Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (*Microsporidia*)

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Summary

**Two microsporidia species have been shown to infect
Apis mellifera, *Nosema apis* and *Nosema ceranae*.
This work present evidence that *N. ceranae* infection
significantly suppresses the honey bee immune
response, although this effect was not observed fol-
lowing infection with *N. apis*. Immune suppression
would also increase susceptibility to other bee patho-
gens and senescence. Despite the importance of both
Nosema species in honey bee health, there is no
information about their effect on the bees' immune
system and present results can explain the different
virulence between both microsporida infecting
honeybees.**

Introduction

Insects have a robust immune system to defend them-
selves against the attack of microbial and eukaryotic
pathogens. Physical barriers are the insects' first lines of
defence, which prevent infectious agents from gaining
entry into the body cavity. These physical barriers include
the exoskeleton cuticle and the peritrophic membranes
lining the digestive tract. As a second line of defence,
the insects' innate immunity is usually considered in
two broad categories, cellular and humoral immunity
(Gillespie *et al.*, 1997; Lavine and Strand, 2002; Boman,
2003). Cellular immunity involves processes such as
phagocytosis, nodulation and encapsulation. Both
nodulation and encapsulation are often accompanied by
melanization, which is catalysed by the (prophenol-)
phenoloxidase (PO) (Ashida and Brey, 1998) and this
PO-mediated melanin synthesis plays a major role in an
insect's immune defence. The cellular response also
requires the participation of glucose dehydrogenase
(GLD), both during the encapsulation reaction and the
insect killing response to fungal invaders. Indeed, GLD
may be used as a marker of the initial activation of the
cellular immune response (Lovallo and Cox-Foster,
1999). In addition, lysozyme (LYS) is also important in
insect immunity against Gram-positive and Gram-
negative bacteria (Daffre *et al.*, 1994; Lavine and Strand,
2001), and it may promote the expression of other anti-
microbial peptides (Imler and Bulet, 2005).

Alternatively, insects humoral immunity involves the
synthesis of a battery of antimicrobial peptides in
response to infection by bacteria, fungi or parasites (Hetru
et al., 1998; Lamberty *et al.*, 1999; Yamauchi, 2001;
Klaudiny *et al.*, 2005). In honey bees (*Apis mellifera*), the
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-Jonsson *et al.*, 1994).

Honey bee vitellogenin (Vg) is a female-specific
180 kDa (Wheeler and Kawooya, 1990) that is synthe-
sized in the fat body of the abdomen, released into the
haemolymph and transported to the ovaries and other
tissues (Amdam *et al.*, 2003). 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), and resistance to
oxidative stress in honey bees has been linked to the
expression of Vg (Seehuus *et al.*, 2006; Corona *et al.*,
2007; Nelson *et al.*, 2007).

Microsporidiosis is caused by infection with microspo-
ridia, obligate intracellular spore-forming parasites that
belong to the order *Microsporidia*. These were reclassified
from the protozoa to the Fungi cluster, rank Opisthokonta
(Adl *et al.*, 2005). These organisms are ubiquitous exhib-
iting an extensive range of hosts that includes honey
bees.

Two microsporidia species have been shown to infect *A. mellifera*, *Nosema apis* and *Nosema ceranae*. Nosemosis due to *N. apis* has been widely studied (Hassanein, 1951; Wang and Moeller, 1969; Liu, 1984; Fries, 1988; De Graaf and Jacobs, 1991; Fries *et al.*, 1992), while nosemosis due to *N. ceranae* was reported for the first time in Europe in 2005 (Higes *et al.*, 2005; 2006) and later, it became evident in Taiwan (Huang *et al.*, 2007) and in other countries (Klee *et al.*, 2007; Martín-Hernández *et al.*, 2007; OIE, 2008). This pathogen has been recently postulated as an important threat to honey bee health (Higes *et al.*, 2005; 2008; Martín-Hernández *et al.*, 2007; Van Ooij, 2008; Higes *et al.*, 2009).

Despite the importance of both *Nosema* species in honey bee health, there is no information about their effect on the bees' immune system. In this work trials were designed to look for differences between *N. apis* and *N. ceranae* and immune response of its host.

Results

The RNA expression levels of seven genes were determined 4 and 7 days after infection. Gene-specific amplification was confirmed for the seven primer pairs as a single peak in the melting curve analysis and through the Tm values.

Effect of N. apis and N. ceranae infection on the expression of antibacterial peptides

Infection of honey bees with *N. apis* and *N. ceranae* produced significant changes in abaecin mRNA expression (ANOVA, $P = 0.0007$; Fig. 1A and B). Indeed, abaecin mRNA expression augmented significantly 4 days after infection with *N. apis* when compared with the controls and *N. ceranae*-infected bees. Seven days after infection by *N. ceranae* the expression of abaecin diminished
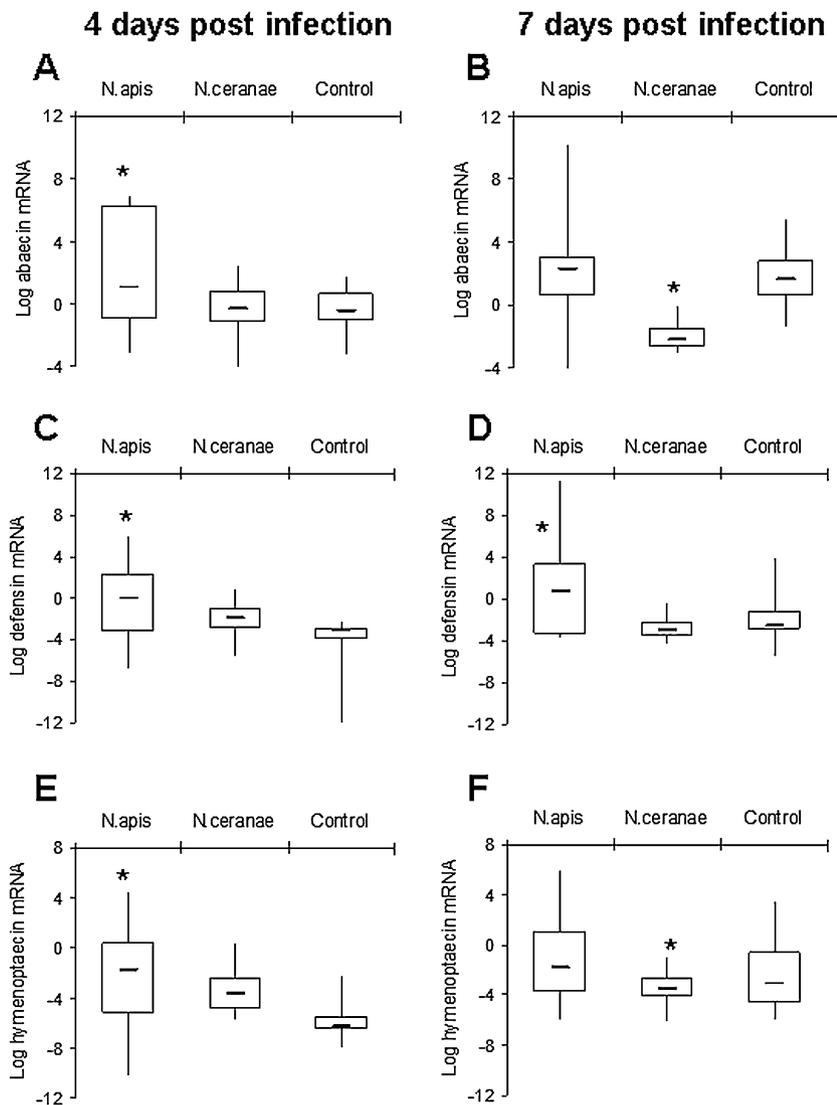

Fig. 1. Effect of *N. apis* and *N. ceranae* infection on the expression of genes encoding the antibacterial peptides abaecin, defensin and hymenoptaecin (box plots graphics). Asterisks indicate statistically significant differences (ANOVA).
A and B. Expression of abaecin at 4 (A) and 7 days (B) post infection. *Nosema apis* significantly elevated the expression of abaecin at 4 days, while *N. ceranae* significantly diminish its expression at 7 days ($P = 0.0007$).
C and D. Expression of defensin at 4 (C) and 7 days (D) post infection. Defensin expression was elevated by *N. apis* infection at 4 and 7 days ($P = 0.002$).
E and F. Expression of hymenoptaecin at 4 (E) and 7 days (F) post infection. Hymenoptaecin mRNA levels were also elevated by *N. apis* infection at 4 and 7 days, while *N. ceranae* significantly diminished its expression ($P = 0.05$).

significantly when compared with control and *N. apis*-infected bees.

Defensin expression also increased significantly following *N. apis* infection when compared with control and *N. ceranae*-infected bees, both 4 and 7 days after infection (ANOVA, $P = 0.002$). By contrast, no changes were observed after infection with *N. ceranae* (Fig. 1C and D).

In the case of hymenoptaecin mRNA levels, significant differences between the groups were found (ANOVA, $P = 0.05$) (Fig. 1E and F). *Nosema apis* increased the hymenoptaecin expression 4 days after infection compared with control bees, while *N. ceranae* decreased its expression level compared with *N. apis*-infected bees.

Effect of infection on the expression of genes encoding immunity-related enzymes

Seven days after *N. ceranae* infection, there was a significant decrease in Vg and GLD expression when compared with control or *N. apis*-infected bees (Kruskal–Wallis, $P = 0.01$ and $P = 0.02$ respectively) (Fig. 2A–D). *Nosema apis* infection did not alter the expression of these genes, although it did increased the mRNA levels of PO 7 days post infection when compared with control or *N. ceranae*-infected bees (Kruskal–Wallis, $P = 0.02$). By contrast, *N. ceranae* did not affect the expression of this gene (Kruskal–Wallis, $P > 0.05$; Fig. 2E and F). Lysozyme expression was not affected by either *N. apis* or *N. ceranae* infection (Kruskal–Wallis, $P > 0.05$; Fig. 2G and H).

Discussion

To our knowledge, this is the first study to address the effects of infection by *N. apis* and *N. ceranae* on the immune response in honey bees. We demonstrated that the honey bee immune system quickly activates defence mechanisms after infection with *N. apis*, which includes the increase in the expression of genes encoding antimicrobial peptides and other immunity-related enzymes. On the other hand, *N. ceranae* infection seems to suppress the immune response by reducing the transcription of some of these genes.

According to previous reports, only a few ventricular epithelial cells are infected 3 days after *Nosema* infection, while the majority of the cells are infected after 7 days and display evidence of degeneration (Higes *et al.*, 2007). Four days after *N. apis* infection, the expression of the antibacterial peptides abaecin, denfesin and hymenoptaecin increases. These peptides are associated with the bee humoral immune system and they present a broad antibacterial activity against Gram-positive and Gram-negative bacteria (Jarosz, 1979; Boman and Hultmark, 1987). The expression of PO also increases 7 days after *N. apis* infection, and it has been proposed that high levels of PO generate opsonic intermediates and favour nodulation, encapsulation and phagocytosis (Glinski and Jarosz, 2001).

By contrast, in *N. ceranae* infection the expression of antibacterial peptides was not affected 4 days after infection, even though the pathogen had already invaded the ventricular epithelium (Higes *et al.*, 2007). Seven days after infection the expression of abaecin, hymenoptaecin and GLD diminishes significantly, suggesting that *N. ceranae* partially suppresses humoral and cellular defence mechanisms.

The decrease in Vg expression after *N. ceranae* infection is consistent with the reduced lifespan reported for worker bees with suppressed Vg expression (Nelson *et al.*, 2007; Remolina *et al.*, 2007). Resistance to oxidative stress in worker honey bees has been linked also to the expression of Vg, which led to the suggestion that Vg also increases the lifespan of queen and worker bees (Nelson *et al.*, 2007). This proposal is based on the fact that oxidative modification of intracellular proteins is a major contributor to senescence (Finkel and Holbrook, 2000; Nyström, 2005) and that the extended lifespan of queens is due to slower senescence (Remolina *et al.*, 2007).

Naturally infected honey bees in field conditions that have expressed Vg more weakly are supposed to be less resistant to oxidative stress (Nelson *et al.*, 2007), which is considered as a cause of senescence by Remolina and colleagues (2007). This mechanism would get a shortened lifespan of worker bees, something that it would not happen in *N. apis*-infected bees.

The present work confirmed the negative impact of *N. ceranae* on bee health as reported previously (Higes *et al.*, 2007; 2008; 2009; Paxton *et al.*, 2007; Van Ooij, 2008). *Nosema ceranae* is a more prevalent and virulent microsporidia than *N. apis* (Klee *et al.*, 2007; Martín-Hernández *et al.*, 2007), producing irreversible lesions to the bee ventricular epithelium (Higes *et al.*, 2007; 2008; 2009). In addition to this direct action on the bee, the suppression of the immune system could favour rapid invasion of ventriculus by *N. ceranae*, also favouring the replication of viruses present in a latent state (such as Deformed Wing Virus, Black Queen Cell Virus, Chronic Bee Paralysis Virus, Kashmir Bee Virus). As *Varroa destructor* mite also produces immune suppression (Gregory *et al.*, 2005; Yang and Cox-Foster, 2005), simultaneous infection with both pathogens could be devastating for honeybee colonies.

This study constitutes an important advance in our understanding of the biology and virulence of a worldwide pathogen implicated in the phenomenon of honey bee loss. In addition, it would be interesting to evaluate whether there are genetic differences in the ability of honey bees to tolerate *Nosema* infection or not.
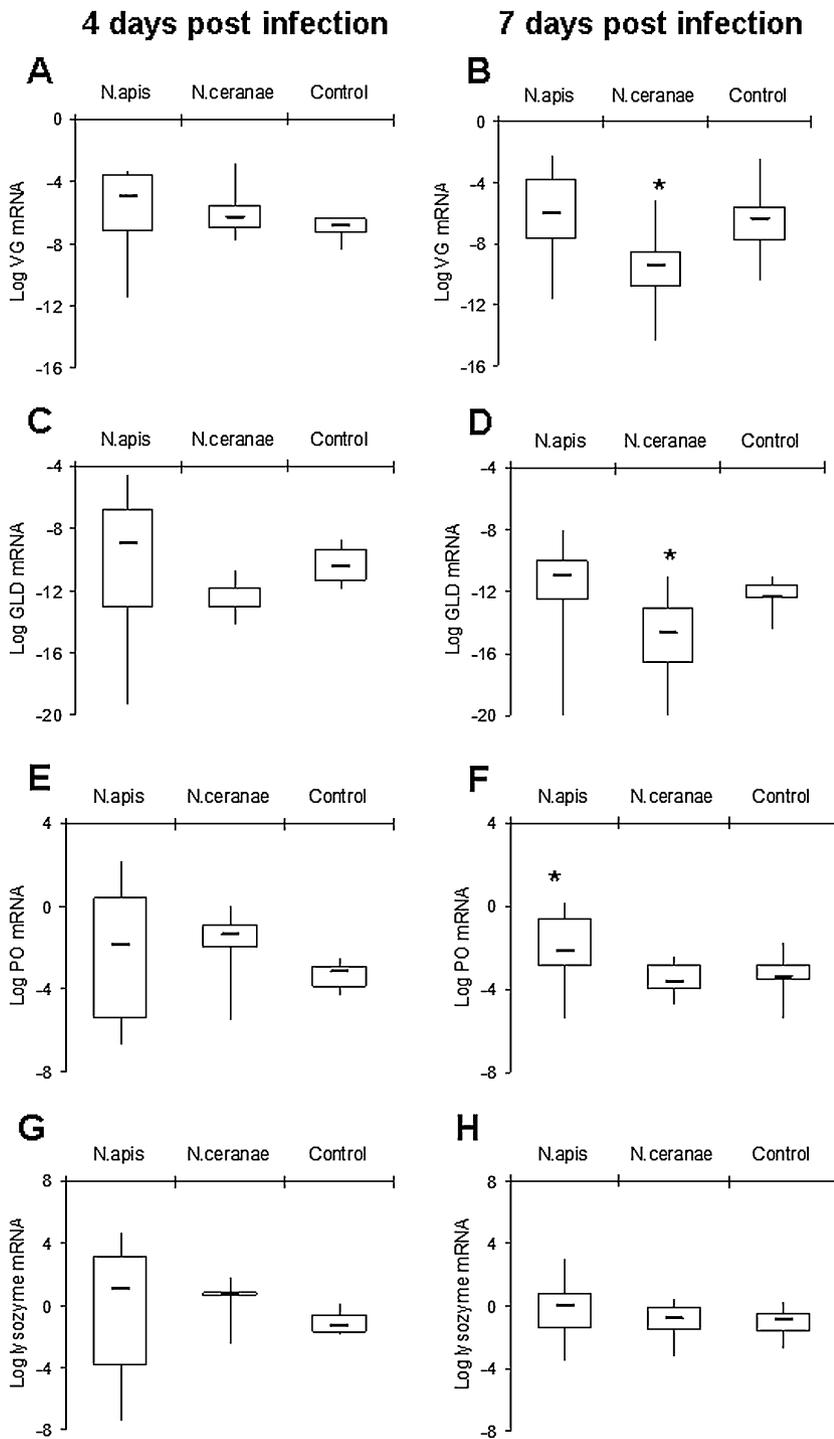
Fig. 2. Effect of *N. apis* and *N. ceranae* infection on the expression of immunity related genes VgMC, GLD, PO and LIS (box plots graphics). Asterisks indicate statistically significant differences (Kruskal–Wallis).
A and B. Expression of VgMC at 4 (A) and 7 days (B) post infection. *Nosema ceranae* infection significantly diminished its expression at 7 days (*P* = 0.01).
C and D. Expression of GLD at 4 (C) and 7 days (D) post infection. *Nosema ceranae* also diminished its expression at 7 days (*P* = 0.02).
E and F. Expression of PO at 4 (E) and 7 days (F) post infection. *Nosema apis* infection elevated the mRNA levels of PO at 7 days (*P* = 0.02).
G and H. Expression of lysozyme at 4 (E) and 7 days (F) post infection. Lysozyme expression was not affected neither by *Nosema apis* or *N. ceranae* infection.

Experimental procedures

Production of N. apis *and* N. ceranae *viable spores*

Nosema apis and *N. ceranae* pure spores with a minimum viability of 99% (as assessed with 4% trypan blue) were obtained from experimentally infected bees by 95% Percoll-purification. The spore number was counted using a haemocytometer and the *Nosema* species was confirmed by PCR (Martín-Hernández *et al.*, 2007), visualizing the amplified PCR products with a QIAxcel System (Qiagen).

Experimental infection

A pure spore suspension of *N. apis* and another of *N. ceranae* were used for experimental infection as described previously (Higes *et al.*, 2007). Frames of sealed brood were

Table 1. Primers used for the amplification of immunity genes.

Primer	Sequence	Amplification target	Reference
RPS5-F	5′-AATTATTTGGTCGCTGGAATTG-3′	Ribosolomal protein S5 (reference gene)	Evans (2006)
RPS5-R	5′-TAACGTCCAGCAGAATGTGGTA-3′		Evans (2006)
β-actina-F	5′-ATGCCAACACTGTCCTTTCTGG-3′	β-Actina (reference gene)	Yang and Cox-Foster (2005)
β-actina-R	5′-GACCCACCAATCCATACGGA-3′		Yang and Cox-Foster (2005)
Abaecin-F	5′-CAGCATTCGCATACGTACCA-3′	Antibacterial peptide abaecin	Evans (2006)
Abaecin-R	5′-GACCAGGAAACGTTGGAAAC-3′		Evans (2006)
Defensin-F	5′-TGTCGGCCTTCTCTTCATGG-3′	Antibacterial peptide defensin	Yang and Cox-Foster (2005)
Defensin-R	5′-TGACCTCCAGCTTTACCCAAA-3′		Yang and Cox-Foster (2005)
Hymenopt-F	5′-CTCTTCTGTGCCGTTGCATA-3′	Antibacterial peptide hymenoptaecin	Evans (2006)
Hymenopt-R	5′-GCGTCTCCTGTCATTCCATT-3′		Evans (2006)
VgMC-F	5′-AGTTCCGACCGACGACGA-3′	Vitellogenin	Corona *et al.* (2007)
VgMC-R	5′-TTCCCTCCCACGGAGTCC- 3′		Corona *et al.* (2007)
GLD-F	5′-CTGCACAACCACGTCTCGTT-3′	Glucose dehydrogenase	Yang and Cox-Foster (2005)
GLD-R	5′-ACCGCCGAAGAAGATTTGG-3′		Yang and Cox-Foster (2005)
PO-F	5′-AATCCATTACCTGAAATTGATGCTTAT-3′	Phenol oxidase	Yang and Cox-Foster (2005)
PO-R	5′-TAATCTTCCAACTAATTCATACGCTCTT-3′		Yang and Cox-Foster (2005)
LYS-F	5′-ACACGGTTGGTCACTGGTCC-3′	Lysozyme	Yang and Cox-Foster (2005)
LYS-R	5′-GTCCCACGCTTTGAATCCCT-3′		Yang and Cox-Foster (2005)

obtained from a healthy colony of *A. m. iberiensis* belonging to the experimental apiary from Regional Apicultural Centre. The colony was *Nosema*-free, as confirmed by PCR and it had not presented any signs of bacterial, viral or fungal infection in the past 3 years.

The brood was incubated at 34°C ± 1°C and the new emerging worker bees were removed, confined to cages in groups of 15 and kept in the incubator. The bees were fed *ad libitum* with a solution of sucrose (50% w/w in water) and Promoter L (Calier Laboratory). Seven days after emersion, two groups of bees were infected with *N. apis* and the other two with *N. ceranae*.

Bees were anesthetized with CO_2 and fed individually with 2 μl of 50% sucrose solution containing 50 000 spores μl^{-1} of *N. apis*, or 3 μl of sucrose solution containing 32 000 spores μl^{-1} of *N. ceranae* (infective doses approximately 100 000 spores), using a micropipette (Malone *et al.*, 1999). Two additional cages of 15 bees were fed with 2 μl of sucrose solution and maintained as controls. The bees were checked and fed daily and the dead bees were removed.

Four days after infection, one cage of *N. apis*-infected bees, one cage of *N. ceranae*-infected bees and one cage of control bees were removed from the incubator and maintained at −80°C. The remaining cages (one for each treatment) were removed from the incubator 7 days after infection and the bees were maintained at −80°C.

RNA isolation and cDNA Synthesis

Each honeybee was individually put in a collection microtube racked (Qiagen, 19560) with 0.2 mm glass beads and 200 μl water (PCR grade). The racks was shaken at 30 Hz for 6 min (Tyssuelyser-Quiagen). Total RNA was isolated from each individual honey bee with the RNeasy 96 Universal tissue Kit (Qiagen), following to manufacturer's instructions, and the samples were then treated with DNase I (Qiagen) in order to remove any contaminating DNA. The total RNA recovered was immediately used to generate first strand cDNAs using the Quantitec Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. Negative controls were run in parallel for each step (RNA extraction and reverse transcription reactions).

Gene and primer selection

Seven immune gene candidates implicated in cellular and humoral immune response were selected (Table 1), and accordingly, the transcript levels for the genes encoding the antimicrobial proteins abaecin, hymenoptaecin, defensin, the immunity related enzymes PO, GLD, LYS and Vg were assessed. Transcripts for the ribosomal protein RPS5 and β-actin, two moderately expressed housekeeping genes were used to normalize for variation in cDNA levels.

Real-time quantitative PCR

All real-time quantitative PCR reactions were carried out in 96-well microtitre plates, using the Quantitec SYBR Green Real Time PCR kit (Qiagen) and specific oligonucleotide primers. Reaction mixes contained 10 μl of 2× QuantiTect SYBR Green PCR MasterMix (Qiagen), 0.5 μM of each primer and 5 μl of 1:10 diluted cDNA in a final volume of 20 μl.

PCR reactions were carried out using a LightCycler 480 Real Time PCR System (Roche), and the cycling programme consisted of an initial pre-incubation step of 95°C for 10 min, and 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 40 s. Fluorescence was measured in the elongation step and negative controls (without DNA) were included in each reaction run. The specificity of the reaction was checked by analysing the melting curve of the final amplified product, which was obtained through continuous reading over increasing temperatures from 70°C to 95°C (5 readings at each °C).

The mRNA level of each gene was measured in 15 separate honey bees infected with *N. apis* or *N. ceranae* and in uninfected (control) bees.

Normalization of the real-time data and statistical analysis

The amplification results were expressed as the threshold cycle number (C_t), which represents the number of cycles needed to generate a fluorescent signal greater than a pre-defined threshold. In order to accurately measure the levels of expression, normalization to the mean of multiple reference genes is recommended rather than to a single gene (Vandesompele *et al.*, 2002). In this case, the reference genes RPS5 and β-actin were used. The mean C_t of the reference genes was calculated, and then the C_t for the different genes was subtracted from the mean of the references genes. The data corresponding to each gene was analysed in order to determine if they fitted a normal distribution and whether there was homogeneous variance. The variation in gene transcript levels between different groups was evaluated by ANOVA, and where the data did not fit the parametric assumptions, the non-parametric Kruskal–Wallis test was applied. *P*-values below 0.05 were considered significant.

Acknowledgements

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