporidian spores, using light microscopy squash preparations of their ventriculi. All bees in one cage were individually fed with 10 μL of a 50% (w/v) sucrose solution containing 10^5 *N. apis* spores originating from Sweden in 1994. This number of spores normally yields infection in all individual worker bees (Fries, 1988b). Those in a second cage were individually fed with 10 μL of a 50% (w/v) sucrose solution containing 10^5 *N. ceranae* spores originating from one infected *A. cerana* colony near Beijing, China, in 1994. Fresh spore solutions for infection were prepared by mass-feeding of each spore type (*N. ceranae* and *N. apis*) to caged worker bees of their respective original host species. Ten days post feeding, the ventriculi from 10 heavily infected worker bees from each cage were dissected out, macerated, and diluted to 10^7 spores per ml using 50% (w/v) sucrose solution. Control bees in the third cage were individually fed with 10 μL of a 50% (w/v) sucrose solution. After treatment, bees could feed ad libitum from a 50% sucrose solution provided in a 15 mL disposable tube with a small hole punched in the bottom. *Nosema* species identity was confirmed by sequencing of the 16S rRNA gene (Fries et al., 1996).

At days 4, 6, 8, 10 and 14 post infection, five living workers were removed from each cage and the number of spores per bee estimated. To do so, we removed a bee's ventriculus, homogenised it in 1 mL H_2O and counted the number of spores at ×256 magnification in 24 squares (0.004 μL per square) diagonally distributed in a Bürker haemocytometer. Multiplication of the average number of spores per square by the dilution factor gave the number of spores per bee (Cantwell, 1970).

Bee mortality was monitored daily from day 3 post infection to day 15 post infection, whereupon the experiment was terminated. Dead bees were removed immediately from a cage.

2.4. Statistical analyses

Differences in the number of spores per bee for field collected samples were analysed by ANOVA, using *Nosema* as a fixed effect factor with three levels (*N. apis*, *N. ceranae* or both species). The significance of differences between means was evaluated using the Tukey a posteriori test. For the infection experiment, differences between treatments (*N. apis*

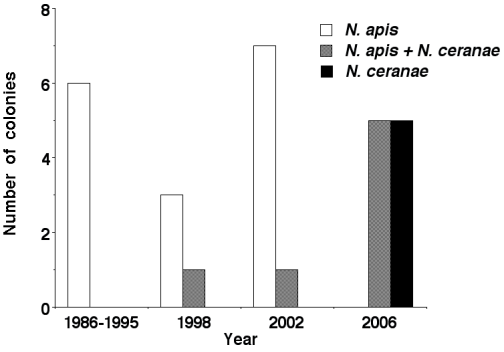

Figure 2. Number of *A. mellifera* colonies (total N = 28) from Southern Finland infected with either *N. apis* (open bars), a mixed infection of *N. apis*/*N. ceranae* (shaded bars) or *N. ceranae* (solid bars) based on RFLP identification. Data for the years 1986 to 1995 were combined.

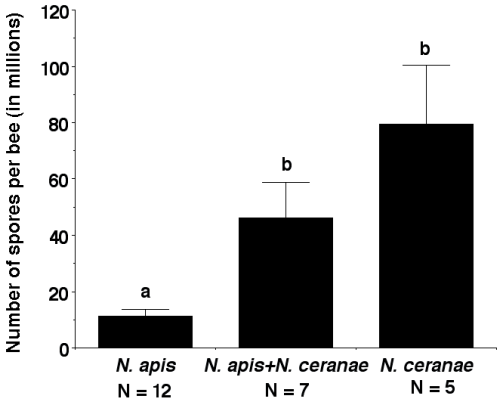

Figure 3. Numbers of spores per bee ($\times 10^6$) ± SE for N = 24 *A. mellifera* colonies from Southern Finland, infected with either *N. apis*, a mixed infection of *N. apis*/*N. ceranae* or *N. ceranae* between 1986 and 2006 and sampled in spring. Spore identification was based on RFLPs. Different lower case letters represent means that differ significantly (ANOVA and Tukey *a posteriori* comparison of means, $P < 0.05$).

versus *N. ceranae*) were also analyzed by ANOVA for each day separately. As data collected on different days post infection from the same cage are not truly independent and as sample sizes are too small to allow us to use a repeated measures ANOVA, we employ the Bonferroni sequential correction to account for multiple tests (days post infection) on data collected from the same cages (Rice, 1989). Differences between experimental infection treatments in total mortality by day 15 post infection were analyzed by Fisher's exact test.

Means are presented throughout ± SE.

3. RESULTS

3.1. *Nosema ceranae* in Finland

Nosema species assignment by RFLPs functioned perfectly for all samples and revealed clear restriction patterns, of either *N. apis* or *N. ceranae* or a mixed infection in which the RFLPs of both species were clearly visible (see Klee et al., 2007, for example gels). There is a clear temporal change in the relative abundance of *N. ceranae* in *Nosema* infected colonies of southern Finland (Fig. 2). Our first recorded case of *N. ceranae* was 1998. In 2006, all *Nosema* infections comprised *N. ceranae*, either with *N. apis* or alone.

Using spore counts per bee for just those 24 colonies sampled in spring (March–May), there was a significant difference among groups infected by *N. apis*, *N. ceranae* or both (ANOVA $F_{2,21} = 11.267$, $P < 0.001$). There were significantly and considerably larger spore loads in bees infected with solely *N. ceranae* (range 1–158 $\times 10^6$ spores per bee) than those infected with solely *N. apis* (range 1–34 $\times 10^6$ spores per bee; see Fig. 3). Bees infected with both microsporidians contained 1–83 $\times 10^6$ spores per bee, not significantly different from those infected solely with *N. ceranae* (Fig. 3).

3.2. Virulence of *N. apis* and *N. ceranae* in a cage experiment

Though identical numbers of spores were fed to every bee in both *N. apis* and *N. ceranae* treatments, the spore load per bee initially built up more rapidly in those bees infected with *N. apis* compared with those infected with *N. ceranae* (Fig. 4; ANOVA results comparing each treatment, with uncorrected *P* values: day 4, $F_{1,8} = 8.491$, $P = 0.020$; day 6, $F_{1,8} = 95.111$, $P < 0.001$; day 8, $F_{1,8} = 83.319$,

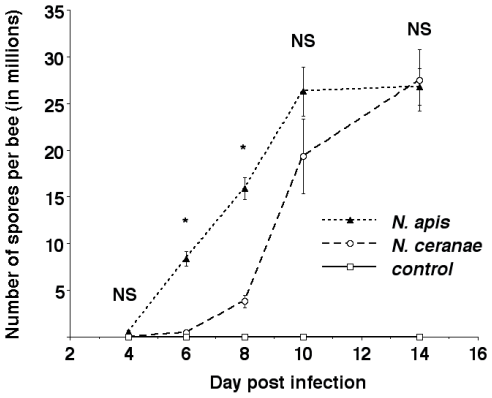

Figure 4. Number of spores per bee ($\times 10^6$) ± SE post infection for *A. mellifera* experimentally infected with either *N. apis* (filled triangles and dotted line), *N. ceranae* (open circles and dashed line) or uninfected (control, open squares and solid line). Each point represents the mean of 5 bees. Results of the ANOVA analyses comparing the two treatments for each day post infection (with Bonferroni correction) are shown above the error bars for that day: *, $P < 0.05$; NS, not significant.

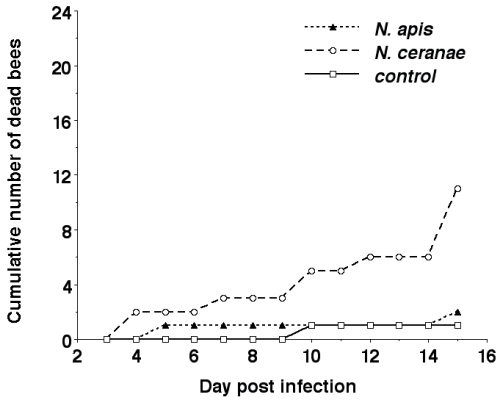

Figure 5. Mortality post infection of 25 *A. mellifera* workers experimentally infected with either *N. apis* (filled triangles and dotted line), *N. ceranae* (open circles and dashed line) or uninfected (control, open squares and solid line).

$P < 0.001$; day 10, $F_{1,8} = 2.113$, NS; day 14, $F_{1,8} = 0.035$, NS). However, at day 14 post infection there were approximately equal numbers of spores ($27 \pm 2 \times 10^6$ spores) per ventriculus for bees treated with both microsporidians. Whereas the number of spores per bee treated with *N. apis* appears to have asymptoted by day 10 post infection. Figure 4 suggests that those with *N. ceranae* would have contained even more spores if the experiment had continued beyond day 14 post infection. Bees in the control cage remained uninfected throughout (Fig. 4).

Cumulative mortality was higher for bees treated with *N. ceranae* versus those treated with *N. apis* or control bees (Fig. 5). By day 15 post infection, only 14 of 25 bees treated with *N. ceranae* were alive compared with 23 of 25 bees treated with *N. apis* and 24 of 25 control (untreated) bees. The difference in mortality between *Nosema* treatments is significant (Fisher exact test, $P = 0.008$), though we urge caution in the interpretation of absolute rates of mortality based, as our results are, on a single cage per treatment.

3.3. DISCUSSION

Our results suggest that *N. ceranae* has been in Finland since at least 1998 and, by implication, elsewhere in Europe for over the past decade. It is also suggested to have been present in the USA since 1995 (Chen et al., 2007). Small sample sizes do not allow a more precise date to be placed on the arrival of *N. ceranae* in Finland or elsewhere in Europe, though it seems clear that *N. ceranae* is an emergent or exotic pathogen of *A. mellifera*, having jumped host from *A. ceranae* in the recent past to have become distributed across much of the world, possibly within the past decade (Klee et al., 2007). If this rate of spread is accurate, it is much faster than the worldwide expansion of the devastating ectoparasitic mite of *A. mellifera*, namely *Varroa destructor* Anderson & Trueman, which is thought to have taken half a century (Breguetova, 1953; Samsinak and Haragsim, 1972; Hicheri, 1978; Sanford, 2001).

How the exotic parasite *N. ceranae* was introduced into Finland is unknown, but it is most likely through the transport of honey bee queens, which Finland imports in large numbers from southern Europe (Korpela, 2002). Similarly, fluvalinate resistant *V. destructor*

mites (Korpela, 1999) and the tracheal mite *Acarapis woodi* (Korpela, 1998) have been imported from southern Europe to Finland and subsequently developed pest status. Understanding the origins and spread of *N. ceranae* through the world populations of *A. mellifera* will now require molecular genetic markers to plot the phylogeography and range expansion of this emergent pathogen. Though some genetic variation exists in the 16S rRNA gene of *N. ceranae* and related microsporidia (Tay et al., 2005; Huang et al., 2007), we urge caution against its use to document *Nosema* phylogeography because rRNA clearly exists in multiple copies per *Nosema* genome and much of the genetic variation detected may represent between-copy, within-spore diversity (O'Mahony et al., 2007).

As worrying as its arrival in Finland is the increase in frequency of *N. ceranae* relative to *N. apis* in the country, which is associated with a higher mean spore load per bee. We found all recent *Nosema* infections in Finland to be caused by *N. ceranae*, either alone or in association with *N. apis*. Recent isolates of *Nosema* from infected Spanish honey bee colonies were likewise primarily *N. ceranae* (Higes et al., 2006). We now require data on the absolute frequency of *N. ceranae* in overtly infected and apparently uninfected colonies to understand its impact on honey bee populations. Bailey (1981) suggested that 30–50×10^6 spores of *N. apis* per ventriculus (i.e. per bee) are typically found in a fully developed *Nosema* infection. That we detected far higher numbers of spores per bee associated with *N. ceranae* infections could be due to our analysis of entire insects including rectal contents, in which spores can accumulate (Lotmar, 1943). On the other hand, our *N. apis* infected colonies contained lower numbers of spores per bee yet were sampled in an identical manner to *N. ceranae* infected colonies. Differences in spore load per bee associated with *Nosema* species certainly demand analysis, in particular controlling carefully for the effect of time of year when sampling.

Although *N. ceranae* built up more slowly in numbers than *N. apis* in an individual bee in our experiment, it may nevertheless go on to reach higher numbers per bee. Infection experiments over a longer time course than 15 days are needed to determine if this is the case. Of great concern is the apparently higher mortality we observed that *N. ceranae* imposes on *A. mellifera* compared to *N. apis*. Unexpectedly high mortality of caged worker bees infected by *N. ceranae* was found by Higes et al. (2006), but no comparison to *N. apis* infected bees was reported. Our results suggest reduced longevity of caged *N. ceranae* infected worker bees compared to bees infected by *N. apis*, although less dramatic than reported by Higes et al. (2006). Further experiments are required to conclusively determine the relative virulence of the two parasites at the individual bee level, both in cage experiments and in the field. There is moreover a need to undertake such experiments with recent infections of *N. ceranae* from Europe or North America, as there may have been a change in the virulence of *N. ceranae* between our cage experiments, undertaken in 1994 in China with spore isolates from *A. cerana*, and *N. ceranae* currently found in *A. mellifera* in Europe or North America. A higher virulence of *N. ceranae*, if conclusively demonstrated to be the case, could account for the unusual reported course of nosema disease in central and southern Europe over the last few years, in which nosema disease is a year-round phenomenon rather than a spring disease, and is associated with higher colony losses (Hatjina and Haristos, 2005; Higes et al., 2005; Imdorf et al., 2006). Colony level infection experiments in the field are now required to demonstrate a causal link between *N. ceranae* infection and colony collapse.

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***Nosema ceranae* infecte *Apis mellifera* en Europe depuis 1998 au moins et peut être plus virulent que *N. apis*.**

Microsporidia / *Nosema ceranae* / parasite émergent / PCR-RFLP

Zusammenfassung – ***Nosema ceranae* kommt bei *Apis mellifera* in Europa bereits seit dem Jahr 1998 vor und ist möglicherweise virulenter als *Nosema apis*.** *Nosema ceranae*, ein Microsporidien-Parasit der früher auf seinen asiatischen Wirt *Apis cerana* beschränkt war, ist kürzlich in Völkern von *Apis mellifera* nachgewiesen worden und hat sich in den vergangenen Jahren nahezu weltweit verbreitet. Wir untersuchten Nosema-Proben aus Finnland (N = 28), die in den Jahren 1986–2006 aus Bienen isoliert wurden (Abb. 1). Wir ermittelten die durchschnittliche Anzahl an Sporen pro Biene und bestimmten über PCR und Restriction Fragment Length Polymorphism (RFLP) des 16S rRNA-Gens auf der Parasiten-DNA die Nosema-Art. Wir konnten nachweisen, dass *N. ceranae* bereits seit mindestens 1998 in Finnland vorkommt, und dass seitdem der Anteil von *N. ceranae* im Vergleich zu *N. apis* zugenommen hat (Abb. 2). Dies hat möglicherweise zu einer insgesamt höheren Sporenbelastung pro Biene geführt (Abb. 3). Neuere Infektionen (2006) bestehen ausschließlich aus *N. ceranae* oder einer Mischinfektion von *N. apis* und *N. ceranae*.
Wir führten daraufhin ein einziges Laborexperiment durch, in dem wir die Virulenz von *N. apis* direkt mit der von *N. ceranae* verglichen. Dazu wurden Käfige mit jeweils 50 Bienen, die mit 10^5 Sporen von *N. apis* bzw. von *N. ceranae* gefüttert wurden, gefüllt. Die negative Kontrolle erhielt Zuckerwasser. Während der nächsten 15 Tage wurden die Anzahl der Sporen im Mitteldarm sowie die Mortalität der Bienen erfasst. Obwohl unsere Ergebnisse wegen der fehlenden Wiederholung nur vorläufigen Charakter haben, scheint bei beiden Parasiten die Anzahl der Sporen pro Biene während der ersten 14 Tage in ähnlichem Umfang zuzunehmen (Durchschnitt $27 \pm 2 \times 10^6$ Sporen, Abb. 4). Allerdings verursachte *N. ceranae* eine signifikant höhere Bienenmortalität im Vergleich zu *N. apis* (Abb. 5). Wir diskutieren unsere Ergebnisse in Zusammenhang mit der beobachteten Zunahme an Völkerzusammenbrüchen in Südeuropa und wir beschreiben Experimente, die notwendig sind um den kausalen Zusammenhang zwischen *N. ceranae*-Infektionen und Völkerzusammenbrüchen nachzuweisen.

Microsporidien / *Nosema ceranae* / Ausbruch / PCR-RFLP

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