Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Period and Time of Harvest Affects the Apitoxin Production in *Apis mellifera* Lineu (Hymenoptera: Apidae) Bees and Expression of Defensin Stress Related Gene

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Article History

Edited by
Celso F Martins, UFPB, Brazil
Received 29 July 2014
Initial acceptance 06 October 2014
Final acceptance 28 October 2014

Keywords
honeybees, collection, venom, welfare

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Abstract
The aims of this study were to determine the period effects (morning and afternoon) and harvest time (30 and 60 minutes) in the apitoxin production as well as the management effects in the expression of a stress related gene. Five *Apis mellifera* L. beehives were used. The harvest of apitoxin and honeybees occurred three times a week (morning and afternoon at 09h00 and 14h00), according to the following treatments (period and apitoxin harvest time): T1: morning/30 minutes, T2: morning/60 minutes; T3: afternoon/30 minutes; T4: afternoon/60 minutes. The apitoxin was collected by electric collectors. The stress level was monitored by defensin gene expression, using actin gene as control. The results were evaluated by ANOVA followed by Tukey-Kramer test ($p \leq 0.05$). It can be concluded that the better period and time to apitoxin harvest is in the morning for 60 minutes, associated to minor stress for honeybees.

Introduction

Apitoxin comes from a Latin word and means: *Apis* - bee and *toxikon* – venom, being produced by bees as a way of defending and protecting the hive. The *Apis mellifera* apitoxin composition varies according to the subspecies development stage and habits. The primary findings are variations in the concentrations of apitoxin proteins throughout the seasons of the year, showing an influence of the environment (Abreu, 2010).

The apitoxin injected into a sting contains about 50 mg of dry matter. The major proteins present are melittin; 50% of the dry weight of apitoxin (DWV), phospholipase A2 (DWV 12%), mast cell factor degranulation (3% DWV), hyaluronidase (3% DWV) and apamin (2% DWV). Moreover, biogenic amines are present, including histamine (1% DWV), dopamine (0.5% DWV) and norepinephrine (0.5% DWV) (Cruz-Landim et al., 2002). Many volatile acetates which presumably stimulate the defensive behavior of other bees are also found in apitoxin (Abreu et al., 2010).

The apitoxin has beneficial effects to the humans, apitherapy (the use of bee products for the benefit of humans) has been used for many health problems, such as, eczema, tropical ulcers, infections such as laryngitis and mastitis, rheumatologic problems, cardiovascular, pulmonary and orthopedic, and help in the inhibition of ovarian cancer, multiple sclerosis and have an antibiotic effect (Leite et al., 2005).

The apitoxin harvest can be performed by electrical collectors placed at the entrance of the hive containing filaments

conducting an electric current, thereby promoting accessory muscle contraction release of the apitoxina, without killing the bees (Leite & Rocha, 2005).

However, even when not promoting the death of bees, there is still no knowledge of the influence of management on the hive. It is assumed that it can promote behavioral changes, interfering with routine activities of the colony, causing an acute or chronic stress.

The main goal of this study was to investigate the effects of period (morning and afternoon) and harvest time (30 and 60 minutes) in apitoxin production by Africanized honeybees and the influence of this management on the defensin gene expression.

Material and methods

The experiment was conducted by the Center for Education, Science and Technology in Rational Beekeeping - NECTAR, the Apicultural Sector, from College of Veterinary Medicine and Animal Science, Experimental Farm Lageado, UNESP, Botucatu, Brazil; with the following coordinates: 22º 49'' S, 48°24'' W and 623 meters high.

In order to perform the experiment 10 Africanized beehives in wooden Langstroth hives model (five as control and five per treatment), externally oil painted light green, kept in 50cm racks numbered for easy identification were used. The selected colonies were standardized to the number of frames of brood and food. During the experimental period, each beehive received sugar syrup (50% water + 50% sugar) weekly through Bordman feeder.

To measure the defensive behavior of the colonies, we performed the defensiveness test, according to the methodology described by Stort (1975) and Brandenburg and Gonçalves (1990). The test consisted of a black suede ball swinging in front of the entrance, attached to a string, for a minute. The first sting time (FST) were recorded and the number of stings left in the black suede ball (SNB). The tests were repeated in triplicate. Values above 36 stings designate defensive colonies.

The bee and apitoxin harvesting to evaluate expression of genes related to stress occurred three times a week, from November 2011 to January 2012 (twelve weeks), and five beehives per treatment were assessed in the same day using the same treatment according to: T1: Harvesting apitoxin in the morning, lasting 30 minutes; T2: Harvest apitoxin in the morning, lasting 60 minutes; Treatment 3: Harvest apitoxin in the afternoon, lasting 30 minutes; Treatment 4: Harvesting apitoxin in the afternoon, lasting 60 minutes. Thus, each treatment was realized in nine replicates

The harvest took place in the morning, always starting at 09:00 and in the afternoon at 14:00, by electric collectors consisted of wire filaments with six volts of electric current, and a glass plate for the fall of the released apitoxin (Leite & Rocha, 2007). At the end of each harvest, the collectors and the glass plates were removed and sent to the Laboratory of Apiculture Sector, kept at room temperature and protected from light, until the evaporation of the volatile phase. After, the apitoxin was scraped with a spatula, mixed (pool of five beehives), weighed and stored at -20ºC.

Climatic variables (mean temperature, wind speed, rainfall and relative humidity) were provided by the Department of Environmental Sciences, College of Agricultural Sciences, UNESP, Botucatu.

The climatic data for the period of the study were as follows: minimum temperature of 17.2 ± 1.9 ºC, maximum temperature of 30.6 ± 2.2, precipitation of 5.0 ± 6.6 mm, relative humidity 60.13 ± 10.4%, solar radiation 477.1 ± 80.0 cal / cm², insolation 8.1 ± 3.4 hours decimal and wind speed 99.7 ± 39.8 km/day.

The data for the analysis of stress-related genes were processed at Laboratory for Genetics Research and Analysis, Parasitology Department, Biosciences Institute, São Paulo State University – UNESP, Botucatu Campus.

For the experiments of *defensin* expression, a stress related gene, the harvest of the after the placement of apitoxin collectors. Ten internal worker bees and ten foragers were used. After harvest, the bees were immediately stored at - 80°C for RNA extraction. Bees were collected also of five beehives control, without apitoxin collect, in the different treatments.

For RNA extraction, the head of each worker bee was separated from the body with the aid of a disposable scalpel (Scharlaken et al., 2008). Each sample consisted of a "pool" of five heads and RNA extraction was performed by using the TRIzol method, using for each sample 500 ul of TRIzol (GIBCO BRL) to disrupt the cells and release their contents. The extraction product was visualized on a 1% agarose gel and quantified using the NanoDrop instrument (ND-1000 Spectrophotometer). Then all samples were stored at -80°C until ready to use.

After, samples were treated with DNase, and cDNA synthesis reaction was set as follows: a mix of oligodT solution (N = 18) 0.75 mM; random oligonucleotides (N = 8) 0.15 mM; 0.75 mM dNTP and 11 µl of RNA treated with DNAse in the previous step , was prepared and incubated at 65°C for 5 minutes and then placed on ice for 1 minute. To this preparation, 0.5mM DTT, 40U of RNase out and 100U of Super Script III were added. The reaction was then incubated at 50°C for 1 hour and then at 70°C for 15 minutes.

The stress level of the bees was monitored by changes in *defensin* gene expression (Scharlaken et al., 2008). As internal control for quantitative PCR reactions, we used the *actin* gene (Scharlaken et al., 2008).

The determination of gene expression was performed by real time polymerase chain reaction in triplicate, on a Real Time ABI 7300 instrument (Applied Byosystems) using the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) under the following conditions: one cycle at 50°C for 2 minutes, a cycle at 94°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. The dissociation curve was obtained as follows: 95°C for 15 seconds, 60°C for 30 seconds and 95°C for 15 seconds.

The oligonucleotides sequences used and details are shown in the Table 1:

Table 1. Genes and oligonucleotide sequences useoto avaluate the defensin expression.

Gene	Gene Bank Number Accession	Oligonucleotides sequences 5'-3'	Amplification (pb)	Ta (°C)[a]	EE (%)[b]
Actina	AB023025	TGCCAACACT GTCCTTTCTG AGAATTGAC CCACCAATCCA	155	61	99,11
Defensina	U15955.1	GTCGGCCTTCT CTTCATGGT GACCTCCAGCT TTACCCAAA	200	61	91,70

[a]Ta optimal annealing temperature specific to each oligonucleotide

[b]E Measuring the efficiency of the reaction Real Time PCR (calculated trough the standard curve)

To calculate the efficiency for the oligonucleotides used, four dilutions of cDNA samples were made: 1:5, 1:25, 1:125 and 1:625. The efficiency (E) was calculated using the formula E = 10 (-1/slope). Relative quantification (R) was determined according to Pfaffl (2001).

Data were calculated using bees taken before placing electric collectors as controls in relation to the other five harvests after the electric collectors were removed.

The analysis of the data obtained was compared by ANOVA followed by Tukey test to check for differences between means. Results were considered statistically different when $p<0.05$ (Zar, 1996).

Results and Discussion

Do not observed difference in the first sting time between the beehives of treatments and control (1.3±0.7 to 1.4±0.8 seconds) and number of stings left in the black suede ball (50.3±17.2 to 51.8±14.4).

There was a higher apitoxin production in the treatment using 60 minutes in the morning (T2), which differed significantly from the other treatments (Table 2).

Table 2. Mean values and standard deviation for apitoxin production (milligrams) of Africanized honeybees according the day time and time of production.

Production Period (min)	Morning (minutes)	Afternoon (minutes)
30	33.7±13.0 aA	24.6±10.0 aA
60	49.1±13.0 bB	24.9±6.0 aA

Different small letters in the same column indicate statistical differences between averages ($p<0.05$).
Different capital letters in the same line indicate statistical differences between averages ($p<0.05$).

The relative quantification (R) results from Defensin gene, using Actin as the endogenous gene in the samples from the different treatments and control are shows in Table 3.

Table 3. Mean and standard deviation of defensin gene relative quantification of foragers workers (FW) and internal workers (IW), in different treatments with Afr canized honeybees.

Treatment	FW	FW - Control	IW	IW - Control
1	2.02±1.6aAα	0.007±0.0aBα	1.84±1.7abAα	0.005±0.0aBα
2	1.23±0.1aAα	0.003±0.0aBα	0.16±0.2aAα	0.06±0.01aAα
3	1.35±1.4aAα	0.002±0.0aBα	2.97±1.6bAα	0.07±0.0aBα
4	0.59±0.4aAα	0.025±0.0bBα	0.56±0.6abAα	0.003±0.0aBβ

Different small letters in the same column indicate statistical differences between averages ($p<0.05$).
Different capital letters in the same line, to group of foraging or internal bees (treatment vs control), indicate statistical differences between averages ($p<0.05$).
Different greek letters in the same line, to foraging vs internal, indicate statistical differences between averages ($p<0.05$)
Treatment 1: Apitoxin production in the morning, during 30 minutes; Treatment 2: Apitoxin production in the morning, during 60 minutes; Treatment 3: Apitoxin production in the afternoon, during 30 minutes , Treatment 4: Apitoxin production in the afternoon, during 60 minutes.

Relative quantification of the *defensin* gene in foragers bees showed no significant difference between treatments and treatments versus control. In control worker bees was observed that treatment using 60 minutes in the afternoon (T4) showed significant difference between others treatments.

Internal workers showed significant difference between treatments 2 and 3 throughout the experimental period. The control beehives do not observed differences. It was observed significant difference between internal works to treatment 4 and the control.

No difference was observed between the foragers and internal workers for all treatments and was observed difference between foragers and internal worker bees from T4 and control.

The colonies used in the experiment can be classified as defensive, regardless the period of the day, during apitoxin production, according to the Stort (1975) classification, which the bees are classified with high defensiveness when they sting 36 more times the black suede ball. We could observe that there was no influence of climatic variables on colonies defensiveness. Previous work, in the same place, found no significant correlations between time and number of first sting in the black suede ball with climatic variables (Lomele et al., 2010).

The higher apitoxin production occurred in the morning period during 60 minutes. A probable cause for this result would be the foraging behavior of bees. Malerbo and Souza (2011) observed in the end of spring and in the beginning of summer, that nectar were collected by the bees throughout the day, with preference for the hottest hours of the day, between 10 and 14h, when the temperature was between 15 and 30°C. Thus, a greater flow of bees results in greater amount of foragers through the collector and consequently a significant increase on the release of apitoxin through electrical stimulation.

However, the apitoxin harvest could promote alterations in all beehives, affecting the grooming, brood care and hygienic behavior, besides could affect the immunity sistem. As the honeybees could be exposed to field pathogens, *defensin* gene related to stressor stimulus like changes in immune system was quantified (Ursic-Bedoya & Löwenberger, 2007).

There was significant increase in the *defensin* gene expression in the internal worker bees in the treatment 2 (morning/60 minutes) compared to the treatment 3 (late/30 minutes). This higher *defensin* gene expression in the internal worker bees could be related to the increase of alarm pheromones released at the time of apitoxin release by the electrical stimulation (isopentilacetate and 2 heptanone) promoting alertness in other worker bees to protect the hive, increasing discomfort and possibly higher stress in the afternoon period than morning.

It was observed that the apitoxin harvest promote a highest defesin expression in treatments when compared as control, with exception for internal worker bees from treatment 2 that do not differ as control. Its result could be explained by the worker bees alarm pheromone adaptation in the collector. This is important because apitoxin harvest would not promote stress in the bees, associated with highest production, and can be used by beekeepers.

It can conclude that the better period and time to apitoxin harvest is in the morning for 60 minutes, associated the minor stress for honeybees.

Acknowledgments

We would like to thank (CAPES), for the master's degree scholarship and FAPESP by financial support (process number 2012/23466-2).

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