Assessment of antibacterial genes of emerging queen *Apis mellifera* L. induced by the bacterium *Paenibacillus larvae* larvae

SAS Goma1, EMS Barakat2, MS Salama2 and EE El Gohary1,2

1Research and Training Centre on Vectors of Diseases, Faculty of Science, Ain Shams University, Abbassia, Cairo, Egypt
2Department of Entomology, Faculty of Science, Ain Shams University, Abbassia, Cairo, Egypt

This study evaluated the expression of antimicrobial abacien and defensin genes in virgin honey-bee queens (*Apis mellifera*) following induction of 4th larval instar with the bacterium *Paenibacillus larvae* larvae (1.07 × 102 CFU/queen); to investigate whether the presence of bacteria affects the immune response as well as gene transcript levels for the immune proteins. The total body proteins of bacteria-treated queens showed a highly significant increase, and the appearance of new proteins patterns and/or disappearance of others. The gene expression profile of treated queens showed up-regulation equalled four-fold of the defensin gene, whereas no progression occurred in the abacien gene. This finding likewise greatly affects the diseases challenging this pollinator.

INTRODUCTION

Honey-bees are social insects that live in a nested environment with at least 50 000 individuals in contact with each other, providing ideal conditions for disease and parasite transmission (Cornman et al. 2012). Social insects have evolved various strategies to minimise disease impacts (Decanini et al. 2007). American foulbrood (AFB) is considered the most fatal bacterial disease of honey-bee brood (Craisheim & Riessberger-Galle 2001) caused by the Gram-positive bacterium, *Paenibacillus larvae* subsp. *larvae* (*P. l. larvae*). These spores are extremely resistant to heat and chemical agents and can remain viable for over 35 years (Haseeman 1961). Larvae are highly susceptible to *P. l. larvae* spores during the first 36 h after egg hatching, older larvae needed a high dose of spores to be infected under natural conditions (Gomaa 2009). It germinates in the larval midgut for several days without destroying midgut epithelium (Yue et al. 2008), later bacteria destroy the peritrophic membrane and invade the haemocoel. Although it only kills larvae, adults serve as vectors within and between colonies, delivering spores to the brood while nursing (Fries et al. 2006), and spores can spread and leads to the collapse of entire colonies.

A primary goal of honey-bee research remains to breed bees that resist or tolerate pests and pathogens (Evan & Lopez 2004). Honey-bees like all insects and other arthropods lack an adaptive immune system. To combat pathogens, they have many lines of defence; cooperative social behaviour (Cremer et al. 2007); physical barriers and finally, innate immune system (Randolt et al. 2008). In comparison to *Anopheles or Drosophila*, the honey-bee genome has one-third of the genes that are related to the existence of social immunity (Cremer et al. 2007). In such a crowded environment, the queen influences the immunity of direct progeny, thus increasing resistance to current infection in the colony (Decanini et al. 2007). In hymenopteran insects such as honey-bees, wasps, bumble-bees, and ants, antimicrobial peptides (AMPs) play a key role in the elimination and destruction of bacteria and fungi in invertebrates (Rowley & Powell 2007; Xu et al. 2009), which they consider important components of the honey-bee immune system. The AMPs of bees are induced by one of two immune responses to insects (Tzou et al. 2002), which are triggered by recognition proteins in the presence of microorganisms and stimulate different signal transduction and modulation pathways (Evans 2004).

In the cellular response to infections, host-defence in insects relies on an inducible systemic humoral immune response to counter infections; it involves the synthesis of a battery of antimicrobial peptides in response to infection by bacteria, fungi, or parasites (Klaudiny et al. 2005; Kwong et al. 2017). The latter studies demonstrated that induced levels of AMPs in offspring are higher when their parents received an immune challenge (Lopez et al. 2014). Synthesis and secretion of different AMPs in the fat body (Angus et al. 2001) compose a general non-specific line of defence in response to oral bacterial infections (Evans 2004). AMPs isolated from honey-bees consist of at least four peptides, including apidaecin, abacien, hymenoptaecin, and defensin (Danilhik et al. 2016), all peptides inhibit bacterial activity in vitro. Each of them was with a rather broad activity spectrum (Tzou et al. 2002).

This study explores whether the presence of bacteria affects the immune response as well as gene transcription levels for the synthesised immune proteins. This investigation, in turn, can help attempts to breed immune honey-bee queens resistant to disease, which influence the immunity of direct progeny, where the queen as a single individual positively impacts the immunological status of the whole colony.
MATERIALS AND METHODS

Rearing of honey-bees

Honey-bees used in this study belonged to two colonies of healthy craniolian hybrid Apis mellifera carnica. They were kept in a private apiary yard in Abo-Yassin, El-Sharquia governorate, Egypt under normal living conditions; tested queens were obtained using the grafting technique (Doolittle 1889).

Source of the bacterium pathogen

The bacterium, P. l. larvae was isolated and kept from ropy remains of honey-bee larvae collected from the Agriculture Research Centre, Plant Protection Institute, Department of Agriculture Research, Cairo, Egypt. Activation and cultivation of bacterial pathogens were performed according to the method mentioned by Hansen & Brodersgaard (1999).

An induction dose of bacterium P. l. larvae

The sub-lethal dose of 1.07 × 10⁵ CFU/queen was determined in a previous study by Gomaa et al. (2018). This dose was enough to induce the immune response of queens and did not cause a high mortality rate. Subsequently, 10 µl of this dose was mixed with the food of the tested group of queen’s larvae (10 individuals), according to the method of Decanini et al. (2007) and another group consisted of untreated 10 individuals that were used as a control. Doses were expressed as the number of CFU/queen.

Estimation of the total body protein concentration of queen

After emergence, queens were crushed in a sterile Eppendorf tube using Ultrasonic Homogenizer, 4710 Series on 30 Hz for 2 min on ice, then 250 µl of phosphate-buffered saline (PBS) (Oxoid) was added and pipetted, then centrifugation (Eppendorf Centrifuge, 5402) at 4000 rpm for 15 min on cooling; the supernatant was decanted into another sterile Eppendorf and stored at −20 °C until use. The total body protein contents of the control and bacteria-treated honey-bee queens were estimated according to the method described by Bradford (1976). This method involved the binding of Coomassie Brilliant Blue G-250 (CBB) dyes to proteins (Compton & Jones 1985). Bovine serum albumin (BSA) solution (0.8 mg/ml) was prepared for the standard protein solution. Samples of queens’ total body were pipetted 20 µl in test tubes, then 1 ml of CBB solution was added, and the contents of the tubes were mixed. The absorbance was measured at a wavelength of 595 nm (Sedmack & Grossberg 1977). This procedure was repeated three times for each.

Calculation

The total protein content was estimated as mg/ml using the following formula derived from the equation of the straight line.

Protein concentration = Absorbency − 0.6241/0.085 (mg/ml)

Electrophoretic analysis of proteins

Proteins of the total body homogenate of the different honey-bee queen samples analysed by SDS-polyacrylamide gel slabs. One-dimensional 15% gel electrophoresis was performed on bee queen samples analysed by SDS-polyacrylamide gel slabs. Proteins of the total body homogenate of the different honey-bee queen samples were extracted using the DNA/RNA Extraction/ Purification Kit (BioFlux, Bior Technology Co.). First-strand cDNAs was generated from approximately 1 µg of total RNA using the Sensifast™ cDNA Synthesis Kit Protocol (Bioline, U.K.). A thermal cycler, Gene Amp PCR System 9700 (Applied Biosystems) was used; the reaction product was stored at −20 °C until used. The thermal cycler program was according to the method of Antunez et al. (2009). Two immune gene candidates implicated in immune response were selected. The PCR reaction was performed in a 0.2 ml Eppendorf tube with a final volume of 25 µl. PCR amplification of antibacterial genes (cDNAs) from untreated and bacterial-fed queens was conducted using oligonucleotide primers specific for β-actin, abaecin, and defensin genes. The reaction mixture consisted of 12.5 µl of green master mix (Fermentas, Dream Tag™ green PCR master mix), 1 µl of 10 µM of each primer, and 5 µl of cDNA in a final volume of 25 µl. All reactions were conducted using a thermal cycling programme; the optimal temperature cycling was adjusted according to Antunez et al. (2009). Primer sequences used for detecting an antimicrobial gene are shown in Table 1. The PCR products were visualised on a UV transilluminator (SYNGENE) on 2% agarose gel electrophoresis using Gene Ruler™ 50-bp-wide range (50–1000 bp) DNA Ladder (Fermentas Life science), at 100 V for 45–50 min.

To study the expression profile of target genes in untreated and bacteria-treated honey-bee queens, quantitative real-time PCR (qPCR) was performed using the Mx3005P qPCR system (Agilent Technologies) with some modifications referring to Green & Sambrook (2012). qPCR reactions were performed using Maxima SYBR Green qPCR Master Mix (Bioline, London, U.K.), Sensifast™ SYBR LO ROX Mix kit (Bioline, London, U.K.), and specific oligonucleotide primers. The reaction mixes contained 10 µl of 1X Sensifast Sybr Lo Rox Mix (Bioline), 0.8 µl of 10 µM of each primer, and 5 µl of cDNA in a final volume of 25 µl. All reactions were conducted using a thermal cycling programme; the optimal temperature cycling was adjusted according to Antunez et al. (2009). Primer sequences used for detecting an antimicrobial gene are shown in Table 1. The PCR products were visualised on a UV transilluminator (SYNGENE) on 2% agarose gel electrophoresis using Gene Ruler™ 50-bp-wide range (50–1000 bp) DNA Ladder (Fermentas Life science), at 100 V for 45–50 min.

Table 1. Primers used for detection of antimicrobial of genes expression

| Primer        | Sequence                        | Target gene                  | Reference      |
|---------------|--------------------------------|------------------------------|----------------|
| β-actina-F    | 5’ATGCCAACAATCTGCTTCTCTGG-3’    | β-Actina (reference gene)    | Yang and Cox-Foster (2005) |
| β-actina-R    | 5’GACCCACATCCATAGGGA--3’        |                              |                |
| Abaecin-F     | 5’CAGCATTCGATCGATCACTCCA-3’     | Antibacterial peptide abaecin | Evans (2006)   |
| Abaecin-R     | 5’GACGCGAAACGCGTGAGAGACG-3’     |                              |                |
| Defensin-F    | 5’TGCGCTGTCACTGCTGTCAG-3’       | Antibacterial peptide Defensin| Evans (2004)   |
| Defensin-R    | 5’ATGCCAACAATCTGCTTCTCTGG-3’    |                              |                |

Extraction of proteins in queen samples was performed according to the protocol of Ekramoddoulah & Davidson (1995), three frozen queens (0.3 mg) were ground to powder in liquid nitrogen with a homogeniser and extracted with 300 µl of extraction buffer (4% SDS, 5% 2-mercaptopethanol and 4% sucrose); vortex for two minutes and then boiled at 95 °C for 5 min. Vortex and boiling were repeated once, and the supernatant was collected by centrifugation at 14 000 rpm for 5 min at new Eppendorf. The supernatant of queen samples (15 µl) was diluted with the same volume of treatment buffer, and then denatured by heating at 95 °C for 1 min in the water bath (Kottermann, D3165, Hanigsen, W. Germany) and chilled on ice for electrophoresis. Scanning and analysis of the protein bands were analysed using software: Gel-Pro Analyzer, version 6, from Media Cybernetics, L.P.; U.S.A.
70 to 95 °C (5 readings at each °C). The mRNA level of each gene was measured in a pool of six control and treated honey-bee queens. Amplification curves and threshold cycle number (Ct) were determined by the Stratagene MX3005P software (Agilent Technologies, Inc. 2009, version 4.10). Differences in gene expression between groups were calculated using the △△Ct (cycle threshold, Ct) method, the Ct of each sample was compared with that of the control group according to the ‘△△Ct’ which was normalised against β-actin gene (housekeeping gene) as the reference gene for each sample and expressed as relative mRNA levels compared with controls (Yuan et al. 2006).

**Data analysis**

Levels of significance for differences in means were estimated using Student’s t-test for paired samples.

**RESULTS**

**Induction of honey-bee queens by P. l. larvae**

Emerging queens of honey-bees were induced by LD20 (1.07 × 102 CFU/queen) of *P. l. larvae*. This dose was found to induce the immune response of queen larvae. The LD20 was estimated for queens as a sub-lethal dose to investigate the subsequent tests.

Effects of sub-lethal dose *P. l. larvae* on the total body protein contents

The concentration of the total body protein contents of bacterial-fed queens showed a highly significant increase (*P* ≤ 0.01) with a 99% confidence level compared with the control. The protein contents of the control and bacterial fed queens were 1.75 ± 0.1 and 2.8 ± 0.2 mg/ml, respectively, as shown in Table 2.

**Electrophoretic analysis of the total body proteins**

Electrophoretic profiles of control-tested queens revealed that 16 protein fragments were found, their molecular weights ranged between 17.1 and 100 kDa. Moreover, in treated queens, six protein fragments disappeared with MW of 100, 61.87, 51.6, 43, 36.47, and 24.94 kDa, while the other six proteins were synthesised, with MW 130, 116.2, 55.49, 28.88, 12.9, and 10.55 kDa (Figures 1 & 2).

**Antibacterial gene expression**

Primer specificity for two genes Ab and Df of *A. mellifera* queens was tested. A single PCR amplicon of the expected size was observed. PCR has detected honey-bee Ab and Df genes, which were 72 and 201 bp, respectively, moreover, the positive control β-actin gene was equal to 151 bp. Quantitative RT-PCR of Ab and Df was addressed to determine the alteration of gene expression in the untreated and induced the immune response bacterial-fed honey-bee queens with a sublethal dose 1.07 × 102 CFU/queen. Levels of expression were normalised with the β-actin mRNA, an internal control (housekeeping gene). The 2-ΔΔCT approach was employed to provide an overview of quantitative gene expression investigations using the CT method. The highest recorded Ct value between the two genes was by defensin at 25.3, whereas the lowest was by abaecin at 29.25. Figure 3 represents the relative expression values of abaecin and defensin gene indicate fold-change compared to the mean value of the control. In emerging queens treated with bacterium *P. l. larvae*, defensin gene expression was highly significant upregulation (*P* < 0.0027) with an average 4.08 fold-change compared with the control. However, no changes occurred in abaecin mRNA expression between the control and treatment samples, an insignificant effect of bacterium *P. l. larvae* on honey-bee queens was found (*P* ≥ 0.1492) with an average of 1.02 fold-change.

**DISCUSSION**

Social insects are conspicuous targets for pathogens ranging from viruses and bacteria to protozoa and fungi (Schmid-Hempel 1998). Because of their social lifestyle with a high population density in their hives, honey-bees are especially vulnerable to infection by pathogens. Since the discovery of the AFB disease, it is the most serious and destructive worldwide bacterial brood disease of honey-bee (Genersch et al. 2006). The susceptibility of honey-bee larvae is related to their hereditary constitutions (Hoage & Rothenbuhler 1966). Several workers proposed using the investigation of honey-bee immunity and different gene transcript levels against *P. l. larvae* (Evans & Lopez 2004). Because it is the most resistant larval instar, the queen 4th larval instar was chosen (Gomaa 2009). The resistance

**Table 2.** Total body protein concentrations (mg/ml) of *A. mellifera* queens in healthy and treated with *P. l. larvae*

| Treatment          | Protein concentration (mg/ml) Mean ± SE |
|--------------------|----------------------------------------|
| Healthy (-ve control) | 1.75 ± 0.1                             |
| Bacteria-treated     | 2.8 ± 0.2*                             |

N = 3 replicates per test, *significant to healthy (*P* ≤ 0.001)
level of honey-bee queens’ 4th-instar larvae to inoculation
*P. l. larvae* was relatively strong in this investigation, with an
LD20 of around 100 CFU/queen on a larval natural diet. This
dose was used to induce the immune response proteins of queen
larvae and in the meantime did not cause a high mortality rate
(Goma et al. 2021). Several authors provide references to some
recognised proteins that are immune responsive (more than 50
factors), start to accumulate in the haemolymph within a few
hours post-treatment of bacteria (Decanini et al. 2007; Randolt
et al. 2008; Guani-Guerra et al. 2010). Antimicrobial peptides
(AMPs) are small molecular weight proteins which are involved
in the defence mechanisms of a broad-spectrum antimicrobial
activity against environmental pathogens including bacteria,
viruses, yeast, and fungi (da Silva & Machado 2012). They are also
known as host defence peptides, and they affect inflammation,
wound healing, and adaptive immune system regulation, as well
as maintaining homeostasis (Auvynet & Rosenstein 2009).
These peptides are evolutionarily conserved compounds that
are involved in most living organisms’ defence mechanisms.
AMPs have various biochemical properties, but they act
against microorganisms via a mechanism involving membrane
disruption and pore formation, which results in cell content
leakage and cell death (Lapis 2008). The defensin-family AMPs
are significant players in the orchestration of the innate response
and the interplay between innate and adaptive immunity (Gomes &
Fernandes 2010). Likewise, abaecin is AMPs with exceedingly
successful, against hymenopterans Gram-negative bacteria
(Kim et al. 2007), where it is delivered quickly in fat bodies
after septic injury or immune challenge, then released into the
haemolymph and act against microorganisms (Choi et al. 2008).
They likewise target intracellular components, such as DNA,
enzymes, and even organelles (Teixeira et al. 2012). The total
body homogenates of honey-bee queens showed antibacterial
activity against *P. l. larvae*, a significant increase in activity was
observed with bacteria-treated queens. This result is due to the
simultaneous induction of bacteria (Evans 2004) and a fast rate
of transcription (Rowley & Powell 2007). Each protein is thought
to reflect the activity of a specific gene by producing an enzyme
that works as a catalyst to produce a protein responsible for a certain
biological characteristic (Cerda 2003). The abaecin and defensin
genes of honey-bees were qualitatively studied in the laboratory
and in the field to determine the ecological and evolutionary
dynamics of insect immunity (Kurtz 2004). When it comes to
bee disease responses, an ecological-genetic approach can help
beekeepers and breeders who are dealing with huge colony
disease losses (Evans 2006). AMPs can be triggered for a brief
period and transported to the infection site (Aerts et al. 2008).
The presence of effective anti-infectious defence mechanisms in
honey-bees is required, with defensins a family of AMPs, being
one of the most significant components. Defensins are inducible
and have a wide antibacterial range (Liyasove et al. 2011).
The differences in immune system end products can reflect changes
in bees’ abilities to recognise infections (Werner et al. 2000).
*P. l. larvae* infection can also activate abaecin and defensin
(Evans 2004). These peptides are related to the bee humoral
immune system and they present a broad antibacterial activity
against Gram-positive and Gram-negative bacteria (Jarosz
1995). The expression levels of Ab and Df genes were assessed
in healthy and bacteria-treated queens with *P. l. larvae*. The
study found no differences in the abaecin gene between treated
and control queens, which is similar to Evans’ (2004) findings
on honey-bee 2nd, 3rd, 4th, and 5th larval instars infected with
*P. l. larvae* spores. However, Antúnez et al. (2009) reported an
increase in abaecin gene after Nosema apis infection. Contrarily,
defensin expression levels showed an up-regulation in bacterial
fed queens four-fold compared to control. The expression level
of the antibacterial peptide defensin gene increases after bees’
infection with *N. apis* (Antúnez et al. 2009) and *P. l. larvae*
spores (Evans 2004).

Characterising heritable components of the immune abilities
of bees will help define costs, trade-offs, and mechanistic
vulnerabilities of disease survival in this species and should
have a general impact on the evolution of immune resistance
(Decanini et al. 2007). The queen’s health, as well as the colony’s
ability to prevent the spreading of certain diseases, is becoming
increasingly important in breeding schemes, demonstrating
the queen’s inheriting ability (Hatjina et al. 2014). From the
previous studies, it was found that challenged queen larvae of
the honey-bee, *A. mellifera* with a sub-lethal dose of *P. l. larvae*
get increasingly a more efficient immunity and resistance to
current infection in individual adult queens. This immunity
was spoken to by various peptides or polypeptides with a
higher rate of antibacterial activity against specific infection
as well as spectrum activity against various other bacterial
species. Expression levels of genes responsible for inducing
this immunity can be utilised as candidate markers at selecting
tolerant honey-bee queen lines in response to infections.

**ORCID IDs**
El-Gohary E El-Gohary – https://orcid.org/0000-0001-9607-3092

**REFERENCES**
Aerts AM, Francois IE, Cmamu BP, Thevissen K. 2008. The mode of
antifungal action of plant, insect and human defensins. Cellular and
Molecular Life Sciences 65(13): 2069–2079. https://doi.org/10.1007/
s00018-008-8035-0

Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J,
Pinsky MR. 2001. Epidemiology of severe sepsis in the United
States: analysis of incidence, outcome, and associated costs
care. Critical Care Medicine 29(7): 1303–1310. https://doi.
go/10.1097/00003246-200107000-00002

Antúnez K, Martín-Hernández R, Prieto L, Meana A, Zunino P,
Higes M. 2009. Immune suppression in the honeybee (*Apis*
mellifera*) following infection by *Nosema ceranae* (Microsporidia).
Environmental Microbiology Journal 11(9): 2284–2290. https://
doi.org/10.1111/j.1462-2920.2009.01953.x

Auvynet C, Rosenstein Y. 2009. Multifunctional host defence peptides:
antimicrobial peptides, the small yet big players in innate and
adaptive immunity. The FEBS Journal. 276(22): 6497–6508. https://
doi.org/10.1111/j.1742-4658.2009.07360.x

Bradford MM. 1976. A rapid and sensitive method for the quantitation
of microgram quantities of protein utilizing the principle of protein-
dye binding. Analytical Biochemistry 72(1-2): 248–254. https://
doi.org/10.1016/0003-9983(76)90527-3

Cerda HP. 2003. Studies on resistance to Bacillus thuringiensis toxins in
insects in relation to resistance management strategies. Ph.D. thesis,
Biological Sciences, London University, London, U.K.

Choi YS, Choo YM, Lee KS, Yoon HJ, Kim I, Je YH, Sohn HD, Jin
BR. 2008. Cloning and expression profiling of four antibacterial
peptide genes from the bumblebee *Bombus ignitus*. Comparative
Biochemistry and Physiology B Biochemistry and Molecular
Biology 150(2):141–146. https://doi.org/10.1016/j.cbpb.2008.02.007

Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Petts JS, van
Engelshorp D, Evans JD. 2012. Pathogen webs in collapsing honey
bee colonies. PLoS One 7(6): e43562. https://doi.org/10.1371/journal.
pone.0043562

Compton SJ, Jones CG. 1985. Mechanism of dye response and
interference in the Bradford protein assay. Analytical Biochemistry
151(2):369–374. https://doi.org/10.1016/0003-9983(86)90190-3

Craileheim K, Riessberger-Gallé U. 2001. Honey bee age-dependent
resistance against American foulbrood. Apidologie 32: 91–103.
https://doi.org/10.1051/apido:2001114

Cremers S, Armitage SAO, Schmid-Hempel P. 2007. Social immunity.
Current Biology 17(16): R693–R702. https://doi.org/10.1016/j.
cub.2007.06.008

Da Silva FP, Machado MC. 2012. Antimicrobial peptides: clinical
relevance and therapeutic implications. Peptides 36(2): 308–314.
https://doi.org/10.1016/j.peptides.2012.05.014
