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Morphometric analysis of some external and internal body characteristics of honey bee *Apis mellifera* queens treated with *Paenibacillus larvae larvae*

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ABSTRACT

The present investigation has been conducted to characterize morphological changes in virgin honey bee *Apis mellifera* queens challenged, in 4th larval instar, with a sub-lethal dose of *Paenibacillus larvae* larvae, causative agent of American foulbrood (AFB) the most threatening bacterial disease of honey bee brood. It provides direct tests to assess the quality of treated queens in terms of their external physical characters of the body (body weight, body length, abdomen length, thorax width, fore wing length and head width) and internal body characteristics (ovary size and number of ovarioles per ovary) in comparison with normal ones.The estimated LD_{20} was 1.07 x10^2 CFU/queen. This dose was enough to immunize queens and did not cause high mortality rate, so it was used in the subsequent tests during this study.

The results indicated that the bacteria-treated queens didn't undergo any significant changes in their external body characteristics than normal ones. This reflects the difficulty of discrimination between normal and disease-tolerant queens in the field. Additionally, the number of ovarioles per ovary didn't show any significant changes between normal and treated queens. In contrast, ovaries of treated queens showed an increase of their sizes as compared with normal queens. These results indicate that the bacterial treatment has no impact on the development of queen`s ovary.

Key words: Honey bee queen, *Apis mellifera*, American foulbroad, External morphology, Ovary and overiols.

INTRODUCTION

The honey bee (*Apis mellifera*) is one of the most important livestock that it plays a vital role in agriculture as a pollinator of many fruits, crops and wild flowers (Morse and Calderone, 2000). Its colony is a complex family group consists of one mother queen, several fathers (drones) present as sperms in queen spermatheca and offspring (workers and drones) of the mother and fathers. These social insects are frequent targets for pathogens and have consequently evolved diverse ways to minimize disease impacts (Decanini *et al*., 2007). The American foulbrood (AFB) is considered as one of the most threatening bacterial disease, it is a fatal and globally spread disease of honey bee brood (Crailsheim and Riessberger-Galle, 2001) and is caused by the spore forming gram positive bacterium, *Paenibacillus* larvae spp. larvae (*P. l. larvae*). The importance of this pathogen comes from its widespread resistance to traditional antibiotics (Evans, 2004). A primary goal of honey bee research remains to breed bees that resist or tolerate pests and pathogens (Evan and Lopez, 2004). In such crowded environment, the

mother queen, upon immunological encounter with a pathogen, could influence the immunity of direct progeny, thus increasing resistance to current infection in the colony (Dicanini *et al.*, 2007). It is very important to employ resistance management strategies to allow bee brood to develop resistance against bacteria.

The present study aims to characterize morphological changes, at external and internal levels, in virgin honey bee queens previously challenged, in 4th larval instar with *P. l. larvae* to explore whether the presence of bacteria affects the immune status of the insect. This, in turn, can help to breed immune honey bee queens that are resistant to this disease which could influence the immunity of direct progeny, where the queen as a single individual could positively influence the immunological status of the whole colony.

MATERIALS AND METHODS

Insects*:*

Two colonies of healthy Craniolian hybrid honey bees; *Apis mellifera carnica* were used in this study and were kept in a private apiary yard under normal living conditions in Abo-Yassin, EL-Sharquia Governorate, Egypt. Tested queens (one mother) were obtained by using the grafting technique (Doollitle, 1889). The routine work for keeping and developing the colonies was carried out during the experimental period.

Bacteria:

Bacteria used in this study, has been isolated from ropy remains of honey bee larvae collected from Agriculture Research Center; Plant Protection Institute; Department of Apiculture Research.The spore suspension was prepared by suspense a pure colony of *P. l. larvae* in distilled water. The concentration of the spore stock suspension was determined by preparation of bacterial serial dilutions (1 ml of the well mixed spore stock suspension was pipetted into the first test tube containing 9 ml sterile distilled water and labeled 10^{-1}, the contents were mixed and 1 ml was pipetted into the second tube containing 9 ml sterile distilled water and labeled 10^{-2}; etc.). Ten plates for each dilution were inoculated using pour plate technique and incubated at 35 ºC in anaerobic conditions for 24 h, where all viable cells form colonies and each colony counted is formed from one bacterial cell. Calculations of total numbers of viable bacteria from these counts were expressed as colony formed unit/milliliter (CFU/ml). The spore suspensions were stored at 4ºC. The concentration of the spore stock suspension was calculated by the following formula:

Con. of the spore stock suspension = Average of viable bacterial counts x dilution factor

Susceptibility of honey bee queen larvae to bacteria:

To determine the susceptibility level and the sub-lethal dose used to immunize honey bee queens, groups of queen 4th larval instar (each containing 10 individuals) were treated with three different bacterial doses: $1.323x10^3$, $1.323x10^4$ and $1.323x10^5$ CFU/queen larvae. Inoculation was made by adding 10 µl of bacterial suspension to the food of bee queen larvae according to the method of Decanini *et al.* (2007).Two groups of controls were used, "+ve control" (queen larvae treated with 10 µl of autoclaved distilled water) and "–ve control"

(untreated insects). Final mortality percentages were scored after queen emergence, and the LD-p line was plotted according to Finney (1972).

A stock suspension of a sub-lethal dose of *P. l. larvae* that produces 20% mortality (LD_{20}) in queens was prepared. Subsequently, 10 µl of this dose was mixed with the food of the tested queen's larvae for investigating the influence of sub-lethal dose of pathogenic infection on the various parameters studied.

Body weight and external morphometric measurements of honey bee queens:

The newly emerged queens were anesthetized in -20 ºC for determining fresh body weight (mg) using electronic balance (AINSWORTH, type Bo41685, U.S.A.) as well as external morphometric measures, lengths (cm) of total body, the right forewing and the abdomen. The widths of the thorax and the head were also measured by using a Vernier caliper to nearest 0.01 mm. Measurements were carried on the right forewing, and the abdomen length at a relaxed position in width of the third segment (Metorima *et al.,* 2015).The measurements were replicated 10 times for controls and treated queens.

Internal morphometric measurements of honey bee queens:

Tested queens were placed alive at -20 ˚C without alcohol, that they can be dissected even months afterwards, wings and legs were removed. Queens were euthanized by decapitation, pinned onto a dissection plate; the abdomen of each queen was then dissected through dorsal midline while being viewed through a stereomicroscope (zoom magnification adjusted as needed). After removal of the spermatheca, the right and left ovaries were clearly appeared.

The numbers of ovarioles of right ovary were counted, as there was no significant difference between numbers in left or right ovaries (Rhodes, 2011), by spreading the ovary in a drop of saline solution (0.9%). The number of ovarioles was then counted by teasing the ovarioles apart and moving each one to the side of the watch glass as it was counted (Anderson, 1999). The freshly dissected ovary viewed and ovarioles counted directly under a stereoscopic (self-illuminated binocular, Carl Zeiss Micro-imaging, Germany) according to Jackson *et al*. (2011).

Statistical analysis:

The correct results of susceptibility tests were represented graphically as probit log-regression line (Bliss, 1935) and analyzed statistically by using software: SPSS 17.0, windows 10. Data of the rest tests were expressed as mean ± standard error (SE). Levels of significance for differences of means were determined using Student's *t*- test for paired samples. The level of significance for each experiment was set at $P \leq 0.05$ and $P \leq 0.01$.

RESULTS

Breeding of honey bee queens*:*

All bred queens were obtained from one mother queen from a certain honey bee colony to avoid the genetic differences between queens studied.The total developmental period of bred honey bee queens was estimated as nearly as 16 days

(three days for egg development, five days for larval duration and eight days for pupal life before emergence of adult queen).

Susceptibility of honey bee queens to *P. l. larvae*:

Data obtained from the susceptibility tests of *A. mellifera* queens by feeding 10 µl of *P. l. larvae* to the queen 4thlarval instar was illustrated graphically in Figure (1). The LD_{50} and LD_{20} values were 4.39 x10^3 and1.07 x10^2 CFU/queen, respectively. The LD_{20} was estimated as sub-lethal dose to investigate the subsequent tests.This dose was found to induce the immune response of queen larvae.

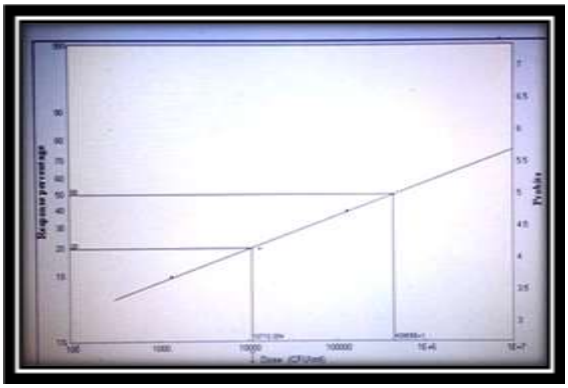

Fig. 1: Susceptibility of *A. mellifera* queens to *P. l. larvae* by feeding through 4thlarval instar.

Effects of *P. l. larvae* on the wet body weight of honey bee queen:

The mean total body weight of the un-treated queens (-ve control) was 152±7.9 mg, the water treated queens (+ve control) was 147±10 mg, and the bacterial treated queens was145±4.5 mg. The fresh body weight value of treated queens showed insignificant changes (P> 0.05) compared to control queens (Fig. 2).

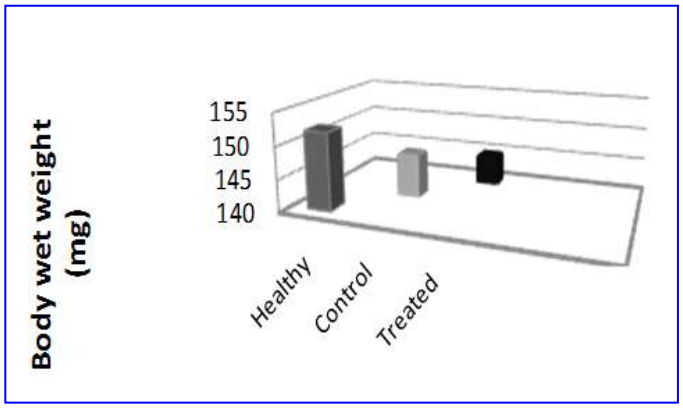

Fig. 2: Body weight (mg) of normal, control and bacterial treated honey bee queens.

Morphometric analysis of some external and internal body characteristics of honey bee *Apis mellifera* queens treated with *Paenibacillus larvae larvae*

Effects of *P. l. larvae* on the external body characteristics of honey bee queen:

The body length, abdomen length, thorax width, forewing length and the head width of *A. mellifera* queens were estimated after emergence of queens treated in the 4th larval instar, with a sub-lethal dose of *P. l. larvae* (1.07X102 CFU/queen). The same parameters were also estimated for untreated and water-treated control insects. The different morphometric values of treated queens showed insignificant differences (P > 0.05) as compared to controls (Fig. 3).

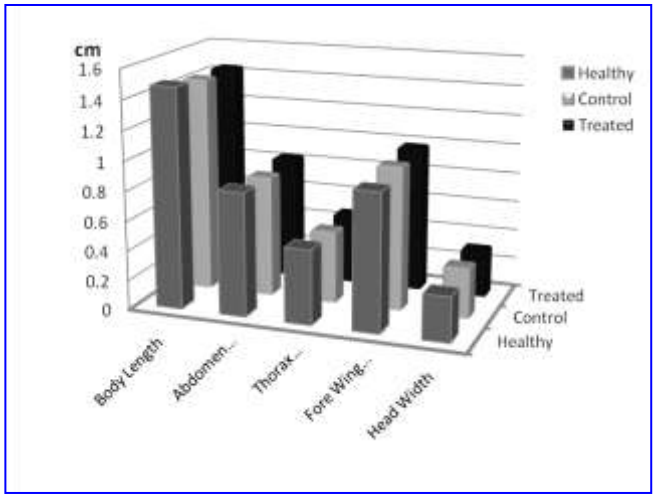

Fig. (3): Variations of the external physical characteristics (body length, abdomen length, thorax width, forewing length and head width (cm) of untreated, water-treated (controls) and bacterial treated honey bee queens

Effects of *P. l.* larvae on the external body characteristics of honey bee queen:

The ovary size (length and width) and the number of ovarioles per ovary of the untreated, water-treated and bacteria-treated *A. mellifera* queens were measured after dissection of virgin queen (Fig. 4). The ovary length of bacteria-treated queens showed a significant increase ($P \leq 0.05$) as compared to controls. But its width showed insignificant change (Fig. 5). The numbers of ovarioles of the un-treated, water-treated and bacteria-treated queens were 116±6, 116.7±1.7 and 133.3±8.8 ovarioles/ovary, respectively. No significant changes (P > 0.05) were detected in treated queens as compared to control queens (Fig. 6).

Fig. (4): Stereomicroscopic photograph showing left and right ovaries of honey bee queen dissected via dorsal abdominal midline (magnification 12x). Scale bar 100 µm.

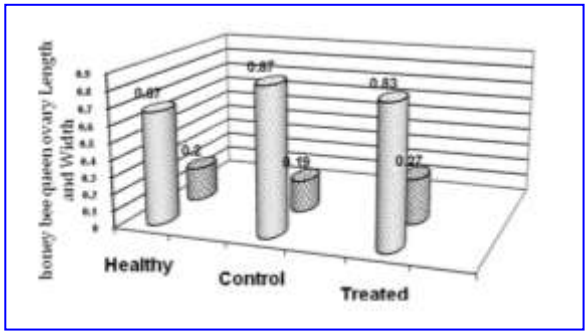

Fig. 5: Length and width (cm) of *A. mellifera*virgin queen`s right ovary determined in untreated, water-treated control queens and treated queens with *P. l. larvae.*

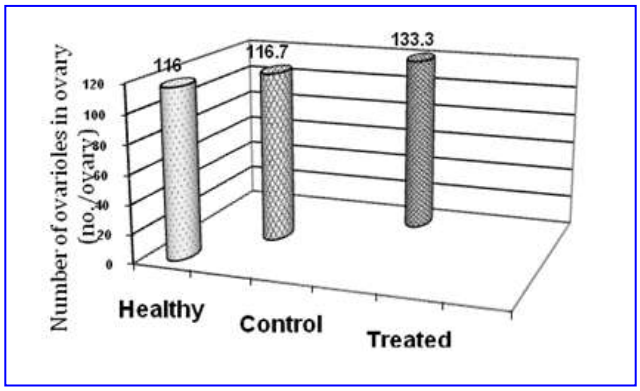

Fig. (6): The total number of ovarioles in ovary (ovariole/ovary) of A. mellifera virgin queens determined in untreated, water-treated control queens and treated queens with P. l. larvae.

DISCUSSION

In the present study, high resistance level of honey bee queens towards inoculated *P. l. larvae* was determined. This was indicated by the relatively high LD_{50} (4.39×10^3 CFU/queen). The queen 4th larval

instar had been selected because it is the most larval resistant instar (Gomaa, 2009). Where infection took place orally by mixing10 µl of *P. l. larvae* stock 100 CFU/µl (Brodsgaard *et al*., 1998; Evans, 2004; Evans and Lopez, 2004; Decanini*et al*., 2007). The LD_{20} is approximately 100 CFU/ queen with larval natural diet.

The performance of a honey bee colony is the result of its queen's function as well as of that of the drones that mated with her. These two approaches are often considered together and give a general view of the queen production technique and selection. (Hatjina *et al*., 2014).

Most of the research works on the quality of the queens refers to physical characters, for example: weight of the queen body, ovaries weight, ovarioles number and spermatheca diameter (Hatijina *et al*., 2014). Some authors have given information for queen reproductive quality, such as standard morphological measures of thorax width, head width, and wing lengths (Dedej *et al*., 1998; Hatch *et al*., 1999; Gilley *et al*., 2003 ; Dodoglu*et al*., 2004); as well as some physiological and reproductive determinations such as vitellogenin amounts and effective paternity frequency (Delaney *et al*., 2011).

Morphological characteristics of honey bees may also affect foraging efficiency; for example, proboscis and forewing lengths play important roles on nectar collection and flight distance, respectively (Gomeh *et al*., 2016). Wing characters were found to be affected by different factors e.g., temperature (Tan *et al*., 2005), season (Mattu and Verma, 1984) and bee age (Herbert *et al*., 1988). In the present study, the treated queens didn't undergo any morphological changes in their body weight, body length, abdomen length, thorax width, fore wing length and head width than normal ones, so the treated queens can complete normal queens in mating flights and laying eggs in the field.

Queens can lay 1500- 2000 eggs per day throughout their lives (Merrill, 1924 ; Nolan, 1925); where queens became sexually mature 6 days after emergence and they mate with 17 drones, on average, in mating flights and store sperms to fertilize eggs in their life span (Woyke, 1960), then they engage in egg- laying activities (Wintson, 1987).

Hymenopteran female ovaries are divided into elongated tubular ovarioles (Martins and Serrão, 2004).The range of varioles number of honey bee queens is wide, from 100 to 180 ovarioles per ovary (Snodgrass, 1956; Jackson *et al*., 2011). Results of the present study, concerning both normal and treated honey bee queens, are in accordance with these findings. Although the total number of ovarioles slightly differs, all tested queens had ovariole counts within the expected range. These results are also in accordance with Jaglarz (1998), Hassona and Mourad (2016), who stated that the number of ovarioles/ovary is variable and shows interspecific differences morphologically and physiologically. On the other hand, other investigators (Avetisyan, 1961; Woyke, 1971; Szabo, 1973; Wen-Cheng and Chong-Yuan, 1985; Gilley *et al*., 2003) stated that the number of ovarioles is

unchanged throughout queen's life which related to its origin and breeding conditions.

Generally, results of the present investigation showed no correlation between ovariole number and any other morphological characters such as thoracic width, wing length, body length and wet weight. These findings are in accordance with Eckert (1934), Hatch *et al*. (1999) and Jackson *et al*. (2011). The more ovarioles, the more eggs the queen can potentially lay (Jackson *et al*., 2011). Also, David (1970) documented a positive relationship between ovariole number and egg production.

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تحليل مورفومترى لبعض خصائص الجسم الخارجية والداخلية لملكات نحل العسل،*إيبس ميليفيرا* المعالجة ببكتيريا بينى باسيلاس لارفى لارفى

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المستخلص

تم إجراء هذا البحث لتوصيف التغيرات المورفولوجية التى تنشأ في ملكات نحل العسل، *إيبس ميليفيرا* التى سبق معالجتها، فى العمر اليرقى الرابع، بجرعة غير مميتة من بكتيريا بينى باسيلاس لارفى لارفى، العامل المسبب لمرض تعفن الحضنة الأمريكى، الذى يعد من أكثر الأمراض تهديداً لليرقات (الحضنة). وقد تم فى هذا البحث إحراء إختبارات مباشرة لتقييم نوعية الملكات المعالجة ومقارنتها بالملكات غير المعالجة، من حيث الخصائص الخارجية للجسم مثل وزن الجسم وطوله، وطول البطن، وعرض الصدر، وأيضاً طول الجناح الأمامي وعرض الرأس. وكذلك خصائص الجسم الداخلية مثل حجم المبايض وعدد الأنابيب المبيضية لكل مبيض. ومن خلال إختبارات الحساسية تم تحديد الجرعة غير المميتة (LD_{20}). هذه الجرعة كافية لتحصين الملكات ولم تتسبب في إرتفاع معدل الوفيات (حيث تسبب نسبة إماتة 20% فقط من الحشرات المعالجة)، لذلك يمكن إستخدامها في الإختبارات التالية خلال هذه الدراسة. وأوضحت النتائج أن الملكات المعالجة بالبكتيريا لم تخضع لأي تغيرات ملحوظة في خصائص الجسم الخارجية مقارنة بالملكات غير المحقونة. وتعكس هذه النتيجة صعوبة التمييز بين الملكات الطبيعية والملكات المحصنة ضد الإصابة المرضية في هذا المجال. بالإضافة إلى ذلك أوضحت النتائج أيضاً عدم حدوث أي تغييرات ملحوظة فى أعداد الأنابيب المبيضية لكل مبيض بين الملكات غير المعالجة والملكات المعالجة. في المقابل، أظهرت مبايض الملكات المعالجة زيادة ملحوظة في أحجامها مقارنة مع الملكات العادية (غير المحقونة). هذه النتائج تشير إلى أن المعالجة البكتيرية ليس لها أي تأثير على تطور ونمو المبايض فى الملكات المعالجة.