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Impact of *Nosema ceranae and Nosema apis* on individual worker bees of the two host species (*Apis cerana* and *Apis mellifera*) and regulation of host immune response

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ABSTRACT

Nosema apis and *Nosema ceranae* are obligate intracellular microsporidian parasites infecting midgut epithelial cells of host adult honey bees, originally *Apis mellifera* and *Apis cerana* respectively. Each microsporidia cross-infects the other host and both microsporidia nowadays have a worldwide distribution. In this study, cross-infection experiments using both *N. apis* and *N. ceranae* in both *A. mellifera* and *A. cerana* were carried out to compare pathogen proliferation and impact on hosts, including host immune response. Infection by *N. ceranae* led to higher spore loads than by *N. apis* in both host species, and there was greater proliferation of microsporidia in *A. mellifera* compared to *A. cerana*. Both *N. apis* and *N. ceranae* were pathogenic in both host *Apis* species. *N. ceranae* induced subtly, though not significantly, higher mortality than *N. apis* in both host species, yet survival of *A. cerana* was no different to that of *A. mellifera* in response to *N. apis* or *N. ceranae*. Infections of both host species with *N. apis* and *N. ceranae* caused significant up-regulation of AMP genes and cellular mediated immune genes but did not greatly alter apoptosis-related gene expression. In this study, *A. cerana* enlisted a higher immune response and displayed lower loads of *N. apis* and *N. ceranae* spores than *A. mellifera*, suggesting it may be better able to defend itself against microsporidia infection. We caution against over-interpretation of our results, though, because differences between host and parasite species in survival were insignificant and because size differences between microsporidia species and between host *Apis* species may alternatively explain the differential proliferation of *N. ceranae* in *A. mellifera*.

1. Introduction

Microsporidia are obligatory intercellular single-cell spore-forming fungal parasites with a wide array of hosts, ranging from invertebrates to vertebrates (Larsson, 1986; Tsai et al., 2003). *Nosema* is a widespread genus of parasitic microsporidia commonly infecting invertebrates (Larsson, 1986; Tsai et al., 2003) such as Amphipoda (Terry et al., 1999), Orthoptera (Henry, 1971), Lepidoptera (Tsai et al., 2003; Higes et al., 2007) and Hymenoptera (Fries et al., 1996; Higes et al., 2006, 2007). In Asia, two cavity-nesting honey bees, the native *Apis cerana* and the introduced *Apis mellifera*, have been promoted as commercial insects for beekeeping (Akratanakul, 1986). However, Asian beekeepers are confronted with several problems, in particular infections of various microorganisms such as bacteria, viruses and fungi as well as microsporidian parasites (Martín-Hernández et al., 2007; Paxton et al., 2007; Higes et al., 2009).

One of the most widespread honey bee diseases around the world is nosematosis, caused by two described species of microsporidia, *Nosema apis* and *Nosema ceranae. N. apis* and *N. ceranae* were originally described respectively in *A. mellifera* and *A. cerana*. In the last two decades, *N. ceranae* has infected *A. mellifera* and spread worldwide, possibly leading to the decline of *N. apis* (Klee et al., 2007; Paxton et al., 2007; Fries, 2010; Martín-Hernández et al., 2012), at least in warmer climates (Natsopoulou et al., 2015). No published study to date has shown unambiguously that *N. apis* can infect *A. cerana*, though Ingemar Fries (pers. comm.) successfully cross-infected *N. apis* in *A. cerana* when

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first describing *N. ceranae* (Fries et al., 1996).

The honey bee host species possess various mechanisms to combat pathogens, including physiological, behavioral and immune defense responses (Evans and Spivak, 2010). In insects, several immune pathways and defense mechanisms have been identified which can be represented in two broad categories: cellular and humoral immune responses (Gillespie et al., 1997; Lavine and Strand, 2002; Boman, 2003).

When *Nosema* spores invade the bee body cavity, cell-mediated defense reactions play a crucial role (Gliński and Jarosz, 2001). Phagocytosis, encapsulation and melanization mechanisms relate to cellular immunity (Osta et al., 2004). Both nodulation and encapsulation are frequently accompanied with melanization, which are catalysed by (pro-phenoloxidase (PO) (Ashida, 1997; Decker and Jaenicke, 2004). The cellular response also requires the participation of glucose dehydrogenase (GLD) (Cox-Foster et al., 1990), both during the encapsulation reaction and also as a host response in killing a fungal invader. GLD may be also used as a marker of the initial activation of a cellular immune response (Lovallo and Cox-Foster, 1999). Eater (AmEater) is a major receptor which plays an important role in the recognition and phagocytosis of bacteria in *Drosophila* (Ertürk-Hasdemir and Silverman, 2005; Kocks et al., 2005). In addition, lysozyme (LYS) plays an important role in insect immune response in the killing of Gram-positive and Gram-negative bacterial invaders (Daffre et al., 1994; Lavine and Strand, 2001), and again may be involved in defense against microsporidia.

Insect humoral immunity, in contrast, involves the synthesis of a set of antimicrobial peptides (AMPs) in response to infection by bacteria, fungi or parasites (Hetru et al., 1998; Lamberty et al., 1999; Yamauchi, 2001; Klaudiny et al., 2005). In the honey bee (*A. mellifera*), the AMP immune repertoire consists of at least four peptides, including apidaecin (Casteels et al., 1989), abaecin (Casteels et al., 1990), hymenoptaecin (Casteels et al., 1993) and defensin (Casteels-Josson et al., 1994). These antimicrobial peptides show a broad spectrum of activity against microorganisms (Chaimanee et al., 2012) and may also be employed by honey bees in the control of *Nosema* invasion.

Apoptosis, or programmed cell-death (PCD), is an additional intracellular defense mechanism used by a variety of invertebrates against viruses (Galluzzi et al., 2008) and other intracellular pathogens (Knodler and Finlay, 2001; Higes et al., 2013). It plays an important role in normal cell proliferation and development and is therefore critical to the functioning of multicellular organisms (Heussler et al., 2001; Knodler and Finlay, 2001). Apoptosis generally occurs during morphological and molecular changes, and in the presence of abnormal cells, such as damaged or infected cells, may cause cell death. Death of an individual cell is an additional and essential form of defense, as it sometimes represents the only way for the immune system to eliminate pathogens by sacrificing the infected cell (Narayanan, 1998). Apoptosis has also been proposed to be involved in host honey bee response to microsporidia infection (Kurze et al., 2015).

Vitellogenin (Vg) in the honey bee is a 180 kDa female-specific protein (Wheeler and Kawooya, 1990) that is synthesized by the fat body of the abdomen and released into the haemolymph and then transported to the ovaries and other tissues. Vg helps to integrate social organization through its pleiotropic effects on the division of labour and foraging specialization (Amdam and Omholt, 2003; Nelson et al., 2007). Vg also functions in reproduction and is thought to regulate honey bee immune function and lifespan (Amdam et al., 2004, 2005). It again may function in defense against *Nosema* infection (Antúnez et al., 2009).

The first objective of this study was to experimentally infect both *N. ceranae* and *N. apis* in *A. mellifera* and *A. cerana* hosts to test for host mortality and specificity of the microsporidia and differential proliferation in hosts. The second objective was to compare the responses of each host to infection. To this end we measured the expression of gene transcripts encoding commonly known antimicrobial peptides associated with humoral immunity (defensin, abaecin, apidaecin, and hymenoptaecin) and cellular immunity (phenol oxidase (PO), glucose dehydrogenase (GLD) and eater gene (AmEater)) in addition to the expression of genes encoding a female-specific protein (vitellogenin (Vg)), lysozyme and apoptosis genes, which might help in explaining variation in host species to microsporidian proliferation.

2. Materials and methods

2.1. Honey bee species

Two honey bee species, *A. mellifera* (three colonies) and *A. cerana* (three colonies), were used. Experiments were conducted in February 2015 at the Bee Protection Laboratory (BEEP), Department of Biology, Faculty of Science, Chiang Mai University, Thailand. The colonies showed no visible clinical symptoms of disease. Before the experiments, fifty bees were randomly collected from each colony, checked for *Nosema* spores under light microscopy (400x) and further confirmed to be free from *Nosema* by PCR, as described by (Chen et al., 2008). Frames of sealed brood from each colony were incubated at 34 ± 1 °C in the laboratory. New emerging worker bees were carefully captured, confined to cages in groups of 30 bees (total 18 cages) and kept in an incubator at controlled temperature (34 ± 1 °C) and humidity (60–70% humidity) (Fries et al., 2013). Bees were fed with a solution of sucrose (50% w/w in water) *ad libitum*.

2.2. Inoculum preparation

N. ceranae spores were isolated from *A. cerana* at the BEEP apiary; *N. apis* spores were obtained from *A. mellifera* at the University of Halle, Germany (original source: Uppsala, Sweden). The midguts of infected bees were removed and crushed in distilled water, filtered through cotton and centrifuged at 5000*g* for ten minutes. The pellet was redissolved and purified via triangulation (Fries et al., 2013). We confirmed their purity by PCR analysis (Table 1). And the number of *Nosema* spores were checked and estimated by counting by light microscopy (Olympus CX31) at a magnification level of 400× following the method of Cantwell (1970). Each inoculum was freshly prepared on the day of inoculation and mixed with 50% sucrose solution to obtain a final concentration of 10^7 spores/ml.

2.3. N. ceranae and N. apis infection experiments

Five days after emerging, groups of bees were assigned to cages, *N. ceranae* (3 cages per host species), *N. apis* (3 cages per host species) or control (sucrose only, 3 cages per host species). To administer an inoculum, bees were starved for two hours before being anesthetized with CO_2 and, upon arousal, fed individually with 10 µL sucrose containing *N. apis* or *N. ceranae* (infective dose 10^5 spores) using a micropipette (Malone et al., 1995), sufficient spores to ensure 100% infection of treated bees (Fries et al., 2013). Control bees were fed likewise using a pathogen-free extract from healthy bees, obtained by triangulation as described above for microsporidia purification, and mixed with sucrose, as for the inoculation. Bees were held individually in 1.5 mL vials after inoculation for 30 min to ensure the inoculum was ingested. Thereafter bees were returned to their cage in the incubator and given *ad libitum* access to 50% sucrose solution as described by Antúnez et al. (2009). Dead bees were counted daily during experiments and removed from cages.

For gene expression study, three worker bees were collected from each cage at 4, 7 and 14 days post inoculations and then stored at −80 °C for further analysis.

2.4. Determining spore loads

The three worker bees per cage were collected at 14 days post inoculation and the *Nosema* spore loads per bee were determined by

Table 1
Primers used for *Nosema* confirmed and qPCR primers used for the amplification of genes related to innate immunity and potentially playing a role in defense in *Apis cerana* and *Apis mellifera* in response to *Nosema* spp. infection.

Primer	Sequence	Target gene amplified	Code	Reference
Napis-SSU-Jf1	5′-CCATGCATGTCTTTGACGTACTATG-3′	*N.apis* (Microsporidium)	–	Klee et al. (2007)
Napis-SSU-Jr1	5′-GCTCACATACGTTTAAAATG-3′			
N. apis F	5′-CCATTGCCGGATAAGAGAGT-3′	*N.apis* (Microsporidium)	–	Chen et al. (2008)
N. apis R	5′-CACGCATTGCTGCATCATTGAC-3′			
NOS-FOR	5′-TGCCGACGATGTGATATGAG-3′	*N.ceranae* (Microsporidium)	–	Higes et al. (2006)
NOS-REV	5′-CACAGCATCCATTGAAAACG-3′			
N. ceranae F	5′-CGGATAAAAGAGTCCGTTACC-3′	*N.ceranae* (Microsporidium	–	Chen et al. (2008)
N. ceranae R	5′-TGAGCAGGGTTCTAGGGAT-3′			
RPS5-F	5′-AATTATTTGGTCGCTGGAATTG-3′	Ribosomal protein S5 (reference housekeeping gene)	Housekeeping gene	Evans (2006)
RPS5-R	5′-TAACGTCCAGCAGAATGTGGTA-3′			
β-actin-F	5′-TTGTATGCCAACACTGTCCTTT-3′	β-actin (reference housekeeping gene)	Housekeeping gene	Simone et al. (2009)
β-actin-R	5′-TGGCGCGATGATCTTAATTT-3′			
AmEater-F	5′-CATTTGCCAACCTGTTTGT-3′	NimC1, Eater-like	AmEater	Simone et al. (2009)
AmEater-R	5′-ATCCATTGGTGCAATTTGG-3′			
ApidNT-F	5′-TTTTGCCTTAGCAATTCTTGTTG-3′	Antibacterial peptide apidectin	Apidaecin	Simone et al. (2009)
ApidNT-R	5′-GTAGGTCGAGTAGGCGGATCT-3′			
Abaecin-F	5′-CAGCATTCGCATACGTACCA-3′	Antibacterial peptide abaecin	Abaecin	Evans (2006)
Abaecin-R	5′-GACCAGGAAACGTTGGAAAC-3′			
Defensin-F	5′-TGCGCTGCTAACTGTCTCAG-3′	Antibacterial peptide defensin	Defensin	Evans (2006)
Defensin-R	5′-AATGGCACTTAACCGAAACG-3′			
Hymenopt-F	5′-CTCTTCTGTGCCGTTGCATA-3′	Antibacterial peptide hymenoptaecin	Hymenoptecin	Evans (2006)
Hymenopt-R	5′-GCGTCTCCTGTCATTCCATT-3′			
VgMC-F	5′-AGTTCCGACCGACGACGA-3′	Vitellogenin	VgMc	Simone et al. (2009)
VgMC-R	5′-TTCCCTCCCACGGAGTCC- 3′			
GLD-F	5′-CTGCACAACCACGTCTCGTT-3′	Glucose dehydrogenase	GLD	Yang and Cox-Foster (2005)
GLD-R	5′-ACCGCCGAAGAAGATTTGG-3′			
PO-F	5′-AATCCATTACCTGAAATTGATGCTTAT-3′	Phenol oxidase	PO	Yang and Cox-Foster (2005)
PO-R	5′-TAATCTTCCAACTAATTCATACGCTCTT-3′			
LYS-F	5′-ACACGGTTGGTCACTGGTCC-3′	Lysozyme	LSY	Yang and Cox-Foster (2005)
LYS-R	5′-GTCCCACGCTTTGAATCCCT-3′			
Rel_F	5′-ATAACACCGCCTCTGTCCAC-3′	NF-kB transcription factor Relish	REL	This study (Unpublished)
Rel_R	5′-TTGCGGTGGTATAGTCGTCA-3′			
NFAT_F	5′-ATAGTATGCACCCAACCGCC-3′	Rel NFAT transcription factor	NFAT	This study (Unpublished)
NFAT_R	5′-GCATTCCCAGTGAGGTTCCA-3′			
R1_F	5′-TCGGGATAGACGAATGCACG-3′	Apoptosis regulator R1-like	R1	This study (Unpublished)
R1_R	5′-ACACAGTTGCTCCCATCCTC-3′			

counting. The whole abdomen of the individual worker was crushed in 1 mL of distilled water and the number of *Nosema* spores was estimated using a haemocytometer (Cantwell, 1970), as for the determination of spore concentration in the inoculums.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from the entire abdomen of individual worker bees using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. DNA was removed using DNAse I incubation at 37 °C for 1 h followed by 10 min at 75 °C. RNA concentration and quality (as absorption ratio at 260 nm/280 nm) were spectrophotometrically measured (Nanodrop, Biodrop DUO). First-strand cDNA synthesis was performed with approximately 2 μg total RNA using a master mix containing 50 U Superscript II (Invitrogen, Carlsbad, CA), 2 nmol dNTP mix, 2 nmol poly(dT)$_{18}$, and 0.1 nmol poly(dT)$_{(12–18)}$ in a final volume of 10 μL. Synthesis was carried out at 42 °C for 50 min followed by 15 min at 70 °C, as described in Evans (2006).

2.6. Real-time quantitative PCR

Real-time quantitative PCR amplification was performed in a 20 μL reaction mixture using 1 μL cDNA, 5 μL SensiMix of the SYBR Fluorescein kit (SYBR-Green, Bioline, Luckenwalde, Germany), DEPC water for qPCR assays and 0.2 μM of each specific primer (Yang and Cox-Foster, 2005; Evans, 2006; Simone et al., 2009) (Table 1). PCR reactions were carried out in 96-well microtiter plates using a Bio-Rad Icycler (Bio-Rad Corp., Hercules, CA). The amplification was programmed as follows: 95 °C for 2 min followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s and 72 °C for 60 s. Fluorescence measurements were taken repeatedly during the 78 °C step. This procedure was followed by a melt-curve dissociation analysis to confirm product size.

The amplification results were expressed as the threshold cycle (Ct) value, which represented the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. Relative quantification was calculated by using threshold cycle numbers for the target gene subtracted from the mean of the two reference genes (β-actin (Simone et al., 2009) and RPS5 (Evans, 2006)) for each sample. To compare expression levels across treatments using the qPCR results, data were transformed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Chen et al., 2005; Chaimanee et al., 2012). The treatment group with the lowest expression level was used as the calibrator and the levels of gene transcripts in all other groups were expressed as n-fold differences relative to the calibrator (Chen et al., 2005; Khongphinitbunjong et al., 2016).

2.7. Statistical analysis

Spore loads per bee at 14 days post infection (p.i.) were $\log_{10}$ transformed to restore normality and homoscedasticity. We then tested for differences in ($\log_{10}$) spore load between host species and *Nosema* species by 2-way ANOVA with 'cage' as a random factor using the function lme in the package nlme (Pinheiro and Bates, 2016) in R v. 3.1.0. (R, Core Team, 2014).

Differences in survival across host and *Nosema* spp. was tested using a Cox proportional hazard model using the R packages 'survival' v. 2.41-2 (Therneau and Lumley, 2016) and 'coxme' v. 2.2-5 (Therneau et al., 2003) in which '*Nosema* species' and '*Apis* host species' were fixed

factors whilst 'Cage' was again used as a random factor. Its inclusion did not improve model fit, as tested by ANOVA and AIC of models with versus without 'cage', though we retained it in the final model because it was an inherent feature of our experimental paradigm. Differences between treatment means were examined *a posteriori* using a Tukey test with Bonferroni correction for multiple testing using the R package 'multcomp' v. 1.4-6 (Hothorn et al., 2008).

For data related to the expression levels of genes, statistical analyses were performed with SPSS version 17.0 for Window (SPSS, Inc.). Normality and homogeneity of variances of the data were checked and, for genes that conformed to the assumptions of parametric tests, statistical significance was analyzed using one-way ANOVAs. Where differences were found, means were compared by a Tukey-HSD test with Bonferroni correction. For genes that did not conform to the assumptions of parametric tests, statistical significance was analyzed using Kruskal–Wallis tests with Bonferroni correction. Data are presented for overall mean transcript levels across all treatments.

3. Results

3.1. Cross infection of N. apis and N. ceranae in A. cerana and A. mellifera

Across the experiment, *N. ceranae* and *N. apis* spores were never detected in bees from the control cages. All 14-day-old bees treated with a *Nosema* inoculum were infected, demonstrating that our experimental protocol had functioned well. Both *Apis* species can therefore act as host to *N. apis* and *N. ceranae*.

At 14 days p.i. with *N. apis*, *A. cerana* contained an average of 14 million spores and, when infected with *N. ceranae*, an average of 23 million spores (Fig. 1). For *A. mellifera* 14 days p.i., *N. apis* proliferated to 17 million spores and *N. ceranae* to 45 million spores (Fig. 1). *N. ceranae* proliferated to a significantly higher spore load than *N. apis* (ANOVA $F_{8,24} = 204.9$, $P < .001$) and both microsporidia proliferated to higher spore loads in *A. mellifera* than *A. cerana* (ANOVA $F_{8,24} = 85.8$, $P < .001$). The interaction between microsporidian species and host *Apis* species was also significant (ANOVA $F_{8,24} = 21.0$, $P < .01$); *N. ceranae* reached a far higher spore load in *A. mellifera* in comparison to its congener *N. apis* than it did in *A. cerana* (Fig. 1).

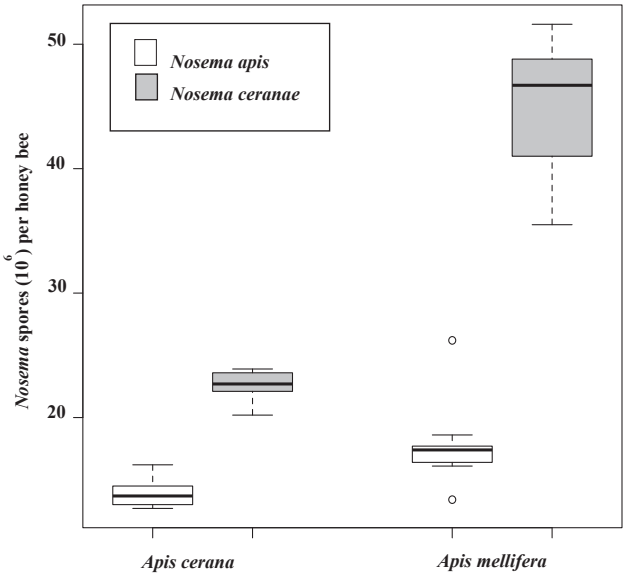

Fig. 1. Boxplot of the number of *Nosema* spores per honey bee, showing the median (central line), interquartile range (box), 95% confidence intervals (whiskers) and outliers (circles). ANOVA revealed a significant effect of both host species and parasite species as well as a significant interaction effect on (log_{10}) spore load ($P < .01$ for all tests, see text for details).

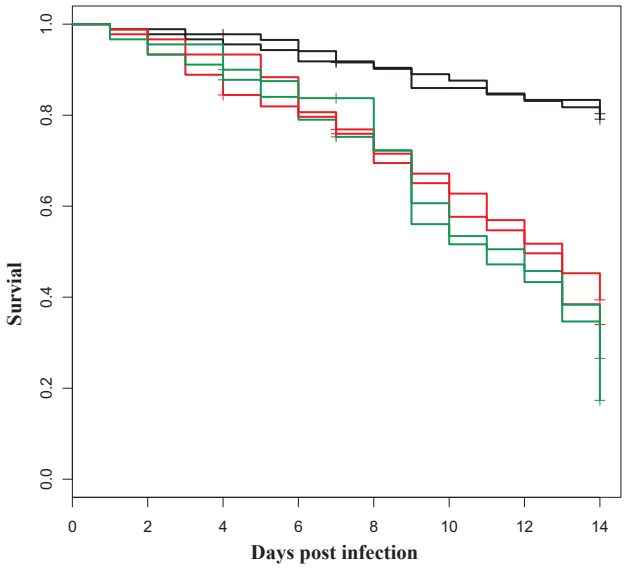

Fig. 2. Survival of *Apis cerana* and *Apis mellifera* after treatment with *Nosema* spp.; red, *Nosema apis;* green, *Nosema ceranae*; black (control); lines for *A. mellifera* and *A. cerana* overlap (see Supplementary Figures S2 and S3 for separate plots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Survival of hosts

Both *N. apis* and *N. ceranae* were pathogenic for hosts *A. cerana* and *A. mellifera* (ANOVA factor '*Nosema* species' chi^2, 102.53, 5 d.f., $P < .001$; Fig. 2; Supplementary Table S1). Though *N. ceranae* induced slightly greater mortality than *N. apis* by the end of the experiment (day 14 p.i., see β coefficients in Supplementary Table S1), differences between *Nosema* spp. were not significant. In addition, *A. cerana* and *A. mellifera* survived similarly in response to infection by either *Nosema* spp. (Supplementary Table S1, Supplementary Figs. S1 and S2).

3.3. Quantitative PCR of host genes

The mRNA expression levels of the 12 host genes were determined at 4, 7 and 14 days (p.i.) following *N. ceranae* or *N. apis* infection of both host species (Supplementary Fig. S3). Gene-specific amplification was confirmed for the 12 primer pairs as a single peak in the melting curve analysis and through correct Tm values.

3.4. Effect of N. apis and N. ceranae infection on the expression of host antimicrobial peptides

Infection of either honey bee species with either *N. apis* and *N. ceranae* caused significant overexpression in four antimicrobial peptide genes, with similar patterns across host species (Table 2). The mRNA transcripts were particularly overexpressed for apidaecin, abaecin and defensin in both host species (Supplementary Fig. S3), though subtle differences between treatments were also apparent (Table 2). For example, expression of apidaecin after infection by *N. apis* and *N. ceranae* was significantly elevated at 4 days post inoculation in both host species ($P < .001$); however, only *A. cerana* exhibited a significant up-regulation at 7 days post inoculation ($P < .001$). Further, only *N. ceranae* infection caused a significant increase in mRNA levels of apidaecin at 14 days p.i. ($P < .001$). Similarly, abaecin mRNA expression increased with time p.i. (4, 7 and 14 days) following *N. apis* and *N. ceranae* inoculation in *A. cerana* compared to controls ($P < .002$ for all treatments; Table 2). However, apidaecin was only significantly up-regulated in *A. mellifera* after infection with *N. apis* at 4 days p.i. and *N. ceranae* at 14 days p.i. ($P < .01$; Table 2). Defensin expression also

Table 2
Heat map shows overexpression (black cells) and underexpression (gray cells) of 12 selected gene transcripts at three time points post infection with either *N. ceranae* (NC) or *N. apis* (NA) compared with controls in two host honey bee species, *Apis cerana* and *Apis mellifera* (see Supplementary Fig. S1 for statistical tests).

Bee species		*A. cerana*						*A. mellifera*					
Days post infection		4		7		14		4		7		14	
Target genes		NA	NC	NA	NC	NA	NC	NA	NC	NA	NC	NA	NC
Humoral response (antimicrobial peptides)	Apidaecin												
	Abaecin												
	Defensin												
	Hymenoptecin												
Cellular response	AmEater												
	PO												
	GLD												
	LSY												
Apoptosis related	NFAT												
	R1												
	REL												
Immunity regulator	VgMc												
Host housekeeping genes	RPS5, β-actin												

= overexpression = significant overexpression (P<0.05 after Bonferroni correction)

= underexpression = significant underexpression (P<0.05 after Bonferroni correction)

significantly increased following *N. apis* and *N. ceranae* inoculation, when compared with controls at 4 and 7 days p.i. in both host species ($P < .001$ and $P < .000$ respectively; Supplementary Fig. S3). At day 14 p.i., this gene showed significantly higher expression only in *A. mellifera* but not in *A. cerana* towards both *Nosema* species. The expression of hymenoptaecin increased in *A. mellifera* across all sampling days ($P < .001$). However, only at day 7 p.i. did *A. cerana* show an increase in hymenoptaecin expression (Table 2).

3.5. Effect of infection on the expression of host genes encoding humoral immunity-related enzymes and Vg

The expression of genes encoding humoral immunity-related enzymes and vitellogenin was mostly affected by *N. ceranae*. Four days after both microsporidia inoculations in *A. cerana*, there was a significant increase in Vg gene expression (ANOVA, $P < .01$; Table 2). At 7 and 14 days p.i. with *N. ceranae*, Vg gene expression of *A. cerana* increased significantly ($P < .041$ and $P < .001$ respectively; Table 2). PO and GLD also showed significant up-regulation in this treatment. In *A. mellifera* 4 days after *N. ceranae* inoculation, there was a significant increase in Vg, PO, GLD and lysozyme gene expression (ANOVA, $P = .01$, $P = .036$, $P = .05$ and $P < .01$ respectively, Table 2). Interestingly, expression of this group of genes was significantly suppressed by *N. apis* in *A. cerana*. In contrast, PO expression in *A. mellifera* was significantly suppressed by both parasites only at 7 days p.i. ($P < .001$; Table 2).

3.6. Effect of Nosema infection on the expression of genes encoding apoptosis-related genes

The three apoptosis-related genes we examined (NFAT, R1 and REL) showed significant down-regulation in response to *Nosema* infection, though down-regulation was inconsistent across treatments (Table 2). Significant down-regulation of R1 mRNA was only seen following *N. apis* infection of *A. mellifera* 14 days p.i. (ANOVA, $P < .05$, Table 2). Interestingly, both parasites were associated with significantly suppression of the REL gene at 4 days p.i. in *A. cerana* (ANOVA, $P < .009$, Table 2). In *A. mellifera* 4 days after *N. apis* inoculation, there was a significant decrease in REL gene expression (Kruskul-Wallis, $P = .030$, Table 2).

4. Discussion

Cross-infection experiments using *N. ceranae* and *N. apis* in *A. mellifera* and *A. cerana* demonstrated that both parasites could successfully infect the two honey bee species, cause mortality, and induce a response in terms of up or down-regulation of host genes associated with innate immune pathways.

N. ceranae infection led to significantly higher spore loads and slightly higher mortality in both *A. mellifera* and *A. cerana* than did *N. apis*. Faster growth by *N. ceranae* has been witnessed in previous studies (Fries and Feng, 1995; Fries, 1997). It may lead to a higher prevalence of *N. ceranae* compared to *N. apis* (Klee et al., 2007; Martín-Hernández et al., 2007; Fries, 2010), particularly in warmer climates (Natsopoulou et al., 2015). Why it multiplies faster than *N. apis* is unclear. Its slightly smaller size compared to *N. apis* (Fries and Feng, 1995) may allow for a more rapid completion of its lifecycle and for more spores to fit into an infected cell compared to *N. apis*.

The more rapid proliferation of *N. ceranae* over *N. apis* has been associated with high colony mortality caused by the former in Spain (Higes et al., 2008). Recent laboratory-based studies, however, have found that *N. ceranae* induces only slightly higher mortality than *N. apis* in *A. mellifera* (Huang et al., 2015; Natsopoulou et al., 2016a), as we also found in both *A. cerana* and *A. mellifera*. Somewhat surprisingly, we found that host species also did not differ in mortality induced by either *N. apis* or by *N. ceranae*.

We found *A. mellifera* to contain more *Nosema* spores of either *Nosema* species for a given time p.i. compared to *A. cerana*. Whether *A. mellifera* is more permissive to *Nosema* spp. proliferation than *A. cerana* is not known. Differences in spore load per bee species may alternatively, for example, reflect the larger size of *A. mellifera* versus *A. cerana*. Nevertheless, our results support the field data of Chaimanee et al. (2013), who compared *N. ceranae* spore loads of four different honey bee species (*A. mellifera, A. cerana, A. dorsata* and *A. florea*) from different geographical origins across Thailand; they (Chaimanee et al., 2013) also found that *N. ceranae* proliferated to a higher spore load in *A. mellifera* populations when compared to the other three native honey bee species (Chaimanee et al., 2010, 2013).

Transcriptome studies have demonstrated that *Nosema* infections in *A. mellifera* lead to significant over-expression in several immune related genes, include genes encoding all the canonical AMPs: abaecin,

apidaecin (*Apid1*), defensins (*Def1* and *Def2*) and hymenoptaecin, all known for their antimicrobial activities (Doublet et al., 2017). These peptides are closely associated with the bee humoral immune response and exhibit broad antibacterial activity against Gram-positive and Gram-negative bacteria (Jarosz, 1979; Boman and Hultmark, 1987). Our data for *A. mellifera* support the results of Doublet et al. (2017) and furthermore show that the patterns of expression of gene transcripts encoding AMPs showed a similar trend in *A. cerana*, and to both microporidia species. Specifically, we found that the mRNA levels of four AMPs (defensin, abaecin, apidaecin and hymenoptaecin) were up-regulated within *N. apis* and *N. ceranae*-infected workers of both *A. mellifera* and *A. cerana*, suggesting that both species use common defense mechanisms against microsporidian infection.

We found that, four days after *Nosema* infection in *A. mellifera* and *A. cerana*, the expression of the AMPs abaecin, defensin and hymenoptaecin markedly increased; however, 14 days p.i. the effect was no longer clearly pronounced. Earlier studies have also reported that the expression of two AMPs (apidaecin and defensin) are up-regulated 4 days and 7 days after infection, though they are less affected at 14 days after infection (Higes et al., 2007). In contrast, Antúnez et al. (2009) demonstrated that transcripts of antimicrobial peptide genes were suppressed in workers when infected by *N. ceranae*. Further research is clearly needed to determine the extent to which AMP transcript expression responds to *Nosema* infection, and how AMPs may help defend the host against *Nosema* spp. infection.

We found that *N. apis* and *N. ceranae* multiplied to lower numbers per bee in *A. cerana* compared to *A. mellifera*. In addition, both we and Xu et al. (2009) found that *A. cerana* displayed high expression of antimicrobial peptides compared to *A. mellifera*. Xu et al. (2009) proposed that domestication of *A. mellifera* may have led to a lower expression of antimicrobial peptides in comparison to the Asian honey bee *A. cerana*. Yet *A. cerana* is also partly domesticated. Domestication *per se* cannot therefore explain the low spores loads in *A. cerana* versus *A. mellifera,* though common beekeeping practice do vary between the two host species (Chantawannakul et al., 2016). Chaimanee et al. (2012) also showed that transcript levels of AMPs in Asian honey bees were significantly higher than those of *A. mellifera* in both control and *N. ceranae* inoculation treatments, suggesting the differences we report here between host species in response to microsporidian infection are robust and consistent. Greater immune response might be one of the factors contributing to the low prevalence of pathogens in *A. cerana* (Chaimanee et al., 2012).

Four days after *N. ceranae* inoculation in *A. mellifera*, there was a significant increase in Vg, PO, GLD and lysozyme expression, suggesting that *N. ceranae* does not suppress humoral and cellular defense mechanisms. A previous study has suggested that decreased lysozyme expression and chronic exposure to *Nosema* might reduce lysozyme transcripts (Garrido et al., 2016). However, *Nosema* infections have more recently been found to induce the expression of genes coding for lysozyme-associated proteins (Doublet et al., 2017), which is consistent with our results.

The expression of Vg, PO and GLD increased at 7 days and 14 days after *N. ceranae* inoculation in *A. cerana*, but Vg expression decreased 14 days after *N. ceranae* inoculation *in A. mellifera*. Vitellogenin is known to be related to the transition from in-hive to out-hive behavior and is therefore a central age pacemaker, reflecting the onset of aging in bees (Page et al., 2012). A decrease in Vg expression after *N. ceranae* infection in *A. mellifera*, as we found and as reported by others (Nelson et al., 2007; Remolina et al., 2007; Antúnez et al., 2009; Chaimanee et al., 2012) is consistent with the reduced lifespan reported for worker bees infected by *N. ceranae* (Natsopoulou et al., 2016b).

Effects of *Nosema* infection on the expression of genes encoding apoptosis-related genes were slight, particularly in comparison to immune related response genes. For example, *REL* gene expression was significantly down-regulated only at 4 days after inoculation with either *N. apis* or *N. ceranae* in *A. cerana*. Also we found a significant decrease in the *R1* mRNA level only at 14 days post inoculation with *N. ceranae* in *A. mellifera*. Previous studies have suggested that *N. ceranae* may suppress host epithelial cell death so as to allow microsporidia proliferation in the gut epithelium (Higes et al., 2013; Kurze et al., 2015). Indeed, a recent report of the expression of genes related to apoptosis following microsporidia infection suggested that *buffy* and *BIRC5* are both up-regulated, supporting the idea that the inhibition of apoptosis is a common host response which benefits this group of intracellular parasitic fungi (Martín-Hernández et al., 2017). These reports are in contrast to our results, which suggest that honey bees may not employ apoptosis as a defense mechanism to combat *Nosema* infection. Further studies are required to understand how altered gene expression translates into a functional response by the host to enhance its defense against microsporidia, or into a functional manipulation by the pathogen to enhance its proliferation in host tissue.

5. Conclusions

N. ceranae proliferated to a higher spore load than *N. apis* in two host *Apis* species following experimental infection. Additionally, spore loads were higher in *A. mellifera* than *A. cerana*. Yet mortality induced by the two microsporidia was similar across host species and for both *Nosema* species. Expression levels in adult honey bees of four antimicrobial peptide encoding genes associated with bee humoral immunity (defensin, abaecin, apidaecin, and hymenoptaecin), cellular immunity (phenol oxidase (PO), glucose dehydrogenase (GLD), eater gene (AmEater)), lysozyme and apoptosis-related genes, as well as the gene encoding the female-specific protein vitellogenin (Vg) varied in response to *Nosema* infection. AMPs were significantly upregulated, while cellular immune encoding genes showed both significant up and down regulation after infection with microsporidia. Moreover, we found that *A. cerana* elicited a higher immune response than *A. mellifera*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2017.12.010.

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