Journal of Insect Physiology 58 (2012) 1090–1095

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Differential expression of immune genes of adult honey bee (*Apis mellifera*) after inoculated by *Nosema ceranae*

Veeranan Chaimanee [a], Panuwan Chantawannakul [a,b], Yanping Chen [c], Jay D. Evans [c], Jeffery S. Pettis [c,*]

[a] Bee Protection Center, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[b] Materials Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[c] USDA-ARS, Bee Research Laboratory, Beltsville, MD 20705, USA

ARTICLE INFO

Article history:
Received 21 February 2012
Received in revised form 25 April 2012
Accepted 27 April 2012
Available online 16 May 2012

Keywords:
Nosema ceranae
Antimicrobial peptides
Immunosuppression
Apis mellifera
Innate immunity

ABSTRACT

Nosema ceranae is a microsporidium parasite infecting adult honey bees (*Apis mellifera*) and is known to affects at both the individual and colony level. In this study, the expression levels were measured for four antimicrobial peptide encoding genes that are associated with bee humoral immunity (defensin, abaecin, apidaecin, and hymenoptaecin), eater gene which is a transmembrane protein involved cellular immunity and gene encoding female-specific protein (vitellogenin) in honey bees when inoculated by *N. ceranae*. The results showed that four of these genes, defensin, abaecin, apidaecin and hymenoptaecin were significantly down-regulated 3 and 6 days after inoculations. Additionally, antimicrobial peptide expressions did not significantly differ between control and inoculated bees after 12 days post inoculation. Moreover, our results revealed that the mRNA levels of eater and vitellogenin did not differ significantly following *N. ceranae* inoculation. Therefore, in this study we reaffirmed that *N. ceranae* infection induces host immunosuppression.

Published by Elsevier Ltd.

1. Introduction

Honey bees, *Apis mellifera*, are challenged by bacteria, viruses, fungi and parasites. *Nosema ceranae* is a microsporidian parasite that infects *A. mellifera* worldwide. *N. ceranae* infection has impacts at both the individual honey bee (Paxton et al., 2007; Higes et al., 2009; Martín-Hernández et al., 2007) and colony level and has been associated with honey bee colony losses (Higes et al., 2008, 2009; Topolska et al., 2008; Bacandritsos et al., 2010; Borneck et al., 2010; Currie et al., 2010; Van der Zee, 2010). However, insects have a variety of methods to combat the effects of pathogens and parasites. Behavioral, physiological and immune defenses are common mechanisms involved in eliminating pathogens (Evans and Spivak, 2010). In honey bees, many immune pathways and defense mechanisms have been identified. They consist of both cellular and humoral immune response. Phagocytosis, encapsulation and melanization are related to the cellular immunity (Osta et al., 2004). These mechanisms are catalyzed by phenol oxidase (PO) (Decker and Jaenicke, 2004) and glucose dehydrogenase (GLD) (Cox-Foster et al., 1990). Moreover, phagocytosis is related to eater which is a major receptor and plays an important role in the recognition and phagocytosis of bacteria in *Drosophila* (Ertürk-Hasdemir and Silverman, 2005; Kocks et al., 2005). While humoral immunity involves antimicrobial peptide (AMPs) synthesis (Evans et al., 2006). Four antimicrobial peptides, abaecin, apidaecin, defensin and hymenoptaecin have been identified in *A. mellifera* when honey bees are infected by bacterial infection (Casteels et al., 1989, 1990, 1993; Casteels-Jonsson et al., 1994). These antimicrobial peptides provide a broad-spectrum of activity against microorganisms.

Honey bee vitellogenin (Vg) is a 180 kDa female-specific protein synthesized by the fat body and released into the heamolymph (Wheeler and Kawooya, 1990). It has a function in reproduction and regulates immune function and become a major regulator of lifespan of honey bees (Amdam et al., 2004, 2005).

Recently, Antúnez et al. (2009) demonstrated that abaecin and hymenoptaecin expressions in *N. ceranae* infected bees were significantly down-regulated as compared to control bees after 7 days infection. Moreover, the mRNA levels of the immunity-related enzyme, glucose dehydrogenase (GLD) and vitellogenin were also significantly decreased following *N. ceranae* infection after 7 days infection. These results suggest that *N. ceranae* infection causes honey bee immune suppression.

Our aim in this study was to determine how bee immunity responds to *N. ceranae* infection in comparison with the previous studies and to compare immune response overtime. To do this, we studied the expression of gene encoding for five antimicrobial peptides (defensin, abaecin, apidaecin, and hymenoptaecin), eater encoding gene and a female-specific protein (vitellogenin) encoding gene in honey bees when inoculated with *N. ceranae* parasite.

* Corresponding author. Address: USDA-ARS Bee Research Laboratory, Bldg. 476 BARC-E, Beltsville, MD 20705, USA. Tel.: +1 301 504 7299; fax: +1 301 504 8736.
E-mail address: jeff.pettis@ars.usda.gov (J.S. Pettis).

0022-1910/$ - see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.jinsphys.2012.04.016

2. Materials and methods

2.1. Worker bees

Frames of sealed brood were obtained from three healthy colonies of *A. mellifera ligustica* in the apiary at the Bee Research Laboratory, USDA in Maryland, USA used as colony-level replication in this study. The colonies had not presented any visible clinical symptoms of disease and they were *Nosema*-free (50 bees from each colony were examined using light microscopy and no *N. ceranae* spores could be detected). The brood was incubated at 34 ± 1 °C. Five groups of 30 newly emerged worker bees in each colony were removed and divided into cages. Each cage of bees was inoculated with different doses of *N. ceranae* spore (control, 1000, 10,000, 100,000 and 1000,000 spores/ml).

2.2. Inoculum preparation

N. ceranae spores were isolated from a colony infected with *N. ceranae* obtained from an apiary at the Bee Research Laboratory, USDA in Maryland, USA. The midguts were removed and crushed in distilled water and *N. ceranae* spores confirmed using PCR amplification after the methods described by Chen et al. (2008). Additionally, the numbers of spores were estimated by counting spores with light microscopy following the method of Cantwell (1970). The inoculum was freshly prepared in various concentrations by mixing with 50% sucrose solution to obtain a final concentration of 1000, 10,000, 100,000 and 1000,000 spores/ml.

2.3. N. ceranae infection experiments

One day after eclosion, workers (n = 30) were placed in standard wooden hoarding cages (12 × 12 × 12 cm), which had a feeder containing sucrose solution (3.5 ml). The sucrose solution containing 1000, 10,000, 100,000 and 1000,000 spores/ml of *N. ceranae* was used to feed bees for the first 2 days. All cages were kept in an incubator at 30 ± 2 °C. After exposure to *N. ceranae*, worker bees were fed *ad libitum* with clean 50% (w/v) of sucrose solution throughout the remainder of the experiment. Control groups were fed with a 50% (w/v) of sucrose solution with no *Nosema* spores. Six bees from each group were collected at days 3, 6 and 12 after infection. The bees were then examined for the presence of *N. ceranae* spores. The individual abdomens were homogenized in 1 ml of distilled water and *N. ceranae* spores were estimated using a haemocytometer (Cantwell, 1970).

2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from the rest of the abdomen of the 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. First-strand cDNA was generated from approximately 2 μg total RNA using a master mix containing of 50 U Superscript II (Invitrogen, Carlsbad, CA), 2 nmol DNTP mix, 2 nmol poly(dT)$_{18}$, and 0.1 nmol poly(dT)$_{(12–18)}$. Synthesis was carried out at 42 °C for 50 min followed by 15 min at 70 °C as described in Evans (2006).

2.5. Real-time quantitative PCR

Real-time quantitative PCR amplification was performed in a 20 μl reaction mixture using the EXPRESS SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen) and 0.2 μM of each specific primer. The oligonucleotide amplification primers are shown in Table 1. The PCR reactions were carried out in 96-well microtiter plates using Bio-Rad Icycler (Bio-Rad Crop., 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 80 s. Fluorescence was measured repeatedly each cycle during the annealing step. This procedure was followed by a melt-curve dissociation analysis to confirm product size.

Table 1
Oligonucleotide primers used in this study for real-time quantitative PCR.

Primer name	Sequence 5′ to 3′	Reference
Actin-F	TTGTATGCCAACACTGTCCTTT	Simone et al. (2009)
Actin-R	TGGCGCGATGATCTTAATTT	
Abaecin-F	CAGCATTCGCATACGTACCA	Evans (2006)
Abaecin-R	GACCAGGAAACGTTGGAAAC	
AmEater-F	CATTTGCCAACCTGTTTGT	Simone et al. (2009)
AmEater-R	ATCCATTGGTGCAATTTGG	
ApidNT-F	TTTTGCCTTAGCAATTCTTGTTG	Simone et al. (2009)
ApidNT-R	GTAGGTCGAGTAGGCGGATCT	
Defensin-F	TGCGCTGCTAACTGTCTCAG	Evans (2006)
Defensin-R	AATGGCACTTAACCGAAACG	
Hymenopt-F	CTCTTCTGTGCCGTTGCATA	Evans (2006)
Hymenopt-R	GCGTCTCCTGTCATTCCATT	
VgMC-F	AGTTCCGACCGACGACGA	Simone et al. (2009)
VgMC-R	TTCCCTCCCACGGAGTCC	

The amplification results were expressed as the threshold cycle (C_t) value, which represented the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. Relative quantifications were calculated by using threshold cycle numbers for the target gene by subtracted from the reference gene (β-actin) for each sample.

2.6. Statistical analysis

Normality and homogeneity of variances of the data was checked using SPSS version 17.0 for window (SPSS, Inc.). Statistical significance was analyzed using a one-way ANOVA and where differences were found, the means were compared by a Tukey-HSD test using SPSS version 17.0 for window (SPSS, Inc.). Data are presented for overall mean transcript levels across all treatments.

3. Results

3.1. N. ceranae infection of A. mellifera

The worker bees in each cage were examined for the presence of *N. ceranae* spores after 6 and 12 days post inoculation. In this experiment, we found that *N. ceranae* spores were not observed in any control samples throughout the experiments.

When inoculated with *N. ceranae* at the spore dose of 10^3, 10^5 and 10^6 spores/ml, only experimental cage No. 3 became infected after 6 days of inoculation with the infection rate of 33.33%, 16.67% and 33.33%, respectively (Fig. 1a). However, after 12 days post inoculation, a large number of spores could be observed when inoculated with *N. ceranae* spore at both 10^5 and 10^6 spores/ml. Spore numbers greatly varied between the experimental cages. There were about $2.51 \pm 1.40 \times 10^6$, $1.4 \pm 0.60 \times 10^5$ and $1.19 \pm 0.66 \times 10^6$ spores/bee with the infection rates of 100%, 66.67% and 66.67% of the cage No. 1, 2 and 3, respectively when inoculated with 10^5 spores/ml (Fig. 1b). Unfortunately, we found that all bees in cage No.1 died at day 9 after dosed with 10^6 spores/ml of *N. ceranae*. The infection rates in cage No.2 was 100% and 50% in cage No. 3 when inoculated with 10^6 spores/ml. The spore numbers were $1.6 \pm 0.53 \times 10^6$ and $1.5 \pm 1.1 \times 10^5$ spores/bee in the cage No. 2 and 3, respectively (Fig. 1b).