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Differential expression of vitellogenin in honey bees (*Apis mellifera*) with different degrees of *Nosema ceranae* infection

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Summary

Nosema apis and *Nosema ceranae* are causative agents of Nosemosis in the honey bee *Apis mellifera*, although *N. ceranae* may cause a more virulent disease. Selection of colonies with high tolerance to *N. ceranae* could be important for reducing problems caused by this pathogen. The aim of the present work was to evaluate the existence of honey bee colonies with different degrees of *N. ceranae* infection and test if this difference could be related to the immune response or vitellogenin expression. Healthy honey bee colonies were relocated to a plantation of *Eucalyptus grandis* to favour natural infection of *N. ceranae*. Fifteen and thirty days after relocation, the proportion of infected bees and the number of *N. ceranae* spores per field were quantified. The colonies with higher and lower levels of infection (HL and LL, respectively) were selected. Newly emerged bees from both colonies were artificially infected with *N. ceranae* and seven days after infection the expression of immune related genes and vitellogenin was evaluated by real time PCR. No significant differences were observed in expression of abaecin, hymenoptaecin, defensin, glucose dehydrogenase or lysozyme mRNA levels between infected bees from HL and LL colonies or between control bees from both colonies. Vitellogenin expression was higher in bees from the LL colony than in bees from the HL colony, when infected or control bees were compared between them. This protein possesses pleiotropic effects and is a central element in the life-history of honey bees. For that reason, its differential expression could be associated with resistance to *N. ceranae*.

Expresión diferencial de la vitelogenina en abejas (*Apis mellifera*) con diferente nivel de infección por *Nosema ceranae*

Resumen

Nosema apis y *Nosema ceranae* son agentes causales de Nosemosis en la abeja *Apis mellifera* aunque *N. ceranae* podría causar una enfermedad más virulenta. La selección de colonias resistentes a *N. ceranae* podría ser importante para la reducción de problemas ocasionados por este patógeno. El objetivo del presente trabajo fue evaluar la existencia de colonias con diferente grado de infección por *N. ceranae* y evaluar si esta diferencia podría estar relacionada a la respuesta inmune o a la expresión de vitelogenina. Colonias de abejas sanas fueron trasladadas a forestaciones de *Eucalyptus grandis* para favorecer la infección natural con *N. ceranae*. A los 15 y 30 días después del traslado, la proporción de abejas infectadas y el número de esporas de *N. ceranae* por campo fueron cuantificados. Las colonias con mayor y menor nivel de infección (HL y LL respectivamente) fueron seleccionadas. Abejas recientemente nacidas de ambas colonias se infectaron artificialmente con *N. ceranae* y siete días después de la infección se evaluó la expresión de genes relacionados con inmunidad y vitelogenina, mediante PCR en tiempo real. No se encontraron diferencias significativas en la expresión de abaecina, himenoptecina, defensina, glucosa deshidrogenasa o lisozima entre abejas infectadas de las colonias HL y LL o entre abejas control de ambas colonias. La expresión de vitelogenina resultó más baja en abejas pertenecientes a la colonia LL que en abejas pertenecientes a la colonia HL, cuando se compararon abejas infectadas o abejas control. Esta proteína posee efectos pleiotrópicos y es central en la vida de las abejas melíferas, por esta razón, su expresión diferencial podría estar asociada a la resistencia a *N. ceranae*.

Keywords: *Apis mellifera*, *Nosema ceranae*, vitellogenin, immune response, real time PCR

Introduction

Nosema apis and *Nosema ceranae* are causative agents of Nosemosis in the honey bee *Apis mellifera* (Fries, 2010; Higes *et al.*, 2010). Both species are obligate intracellular parasites of adult honey bees, although *N. ceranae* apparently is an exotic pathogen of *Apis mellifera* (Klee *et al.*, 2007) and causes a more virulent disease (Martin-Hernandez *et al.*, 2011). As an example, *N. ceranae* increases energetic stress in honey bee (Mayack and Naug, 2009; Martin-Hernandez *et al.*, 2011), suppresses the immune response (Antunez *et al.*, 2009; Chaimanee *et al.*, 2012), shortens the life-span of infected honey bees (Higes *et al.*, 2006) and has been related to colony losses specially in Spain (Martin-Hernández *et al.*, 2007; Higes *et al.*, 2008; Higes *et al.*, 2010). However, the potential role of *N. ceranae* in colony losses is still debated (Cox-Foster *et al.*, 2007; Forsgren and Fries *et al.*, 2010; Fries, 2010; Paxton, 2010).

In Uruguay, according to Invernizzi *et al.* (2009), *N. ceranae* is present and widely distributed and causes an important health problem especially among colonies located in *Eucalyptus grandis* plantations (Invernizzi *et al.*, 2011a; Invernizzi *et al.*, 2011b; Mendoza *et al.*, 2012). Uruguayan beekeepers exploit these plantations moving their colonies at the end of summer (February or March). Although honey production can reach up to 50 kg per colony at the end of the flowering period, colonies are weakened and can have significant losses unless they are quickly removed (Invernizzi *et al.*, 2011b). On the other hand, *N. apis* has not been detected (Invernizzi *et al.*, 2011a; Anido *et al.*, *pers. comm.*)

The only effective treatment against this microsporidium is fumagillin, but this compound is no longer licensed in many countries, including Uruguay (Higes *et al.*, 2008; MGAP, 2010; Williams *et al.*, 2008). The selection of honey bee colonies resistant to *N. ceranae* could be an important route to solve the problem caused by this pathogen.

Insects' immunity comprises the integument and the gut as physical barriers, a cellular response that act when these barriers are breached, and a humoral response, constituting important defence lines against pathogens (Gillespie *et al.*, 1997). Cellular immunity involves processes such as phagocytosis, nodulation, and encapsulation, which are related to enzymes phenoloxidase, glucose dehydrogenase, and lysozyme (Daffre *et al.*, 1994; Gillespie *et al.*, 1997; Lovallo and Cox-Foster, 1999; Lavine and Strand, 2002). Humoral immunity involves the synthesis of antimicrobial peptides such as apidaecin, abaecin, hymenoptaecin and defensin (Casteels *et al.*, 1989; Casteels *et al.*, 1990; Casteels *et al.*, 1993; Casteels-Josson *et al.*, 1994).

Vitellogenin is another important insect protein. It is a female-specific 180 kDa protein that possesses pleiotropic effects being a central element in the life-history regulation of honey bees (Nelson *et al.*, 2007). It is involved in the division of labour and foraging specialization, main characteristics of honey bee social organization, as in resistance to oxidative stress (Wheeler and Kawooya, 1990; Amdam and Omholt, 2003; Seehuus *et al.*, 2006; Corona *et al.*, 2007; Nelson *et al.*, 2007).

Bees with high vitellogenin expression have been associated to an enhanced lifespan (Corona *et al.*, 2007; Remolina *et al.*, 2007).

The aim of the present work was to evaluate the existence of *A. mellifera* colonies with different degree of *N. ceranae* infection and test if this difference could be related to the bee immune response or vitellogenin expression.

Materials and methods

Colony selection

An experimental apiary with 63 asymptomatic colonies of Africanized bees (resulting from crosses of *A. m. mellifera* with *A. m. scutellata*) located in the Instituto de Investigación Agropecuaria "La Estanzuela" (Colonia, Uruguay) was relocated in March 2011 (end of summer) to an artificial plantation of *E. grandis* in Rivera (Uruguay). At the beginning of the experiment, colonies were free of *Nosema* spp. spores.

Fifteen and 30 days after relocation of the apiary, 30 foraging bees were sampled from each colony and individually analysed to detect and quantify the number of *Nosema* spp. spores, following the criteria set by Pickard and El Shemy (1989). For this purpose, the abdomen of each bee was macerated in 0.5 ml of water and an aliquot of the suspension was observed under a microscope at 400 x magnification registering the number of spores found in 10 microscopy fields, as described by Invernizzi *et al.* (2011b). The proportion of infected bees and media of spores/field were calculated.

The colony that presented the highest level of infection, estimated by percentage of infected bees and number of spores per field, at 15 and 30 days of installation was named HL while the colony that presented the lowest level of infection was named LL. These two colonies were chosen for further assays. Presence of *N. ceranae* spores and absence of *N. apis* spores was confirmed by PCR, as described by Martin-Hernandez *et al.* (2007).

Purification of *N. ceranae* spores

Foraging bees were collected from a naturally infected colony of the experimental apiary located in Rivera, Uruguay, and purified by centrifugation in a Percoll gradient, as described by Higes *et al.* (2008). The spore number was determined using a haemocytometer under a contrast phase microscopy. Presence of *N. ceranae* spores and absence of *N. apis* spores was confirmed by PCR (Martin-Hernandez *et al.*, 2007). The obtained suspension was immediately used for experimental infection.

Experimental infection

Frames of sealed brood were obtained from colonies HL and LL. Since both colonies were located in the same apiary, and brood was taken in the middle of the flowering period (not at the end, where losses related to nutritional can be detected), it can be assumed that there were not nutritional differences between both colonies.

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

Amplification target	Primer	Sequence	Tm (°C)	Reference
ribosomal protein S5	RPS5-F	5'-AATTATTTGGTCGCTGGAATTG-3'	75.8	Evans, 2006
	RPS5-R	5'-TAACGTCCAGCAGAATGTGGTA-3'		
antibacterial peptide abaecin	Abaecin- F	5'-CAGCATTGCGATACGTACCA-3'	79.3	Evans, 2006
	Abaecin- R	5'-GACCAGGAAACGTTGGAAAC-3'		
antibacterial peptide defensin	Defensin- F	5'-TGTCGGCCTTCTCTTCATGG-3'	82.3	Yang & Cox-Foster, 2007
	Defensin- R	5'-TGACCTCCAGCTTTACCCAAA-3'		
antibacterial peptide hymenoptaecin	Hymenopt- F	5'-CTCTTCTGTGCCGTTGCATA-3'	80.5	Johnson <i>et al.</i> , 2009
	Hymenopt- R	5'-GCGTCTCCTGTCAATCCATT-3'		
stress protein vitellogenin	VgMC-F	5'-AGTTCGACCGACGACGA-3'	83	Johnson <i>et al.</i> , 2009
	VgMC-R	5'-TTCCCTCCCACGGAGTCC-3'		
glucose dehydrogenase	GLD- F	5'-CTGCACAACCAGTCTCGTT-3'	86.5	Yang & Cox-Foster, 2007
	GLD- R	5'-ACCGCCGAAGAAGATTTGG-3'		
(prophenol-) phenoloxidase	PO- F	5'-AATCCATTACCTGAAATTGATGCTTAT-3'	75.7	Yang & Cox-Foster, 2007
	PO- R	5'-TAATCTTCCAATAATTCATACGCTCTT-3'		
lysozyme	LYS- F	5'-ACACGTTGGTCACTGGTCC-3'	84.5	Yang & Cox-Foster, 2007
	LYS- R	5'-GTCCACGCTTTGAATCCCT-3'		

Brood from both colonies were incubated at 34°C and new emerging worker bees were removed, confined to special cages (16 cm x 12 cm x 6 cm) in groups of 30 bees and kept in the incubator. Bees were fed *ad libitum* with a solution of sucrose (50% w/w in water).

Five days after emergence, experimental infections were carried out as described by Rinderer (1976) and modified by Porrini *et al.* (2011). Two groups of 30 bees from colony HL and two groups of 30 bees from colony LL were infected with fresh *N. ceranae* spores. Bees were fed individually with 3 µl of 50% sucrose solution containing 33,000 spores/µl of *N. ceranae* (infective doses approx. 100,000 spores). Two additional cages of 30 bees from each colony (HL and LL) were treated as previously described, but fed with 3 µl of sucrose solution and maintained as controls. Bees were incubated at 34°C and were checked and fed daily. Dead bees were removed.

Four days after infection one cage with infected bees from colony HL, one cage with infected bees from colony LL and one cage of control bees from each colony (HL and LL) were removed from the incubator and maintained at -80°C until analysis. Seven days after infection, the rest of the bees were removed from the incubator and maintained at -80°C until analysis.

RNA isolation and cDNA Synthesis

Fifteen bees belonging to each group (infected and control groups from colonies HL and LL) were individually macerated in 600 µl of RLT buffer (RNeasy Plus Mini Kit, Qiagen) using a sterile glass rod and a sterile plastic tube. An aliquot of the macerate from every bee (infected and control) was used to determine the number of *N. ceranae* spores per bee, by using a haemocytometer and a contrast phase microscopy.

Total RNA was isolated from each individual bee using RNeasy Plus Mini Kit (Qiagen), following to manufacturer's instructions. This kit includes a column for elimination of genomic DNA. Total RNA recovered was immediately used to generate first strand cDNAs using Quantitec

Reverse Transcription Kit (Qiagen), according to manufacturer's instructions, using a mix of oligo-dT and random primers. This kit also includes an incubation step with gDNA Wipeout Buffer to remove contaminating genomic DNA prior to retrotranscription. cDNA was stored at -20°C.

Gene and primer selection

In order to compare the immune response of bees from colonies HL and LL to *N. ceranae*, transcript levels for genes encoding antimicrobial proteins abaecin, hymenoptaecin, defensin, the immunity related enzymes phenoloxidase, glucose dehydrogenase, lysozyme and vitellogenin were assessed using primers previously described (Evans, 2006; Cox-Foster *et al.*, 2007; Yang and Cox-Foster, 2007). Transcript levels for ribosomal protein RPS5, a moderately expressed housekeeping gene was used to normalize for variation in cDNA levels (Evans, 2006). Table 1 summarizes the primers used.

Real-time PCR

mRNA level of each gene was measured in 15 individual bees infected with *N. ceranae* and in 15 control bees, belonging to colonies HL and LL. In a first approach only bees after seven days of infection (12 days old bees) were analysed. Bees at four days after infection (9 days old bees) were analysed only when necessary. Real time PCR reactions were carried out using QuantiTect SYBR PCR Kit (Qiagen), according to manufactures recommendations, and specific primers for amplification of different genes (Table 1).

Reaction mixture consisted in 1x QuantiTect SYBR Green PCR Master Mix, 0.5 µM of each primer (one pair of primers per reaction), RNase free water and 5 µl of 1:10 diluted cDNA in a final volume of 25 µl. PCR reactions were carried out using a Rotor Gene 6000 (Corbett Research) and cycling program consisted in an initial activation step at 50°C for 2 min and 95°C for 15 min, and 40 cycles of 94°C for 15 sec,

50°C for 30 sec and 72°C for 30 sec. Fluorescence was measured in the elongation step and negative controls (without DNA) were included in each reaction run. Specificity of reactions was checked by analysis of melting curves of the final amplified product (from 65 to 95°C). Amplification results from different genes 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 of different genes were performed using comparative Ct method, as detailed described by Chen *et al.* (2005) and Prisco *et al.* (2011), using RPS5 for normalization. Comparative CT method was validated by showing that amplification efficiencies of different genes and RPS5 were equal.

Statistical analysis

In case that data fit the parameters for the use of parametric statistic, ANOVA test was used. In other cases, Test of Equal or Given Proportions, Mann Whitney or Kruskal Wallis test were used. P values below 0.05 were considered significant. All statistical analyses were performed using STATS package (R Development Core Team, 2010).

Results

Selection of colonies

Fifteen and thirty days after relocation of healthy *A. mellifera* colonies in an artificial plantation of *E. grandis*, a wide range of *N. ceranae* infection values were observed in different colonies considering proportion of infected bees (0 to 77%) and number of spores per field (0 to > 200). The colony that presented the highest proportion of infected bees and a high number of spores per field (HL colony) and the colony that presented the lower proportion of infected bees and a low number of spores per bee (LL colony) were selected for further studies (Fig. 1). Differences between proportions of infected bees in both colonies were statistically significant at 15 and 30 days after installation ($\chi^2 = 11.82$, $p < 0.001$ and $\chi^2 = 11.47$, $p < 0.001$ for 15 and 30 days respectively). Differences between the number of spores per field also resulted statistically significant, according to Mann Whitney test, at 15 and 30 days after installation ($W = 246$, $p < 0.001$; $W = 239$, $p < 0.001$).

When newly emerged bees from both colonies (HL and LL) were artificially infected with *N. ceranae* spores, infected bees from colony HL presented significantly higher number of spores than infected bees from colony LL ($F = 4.66$; $p = 0.035$). At 4 days after infection, values were 68800 ± 49781 spores per bee and 44800 ± 23333 spores/bee, and 7 days after infection, values were 171000 ± 104000 spores/bee and 126000 ± 133000 spores per bee, for colonies HL and LL respectively. Control bees (non-infected) did not present *N. ceranae* spores.

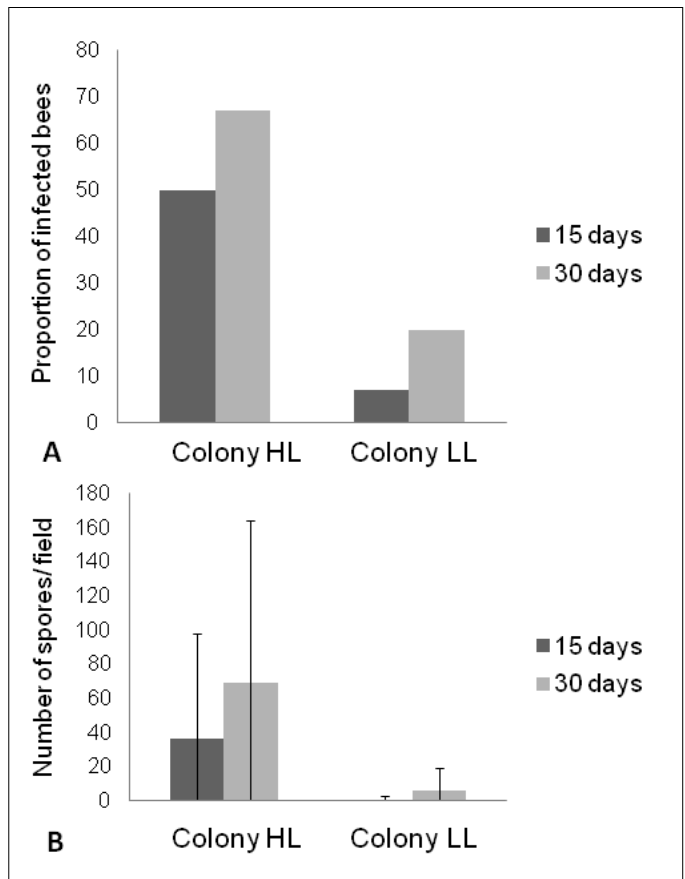


Fig. 1. Proportion of infected bees (A) and number of spores/field (B) of colonies with the highest and lowest level of *N. ceranae* infection (HL and LL, respectively), 30 days after relocation to *E. grandis* plantation.

Evaluation of immune response and vitellogenin expression

RPS5, immune related genes and vitellogenin were successfully amplified in all cases and specificity of amplified products was confirmed by single peaks in the melting curve analysis and analysis of Tm of amplified fragments. In a first analysis, we compared the expression levels of abaecin, hymenoptaecin, defensin, glucose dehydrogenase, lysozyme, phenoloxidase and vitellogenin mRNA levels between bees from colonies HL and LL. No significant differences were obtained for abaecin, hymenoptaecin, defensin, glucose dehydrogenase and lysozyme mRNA levels (Fig. 2). The same results were obtained when infected or control bees from both colonies were compared. However, the expression of phenoloxidase resulted significantly lower in bees from colony LL compared to bees from colony HL ($p = 0.03$) in infected bees, although this result was not reproduced in control bees (Fig. 2).

Vitellogenin expression resulted higher in bees from colony LL than in bees from colony HL, when infected or control bees were compared ($p = 0.04$ and $p = 0.02$ respectively, Fig. 3). According to $\Delta\Delta Ct$ method, vitellogenin expression was 2.9 times higher in infected bees from colony LL than in infected bees from colony HL, and 4.0 times higher in control bees from colony LL than in control bees from colony HL.

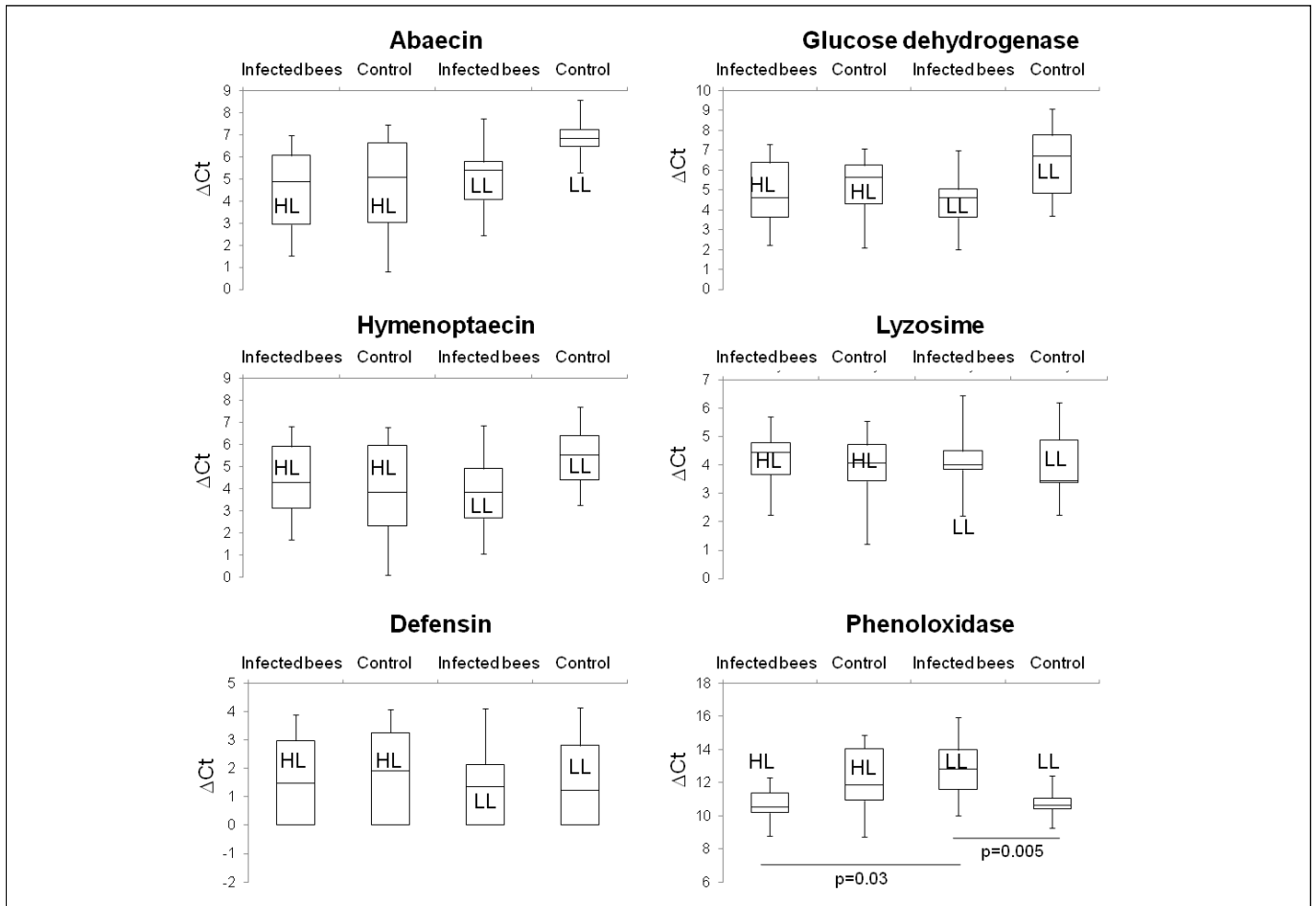


Fig. 2. Effect of *N. ceranae* infection on the expression of antibacterial peptides and other immunity related genes (box plots graphics). Higher values of ΔCt indicate lower expression. HL indicates bees from the colony with the highest level of infection, while LL indicates bees from colony with lowest level of infection.

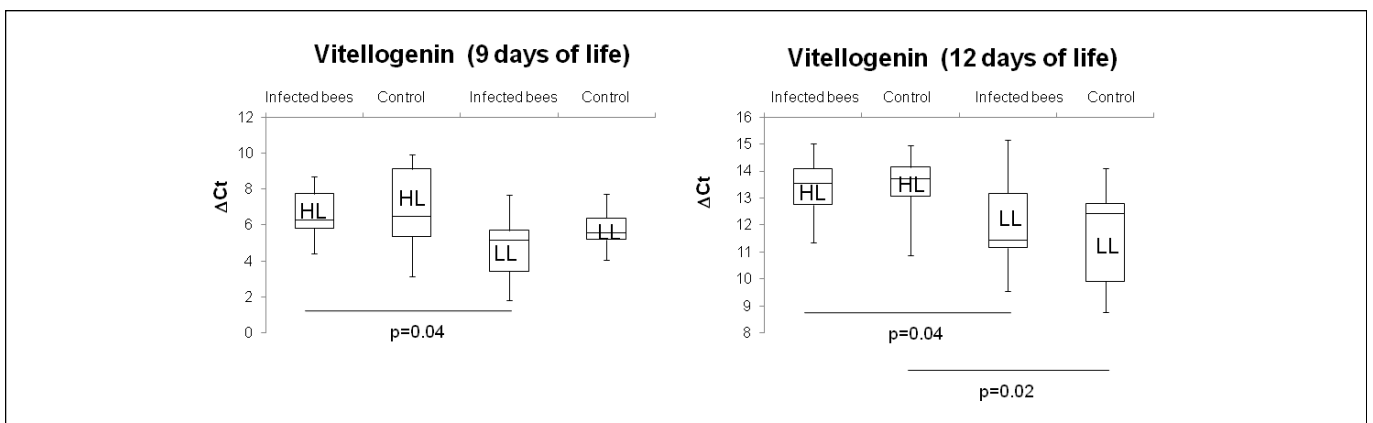


Fig. 3. Effect of *N. ceranae* infection on the expression of vitellogenin (box plots graphics). Higher values of ΔCt indicate lower expression. HL indicates bees from the colony with the highest level of infection, while LL indicates bees from the colony with the lowest level of infection.

In order to confirm these results, the same procedure was carried out four days after infection (9 days old bees), and similar results were found. Vitellogenin expression was 3.5 times higher in infected bees from colony LL than in infected bees from colony HL, and 2.5 times higher in control bees from colony LL than in control bees from colony HL.

New real time PCR analysis were carried out in order to compare vitellogenin expression levels at four and seven days after infection (9 and 12 days of age), but no significant difference was obtained when comparing them, indicating that vitellogenin expression level remains constant during those days. This was confirmed using bees from colony HL and from colony LL (data not shown). Then, we

compared the effect of *N. ceranae* infection on the expression of different genes in bees from colonies HL and LL independently. Infection of bees did not produced significant changes in abaecin, hymenoptaecin, defensin, glucose dehydrogenase, lysozyme or vitellogenin mRNA levels, when compared to control bees. The same results were obtained when colonies HL or LL were used (Fig. 2). On the other side, infection with *N. ceranae* significantly reduced the expression of phenoloxidase, compared to control bees, only when bees from colony LL were used ($p = 0.005$).

Discussion

In the present study we show evidence that *A. mellifera* colonies presented different degrees of *N. ceranae* infection, suggesting that there are different levels of resistance against the pathogen. According to the obtained results, these differences were not associated to the immune system response, because similar expression of abaecin, defensin, hymenoptaecin, lysozyme and glucose dehydrogenase were detected in bees from the colony with high level of *N. ceranae* infection than in bees from colony with low level of infection.

On the other hand, differential expression of vitellogenin was seen in bees from those colonies; its expression resulted significantly higher in bees from colony with low level of *N. ceranae* infection than in bees from colony with high level of infection. The same results were obtained when comparing infected bees between both colonies and control bees between both colonies. These results suggest that the expression of this protein can be related to the infection level. Since only one HL colony was compared with one LL colony, we can only suggest this association, but it is important to state that results were consistent at nine and twelve days of life of bees. On the other side, the difference found can't be explained by nutritional deficiencies causing problems in fat body development (place where vitellogenin is produced), since all bees were born in laboratory conditions, and all were fed in the same conditions. The higher vitellogenin expression level detected in bees from the low level of infection colony could be constitutive in those bees and not induced by infection, since no significant change was detected after infection.

Since vitellogenin is involved in coordination of behaviour and age-associated shift from nest tasks to foraging duties, differences found in vitellogenin expression levels in bees from colonies with different degrees of infection may be related to differences in behaviour of those bees, time to perform foraging activities, life span, between others (Amdam and Omholt, 2003; Guidugli *et al.*, 2005; Amdam *et al.*, 2007).

The second interesting result of the present work is the host immune response to *N. ceranae* infection. In the case of vitellogenin, it was reported that after infection of *A. mellifera iberiensis* with *N. ceranae*, expression of vitellogenin was suppressed (Antunez *et al.*,

2009). However, these results were not reproduced using *A. mellifera ligustica* (Chaimanee *et al.*, 2012a), or in the present work, using hybrids of *A. mellifera mellifera* and *A. mellifera scutellata*. These differences can be related to the use of different honey bee races or to the possible existence of different *N. ceranae* strains with differential virulence. Both hypothesis were confirmed by Chaimanee *et al.*, (2013), who reported differences in the susceptibility of *A. mellifera*, *Apis cerana*, *Apis dorsata* and *Apis florea* when were infected with two different isolates of *N. ceranae*.

A Thailand isolate of *N. ceranae* had high infectivity in *A. mellifera*, *A. cerana* and *A. dorsata* in contrast with *A. florea*; while a Canadian isolate of *N. ceranae* was only able to infect *A. mellifera* and *A. dorsata*. These authors also demonstrated that there are differences in the immune response of bees from different species, when inoculated with the same *N. ceranae* isolate. In addition, Mendoza *et al.*, 2013 also obtained differences in the *N. ceranae* infection level when comparing *A. mellifera ligustica* and hybrids of *A. mellifera mellifera* and *A. mellifera scutellata*.

In the case of other immune related genes, infection by *N. ceranae* did not produced changes in the expression levels of neither in bees from HL or LL. These results are according to those previously described by Antúnez *et al.* (2009) except for abaecin and glucose dehydrogenase, where a decreased in their expression was previously observed (Antunez *et al.*, 2009). On the other side, Chaimanee *et al.*, (2012) recently reported that *N. ceranae* infection down-regulated the expression of abaecin, defensin and hymenoptaecin. Differences found between present results and previous work (Antunez *et al.*, 2009; Chaimanee *et al.*, 2012) can also be related to the use of different honey bee races and *N. ceranae* strains. Although in the present study down regulation of the expression of immune related genes after *N. ceranae* infection was not evidenced, we confirm that the pathogen is able to avoid the immune system defence. This could favour rapid invasion of different tissues by *N. ceranae*.

In conclusion, in the present study we present evidence that there are honey bees with different degrees of *N. ceranae* infection, and this resistance could be associated to a differential expression of vitellogenin.

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