

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

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This study evaluated the expression of antimicrobial abaecin and defensin genes in virgin honey-bee queens (*Apis mellifera*) following induction of 4th larval instar with the bacterium *Paenibacillus larvae larvae* (1.07×10^2 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 abaecin 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 (Crailsheim & 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 (Haseman 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, abaecin, hymenoptaecin, and defensin (Daníhlík 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.

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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 Apiculture Research, Cairo, Egypt. Activation and cultivation of bacterial pathogens were performed according to the method mentioned by Hansen & Brødsgaard (1999).

An induction dose of bacterium *P. l. larvae*

The sub-lethal dose of 1.07×10^2 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.

$$\text{Protein concentration} = \text{Absorbency} - 0.6241/0.085 \text{ (mg/ml)}$$

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 vertical polyacrylamide gels. Electrophoresis conditions and procedures were as described by Laemmli (1970). Extraction

of proteins in queen samples was performed according to the protocol of Ekramoddoullah & 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-mercaptoethanol 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.

Molecular quantitative test for target genes

Extraction of total RNA of the antibacterial target genes abaecin (Ab) and defensin (Df) for control, and bacteria-treated honey-bee queens were extracted using the DNA/RNA Extraction/Purification Kit (BioFlux, Bioer Technology Co.). First-strand cDNA's 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 Taq™ 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 in a final concentration of 400 nM, 1 µl of total RNA, and 7.4 µl RNase-free water: in a final volume of 20 µl. 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

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

Primer	Sequence	Target gene	Reference
β-actina-F	5'ATGCCAACACTGTCCTTCTGG-3'	β-Actina (reference gene)	Yang and Cox-Foster (2005)
β-actina-R	5'GACCCACCAATCCATACGGA--3'		
Abaecin-F	5'-CAGCATTTCGCATACGTACCA-3'	Antibacterial peptide abaecin	Evans (2006)
Abaecin-R	5'-GACCAGGAAACGTTGGAAAC-3'		
Defensin-F	5'-TGGCGTGCTAACTGTCTCAG-3'	Antibacterial peptide Defensin	Evans (2004)
Defensin-R	5'AATGGCACTTAACCGAAACG-3'		

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 $\Delta\Delta Ct$ (cycle threshold, Ct) method, the Ct of each sample was compared with that of the control group according to the ' $\Delta\Delta Ct$ ' 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 the (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. I. larvae*

Emerging queens of honey-bees were induced by LD20 (1.07×10^2 CFU/queen) of *P. I. 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. I. larvae* on the total body protein contents

The concentration of the total body protein contents of bacterial-fed queens showed a highly significant increase ($P \leq 0.01$) 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 24.49 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 1.07×10^2 CFU/queen. Levels of expression were normalised with the β -actin mRNA, an internal control (housekeeping gene). The $2^{-\Delta\Delta Ct}$ 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. I. larvae*, defensin gene expression was highly significant upregulation ($P < 0.0027$)

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

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

N = 3 replicates per test; *significant to healthy ($P \leq 0.01$)

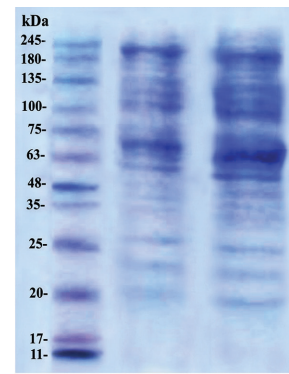


Figure 1. 15% SDS-PAGE electrophoresis of the total body protein patterns. Lane 1; control and Lane 2; treated queens of *A. mellifera* with bacterium *P. I. larva*.

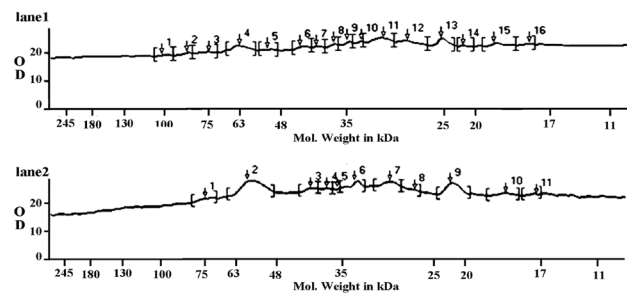


Figure 2. Densitometric scanning of 15% SDS-PAGE electrophoresis of the total body protein patterns of control and treated queens of *A. mellifera* with bacterium *P. I. larva*. Lane 1: control queens and Lane 2: treated queens.

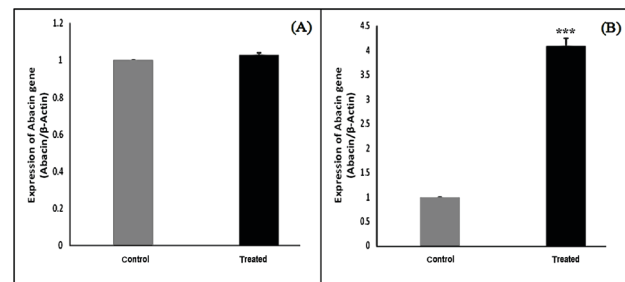


Figure 3. Effect of bacterium *P. I. larva* on queens of *A. mellifera* mRNA expression of (A) abaecin and (B) defensin genes. Values were presented as fold changes relative to control and treated queen after normalisation with the expression of a house-keeping gene (β -Actin). *** ($P < 0.001$) statistically significant compared with the control.

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. I. larva* on honey-bee queens was found ($P \geq 0.1492$) 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-bees (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. I. larva* (Evans & Lopez 2004). Because it is the most resistant larval instar, the queen 4th larval instar was chosen (Gomaa 2009). The resistance

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 (Gomaa 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 hymenoptera 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 (Cerdeja 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, Antunez 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* (Antunez 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.

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