doi:10.1111/j.1462-2920.2009.01953.x

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

Karina Antúnez,[1] Raquel Martín-Hernández,[2] Lourdes Prieto,[3] Aránzazu Meana,[4] Pablo Zunino[1] and Mariano Higes[2]*

[1]*Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay.*
[2]*Bee Pathology Laboratory, Centro Apícola Regional, JCCM, 19180 Marchamalo, Spain.*
[3]*Instituto Universitario de Investigación en Ciencias Policiales (IUICP), Comisaría General de Policía Científica (Forensic Police), DNA Laboratory, Madrid, Spain.*
[4]*Animal Health Department, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.*

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 following infection with *N. apis*. Immune suppression would also increase susceptibility to other bee pathogens 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 themselves 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 antimicrobial 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 synthesized 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 microsporidia, 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 exhibiting an extensive range of hosts that includes honey bees.

Received 2 March, 2009; accepted 13 April, 2009. *For correspondence. E-mail mhiges@jccm.es; Tel. (+34) 949250 026; Fax (+34) 949250 176.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd

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

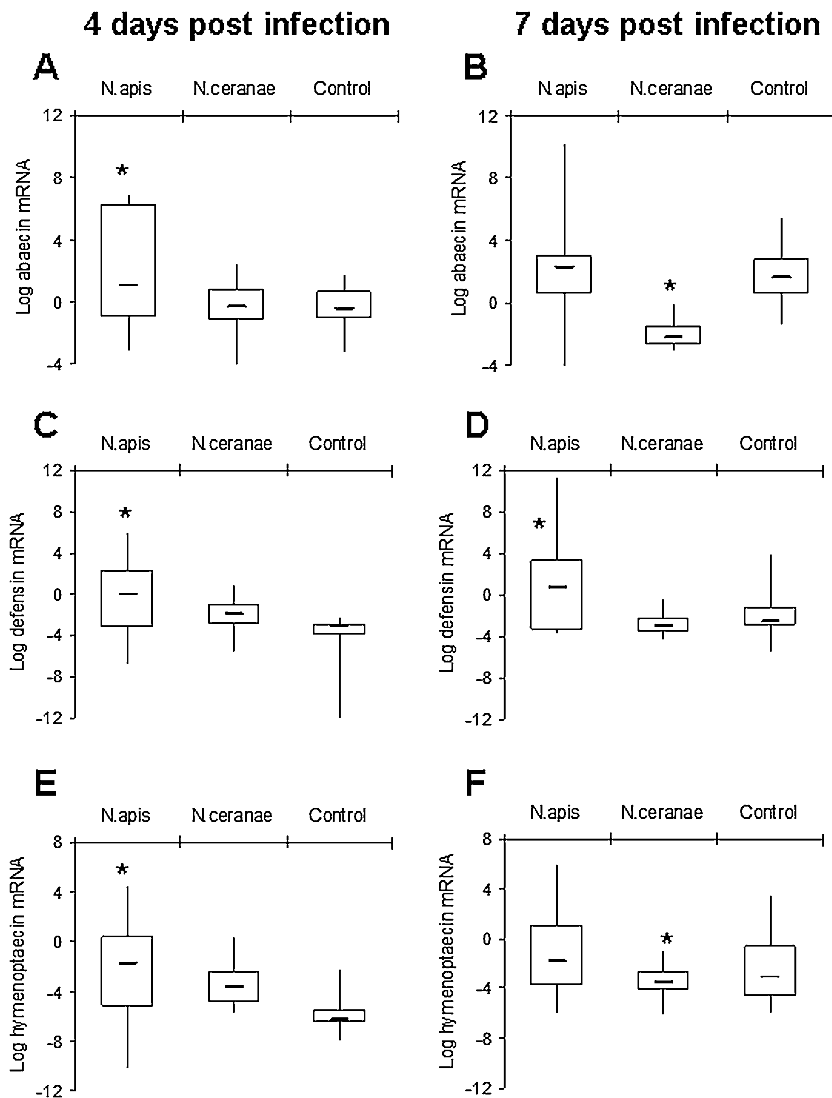

Fig. 1. Effect of *N. apis* and *N. ceranae* infection on the expression of genes encoding the antibacterial peptides abaecin, defensin and hymenoptaecin (box plots graphics). Asterisks indicate statistically significant differences (ANOVA).
A and B. Expression of abaecin at 4 (A) and 7 days (B) post 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 ($P = 0.05$).

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

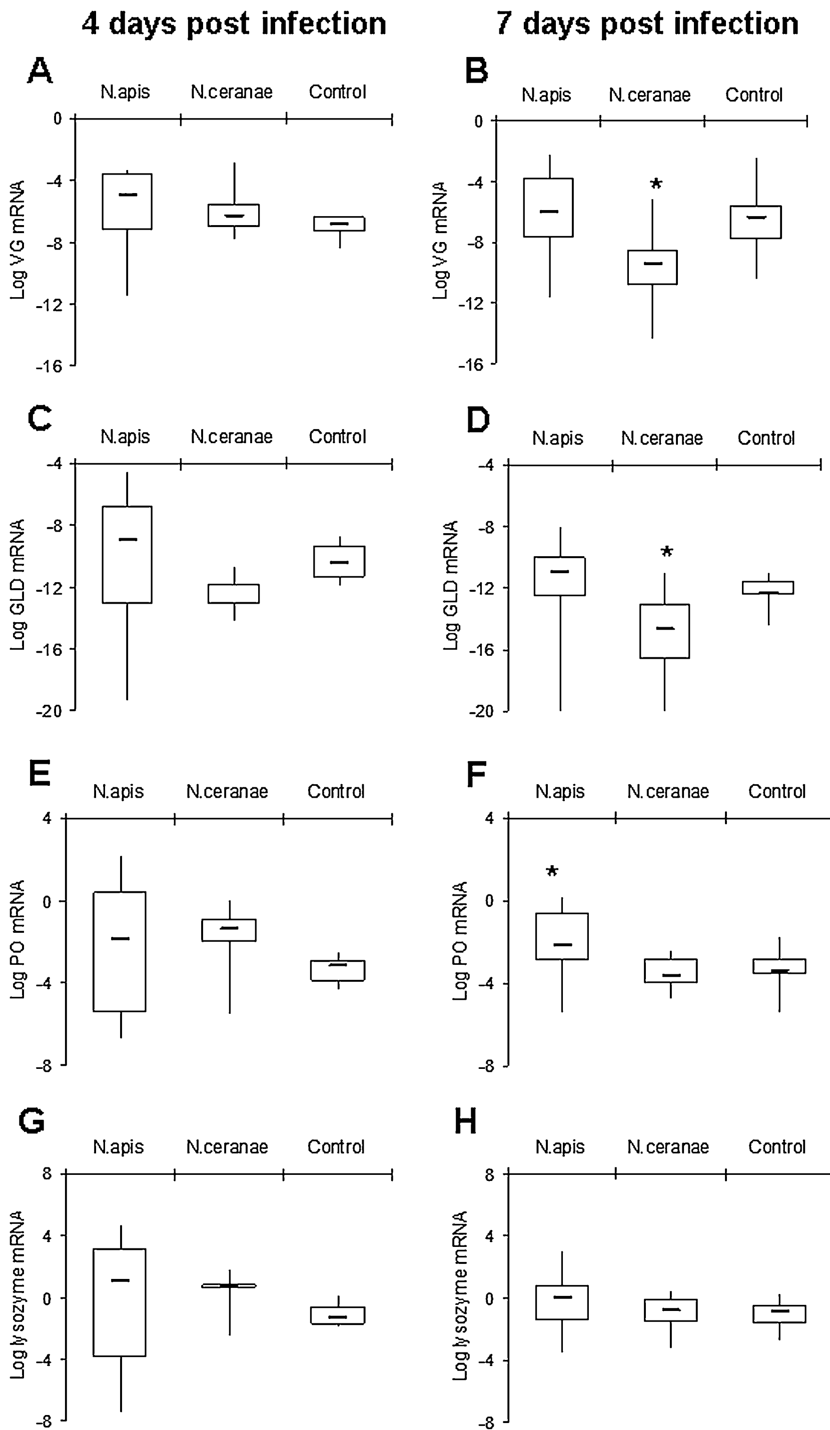

Fig. 2. Effect of *N. apis* and *N. ceranae* infection on the expression of immunity related genes VgMC, GLD, PO and LIS (box plots graphics). Asterisks indicate statistically significant differences (Kruskal–Wallis).
A and B. Expression of VgMC at 4 (A) and 7 days (B) post infection. *Nosema ceranae* 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

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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 predefined 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

This work was supported by Junta de Comunidades de Castilla-La Mancha (Consejería de Agricultura y Medio Ambiente) and Ministerio de Ambiente y Medio Rural y Marino (API06-09/FEGA-MAMR FOUNDS). Authors wish to thank to Dr C. Bartolomé for constructive revision of the text. R. Martín-Hernández was co-financed by the Junta de Comunidades de Castilla-La Mancha (JCCM) and INIA-European Social Fund.

References

Adl, S.M., Simpson, A.G.B., Farmer, M.A., Andersen, R.A., Anderson, O.R., Barta, J.R., *et al.* (2005) The new higher level classification of *Eukaryotes* with emphasis on the taxonomy of *Protists*. *J Eukaryot Microbiol* **52:** 399–451.

Amdam, G.V., and Omholt, W. (2003) The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. *J Theor Biol* **223:** 451–464.

Amdam, G.V., Simoes, Z.L.P., Guidugli, K.R., Noraberg, K., and Omholt, S.W. (2003) Disruption of vitellogenin gene function in adult honeybees by intra-abdominal infection of double-straned RNA. *BMC Biotech* **3:** 1–8.

Ashida, M., and Brey, P. (1998) Recent advances in research on the insect prophenoloxidase cascade. In *Molecular Mechanisms of Immune Responses in Insects*. Brey, P.T., and Hultmark, D. (eds). London, UK: Chapman and Hall, pp. 135–172.

Boman, H.G. (2003) Antibacterial peptides: basic facts and emerging concepts. *J Intern Med* **254:** 1014–1018.

Boman, H.G., and Hultmark, D. (1987) Cell-free immunity in insects. *Annu Rev Microbiol* **41:** 103–126.

Casteels, P., Ampe, C., Jacobs, F., Vaek, M., and Tempst, P. (1989) Apidaecins: antibacterial peptides from honeybees. *EMBO J* **8:** 2387–2391.

Casteels, P., Ampe, C., Riviere, L., Van Damme, J., Elicone, C., Fleming, M., *et al.* (1990) Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). *Eur J Biochem* **187:** 381–386.

Casteels, P., Ampe, C., Jacobs, F., and Tempst, P. (1993) Functional and chemical of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). *J Biol Chen* **268:** 7044–7054.

Casteels-Jonsson, K., Zhang, W., Capaci, T., Casteels, P., and Tempst, P. (1994) Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire post-translational conversion of the precursor structures. *J Biol Chem* **269:** 28569–28575.

Corona, M., Velarde, R., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K.A., and Robinson, G.E. (2007) Vitellogenin, juvenile hormone, insulin signalling, and queen honey bee longevity. *PNAS* **104:** 7128–7133.

Daffre, S., Klysten, P., Samakovlis, C., and Hultmark, D. (1994) The lysozyme locus in *Drosophila melanogaster*: an expanded gene family adapted for expression in the digestive tract. *Biochem Biophys Res Commun* **194:** 152–162.

De Graaf, D.C., and Jacobs, F.J. (1991) Tissue specificity of *Nosema apis*. *J Invertebr Pathol* **58:** 277–278.

Evans, J.D. (2006) Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. *J Invertebr Pathol* **93:** 135–139.

Finkel, T., and Holbrook, J. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408:** 239–247.

Fries, I. (1988) Infectivity and multiplication of *Nosema apis* Z. in the ventriculus of the honey bee. *Apidologie* **19:** 319–328.

Fries, I., Granados, R.R., and Morse, R.A. (1992) Intracellular germination of spores of *Nosema apis* Z. *Apidologie* **23:** 61–70.

Gillespie, J.P., Kanost, M.R., and Trenczeck, T. (1997) Biological mediators of insect immunity. *Annu Rev Entomol* **42:** 611–643.

Glinski, Z., and Jarosz, J. (2001) Infection and immunity in the honey bee *Apis mellifera*. *Apiacta* **36:** 12–24.

Gregory, P.G., Evans, J.D., Rinderer, T., and de Guzmán, L. (2005) Conditional immune-gene suppression of honeybees parasitized by *Varroa* mites. *J Insect Scien* **5:** 1–5.

Hassanein, M.H. (1951) Studies of the effect in infection with *Nosema apis* on the physiology of the queen honey bee. *J Cell Sci* **92:** 225–231.

Hetru, C., Hoffmann, D., and Bullet, P. (1998) Antimicrobial peptides from insects. In *Molecular Mechanisms of Immune Responses in Insects*. Brey, P.T., and Hultmark, D. (eds). London, UK: Chapman and Hall, pp. 40–66.

Higes, M., Martín-Hernández, R., Sanz, A., Alvarez, N., Garcia, M.P., and Meana, A. (2005) El síndrome de despoblamiento de las colmenas en España. Consideraciones sobre su origen. *Vida Apícola* **133:** 15–21.

Higes, M., Martín, R., and Meana, A. (2006) *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. *J Invertebr Pathol* **92:** 93–95.

Higes, M., García-Palencia, P., Martín-Hernández, R., and Meana, A. (2007) Experimental infection of *Apis mellifera* honeybees with the *Microsporidia Nosema ceranae*. *J Invertebr Pathol* **94:** 211–217.

Higes, M., Martín-Hernández, R., Botías, C., Garrido-Bailón, E., González-Porto, A.V., Barrios, L., *et al.* (2008) How

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environ Microbiol* **10:** 2659–2669.

Higes, M., Martín-Hernández, R., González-Porto, A.V., García-Palencia, P., Meana, A., del Nozal, M.J., *et al.* (2009) Honey bee colony collapse due to *Nosema cernae* in professional apiaries. *Environ Microbiol Rep* **1:** 110–113.

Huang, W.F., Jiang, J.H., Chen, Y.W., and Wang, C.H. (2007) A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. *Apidologie* **38:** 30–37.

Imler, J.-L., and Bulet, P. (2005) Antimicrobial peptides in *Drosophila*: structures, activities, an gene regulation. In *Mechanism of Epithelial Defense*, Vol. 86. Kabelitz, D., and Schorder, J.M. (eds). Basel, Switzerland: Karger, pp. 1–21.

Jarosz, J. (1979) Simultaneous induction of protective immunity and selective synthesis of haemolymph lysozyme protein in larvae of *Galleria mellonella*. *Biol Zentral* **98:** 459–471.

Klaudiny, J., Albert, S., Bachanová, K., Kopernicky, J., and Simúth, J. (2005) Two structurally different defensin genes, one of them encoding a novel defensin isoform, are expressed in honeybee *Apis mellifera*. *Insect Biochem Mol Biol* **35:** 11–22.

Klee, J., Besana, A.M., Genersch, E., Gisder, S., Nanetti, A., Tam, D.Q., *et al.* (2007) Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *J Invertebr Pathol* **96:** 1–10.

Lamberty, M., Ades, S., Uttenwiler-Josehp, S., Brookharts, G., Bushey, D., Hoffman, J.A., and Bulet, P. (1999) Insect immunity: isolation from the lepidopteran *Heliothis virescens* of a novel insect defensin with potent antifungal activity. *J Biol Chem* **274:** 9320–9326.

Lavine, M.D., and Strand, M.R. (2001) Surface characteristics of foreign targets that elicit an encapsulation response by the moth *Pseudoplusia includes*. *J Insect Physiol* **47:** 965–974.

Lavine, M.D., and Strand, M.R. (2002) Insect haemocytes and their role in immunity. *Insect Biochem Mol Biol* **32:** 1295–1309.

Liu, T.P. (1984) Ultrastructure of the midgut of the worker honey bee *Apis mellifera* heavily infected with *Nosema apis*. *J Invertebr Pathol* **44:** 282–291.

Lovallo, N.C., and Cox-Foster, D.L. (1999) Alteration in FAD-glucose dehydrogenase activity and hemocyte behaviour contribute to initial disruption of *Manduca sexta* immune response to *Cotesia congregata* parasitoids. *J Insect Physiol* **45:** 1037–1048.

Malone, L.A., Giacon, H.A., and Newton, M.R. (1999) Comparison of the responses of some New Zealand and Australian honey bees (*Apis mellifera* L.) to *Nosema apis* Z. *Apidologie* **26:** 495–502.

Martín-Hernández, R., Meana, A., Prieto, L., Martínez-Salvador, A., Garrido-Bailón, E., and Higes, M. (2007) Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Appl Environ Microbiol* **73:** 6331–6338.

Nelson, C.M., Ihle, K.E., Fondrk, M.K., Page, R.E., and Amdam, G.V. (2007) The gene vitellogenin has multiple coordinating effects on social organization. *Plos Biol* **5:** 673–677.

Nyström, T. (2005) Role of oxidative carbonylation in protein quality control and senescence. *EMBO J* **24:** 1311–1317.

Office International des Epizooties (2008) Chapter 2.2.4, Nosemosis of honeybee. In *Manual of Standards for Diagnostic Test and Vaccines.* [WWW Document] URL http://www.oie.international/eng/normes/mmanual/2008/pdf/2.02.04_nosemosis.pdf.

Paxton, R.J., Klee, J., Korpela, S., and Fries, I. (2007) *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. *Apidologie* **38:** 558–565.

Remolina, S.C., Hafez, D.M., Robinson, G.E., and Hughes, K.A. (2007) Senescence in the worker honey bee *Apis mellifera*. *J Insect Physiol* **53:** 1027–1033.

Seehuus, S.C., Norberg, K., Gimsa, U., Krekling, T., and Amdam, G.V. (2006) Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *PNAS* **103:** 962–967.

Van Ooij, C. (2008) Buzzkill. *Nat Rev Microbiol* **6:** 650–651.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Roy, N.V., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Gen Biol* **3:** 34.1–34.34.11.

Wang, D.I., and Moeller, F.E. (1969) Comparison of the free amino acid composition in the hemolymph of the healthy and *Nosema*-infected female honey bees. *J Invertebr Pathol* **15:** 202–206.

Wheeler, D.E., and Kawooya, J.K. (1990) Purification and characterization of honeybee vitellogenin. *Arch Insect Biochem Physiol* **14:** 73–79.

Yamauchi, H. (2001) Two novel insect defensin from larvae of the cupreous chafer, *Anomala cupra*: purification, amino acid sequences and antibacterial activity. *Insect Biochem Mol Biol* **32:** 75–78.

Yang, X., and Cox-Foster, D.L. (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. *PNAS* **21:** 7470–7475.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*