



Africanized honey bee (*Apis mellifera*) venom profiling: Seasonal variation of melittin and phospholipase A₂ levels

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ABSTRACT

Apis mellifera venom is comprised basically of melittin, phospholipase A₂, histamine, hyaluronidase, catecholamine and serotonin. Some of these components have been associated with allergic reactions, amongst several other symptoms. On the other hand, bee mass stinging, caused by Africanized honey bee (AHB), is increasingly becoming a serious public health issue in Brazil; therefore, the development of efficient serum-therapies has become necessary. In this work, we have analyzed the venom composition of AHB in Brazil through one year. In order to verify the homogeneity of this venom, one specific hive was selected and the correlation with climatic parameters was assessed. It was possible to perceive a seasonal variation on the venom contents of melittin and phospholipase A₂. Moreover, both compounds presented a synchronized variation of their levels, with an increased production in the same months. This variation does not correlate or synchronize with any climatic parameter. Data on the variation of the AHB venom composition is necessary to guide future intra and inter species studies.

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1. Introduction

In October 1957, 26 swarms of African honey bees – *Apis mellifera scutellata* previously *A. m. adansonii* – escaped from an apiary in Rio Claro, Brazil (22°24'48"S; 47°34'10"W). Due to their defensive behavior (among other successful characteristics of these strains), they have spread through the Americas at a rate of 250–300 Km per year, having reached Mexico in 1985, the Lower Rio Grande Valley (Texas, USA) in October 1990, and Phoenix (Arizona, USA) in October 1993 (33°26'54"N; 112°04'26"W) (França et al., 1994; Schumacher et al., 1995). This event made cross-breeding

between the already introduced European bees and the free African bees possible, leading to the appearance of the Africanized honey bee (AHB). These bees are more aggressive and able to release higher amounts of venom upon stinging, which, in an accident, may cause significant injuries to humans and domestic animals (Brizola-Bonacina et al., 2006).

Studies conducted by Schumacher et al. (1989) showed differences in the lethality of this venom in comparison with two populations of European honey bees (EHB). Nevertheless, Schumacher et al. (1990) also demonstrated that the lethality among mice treated with AHB venom may be similar to those treated with the EHB venom. Moreover, Schumacher et al. (1992) showed that AHB tends to carry slightly less venom in their venom reservoirs than the EB (98.2 ± 35.9 and 134.1 ± 53.5 µg, respectively). This corroborates the findings that the AHB possesses smaller

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venom glands and lower hyaluronidase content (Owen, 1983). Funari et al. (2001) verified that, upon stinging, the AHB is able to release more venom than the EB, despite the smaller capacity of its venom reservoir.

The main component, ~50–60% by dry weight, in AHB venom is melittin (Brochetto-Braga et al., 2006), which has also been shown to be the main lethal component in the venom (Schmidt, 1995). The active 26 amino acid peptide is released from its precursor, promelittin, during biosynthesis and has hemolytic activity, as well as being the primary allergen in bee venom (de Graaf et al., 2009). It is an amphiphilic peptide, with a predominantly hydrophobic N-terminal region and a hydrophilic C-terminal region, with the characteristic structure of membrane bound cytolytic, channel-forming peptides and trans-membrane protein helices (Glättli et al., 2006; Raghuraman and Chattopadhyay, 2006). It acts synergistically (Vogt et al., 1970; Ownby et al., 1997) with phospholipase A₂ (PLA₂), an enzyme constituting approximately 11% of the venom dry weight (Owen, 1979). It has been shown that the levels of different *A. mellifera* venom components, such as histamine (Owen and Braidwood, 1974), hyaluronidase (Owen, 1979), dopamine and noradrenaline (Owen and Bridges, 1982), 5-hydroxytryptamine (5-HT) (Owen and Soley, 1988) and PLA₂ (Owen et al., 1990) may change with the age of the individuals or the season of the year (Owen and Braidwood, 1974). Specifically, Owen and Pfaff (1995), showed that melittin content changes as the summer (Northern hemisphere) progresses.

Bee venom composition has been studied previously. However, the literature deals mainly with inter-race differences, quantity and quality (e.g., composition) of the venom, and influence of the method of collection on the venom composition. There has been no longterm (12 months) study comparing the venom composition within the same beehive and its possible correlations with climatic parameters. The present work focused on the biochemical characterization of the seasonal variation of the major components of the AHB venom, by analyzing the pooled venom composition of individuals pertaining to one specific hive for the course of one year. Here we report the seasonal variation of melittin and PLA₂ in the contents of the venom. The relevance of dealing with venom composition variation lies on the need of the development of an effective serum-therapy for the increasing bee mass-stinging accidents.

2. Material and methods

2.1. Reagents

All reagents were of analytical grade and were purchased from Sigma Co (St Louis, MO, USA).

2.2. Venom collection

AHB (*A. mellifera*) workers, age 30–40 days, were obtained from a specific hive at the Apiary of Botucatu School of Veterinary Medicine and Animal Husbandry (UNESP, State of São Paulo, Brazil) once a month, every 3rd week of the month, from October 2007 to September 2008.

2.2.1. Manual stimulation

Manual (or reservoir disrupting) venom extraction, was performed as follows: one hundred foraging bees were captured near the entrance of the colony and immobilized by quick freezing at –20 °C. After that, each individual was dissected and the sting apparatus and the venom reservoirs were removed and the venom was collected through reservoir disruption, i.e., venom reservoirs (or sacs) were carefully opened onto a glass slide, rinsed out in distilled water, centrifuged, lyophilized and stored at –20 °C.

2.2.2. Electrical stimulation

Venom was collected according to a slight modification of the Benton protocol (Benton et al., 1963). Briefly, a wired glass plate was placed in the entrance of the hive so that the homing bee had to land on the plate in order to gain access to the interior of the colony. The wires are electrified and apply mild shocks to the bees that respond by stinging the surface on which they are walking. The venom dries rapidly on the glass plate and can be scraped off and processed as described above. Approximately one hundred bees were allowed to land on the plate.

2.3. Chromatographic analyses

2.3.1. RP-HPLC profiling and purification

A reversed-phase binary HPLC system (20A Prominence, Shimadzu Co., Japan) was used for sample profiling and separation. The lyophilized crude venom powder was solubilized into 0.1% trifluoroacetic acid and the twelve-month collections were normalized to 1 mg mL^{–1} solutions, based on sample mass. These solutions were centrifuged and the supernatant was separated for subsequent chromatographic analyses. Twenty-microliter aliquots were loaded in an ACE C8 column (ACE 3 µm, C8, 300 Å, 100 × 2.1 mm) in a two-solvent system: (A) trifluoroacetic acid/H₂O (1:1000) and (B) trifluoroacetic acid/acetonitrile/H₂O (1:900:100). The column was eluted at a constant flow rate of 0.2 mL min^{–1} with a 10–100% gradient of solvent B over 31 min, after a 5 min isocratic elution with 10% B. The HPLC column eluates were monitored by a Shimadzu SPD-M20A PDA detector scanning from 200 to 500 nm (1 nm steps). Background subtraction was performed for chromatogram superimposition and integration.

2.4. Mass spectrometry

The mass spectrometry analyses were performed in an ESI mass spectrometer (LCQ Duo™, ThermoFinnigan, USA), equipped with a nanospray source and connected to nanoHPLC system (UltiMate HPLC System, LC Packings, Dionex, USA). The samples were introduced in the spectrometer by flow rate at 1 µL/min and diluted in a solution of 5% acetonitrile and 0.2% formic acid. The spray voltage was kept at 1.8 kV, the capillary voltage at 46 V, the capillary temperature at 180 °C and the tube lens offset was kept at –5 V. MS spectra were collected in centroid mode in the 50–2000 *m/z* range. Instrument control, data acquisition and data processing were performed with the Xcalibur Suite.

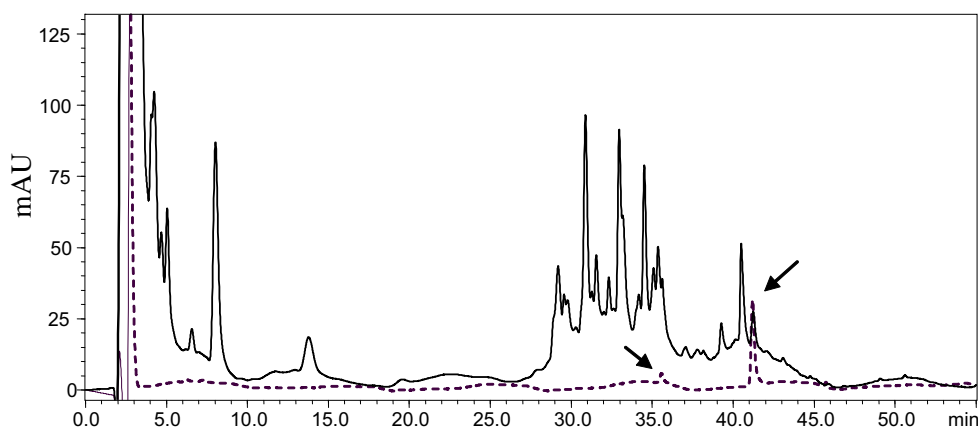


Fig. 1. Superimposed RP-HPLC profiles ($\lambda = 214$ nm) of *A. mellifera* venoms obtained by manual (continuous trace) and electrical (dashed traced) stimulations. The arrows at 35.8' and 40.4' denote the most abundant peaks detected in venom collected by the electrical stimulation and that were analyzed through this work.

Molecular mass analyses were performed on an Ettan MALDI-TOF/Pro system (Amersham Biosciences, Sweden), using α -cyano-4-hydroxycinnamic acid as matrix, under positive reflectron mode, using external calibrants.

2.5. Peptide sequencing

Complete peptide sequencing and/or N-terminal protein determinations were/was performed by Edman degradation using a Shimadzu PPSQ-21 automated protein sequencer, following the manufacturer's standard instructions. Amino acid analyses were performed to quantify the different peptide batches used through this work.

2.6. Climatic parameters

Data used in the present work were collected in Botucatu, SP, Brazil (22°51'S, 48°26'W, elevation 786 m) at the UNESP Agrometeorological station (Environmental Sciences Department; Agronomic Sciences Faculty) that continuously monitor the region. The chosen parameters were: air temperature in Celsius degrees (°C) (minimal, maximal and average), precipitation rate (in mm of rain), relative humidity of air (percent) and hours daylight (measured by a photocell).

2.7. Data fitting, statistical analyses and sequence alignment

When data fitting was performed, results were presented as the calculated value $\pm$ standard deviation (SD). Otherwise, data correspond to the mean of three individual experiments. Peptide sequence alignment was performed using ClustalW software (Larkin et al., 2007).

3. Results

Collection by means of electrical stimulation provided a much more consistent and clearer venom solution than manual collection. Significant differences in the shape, distribution, intensity and presence or absence of peaks are also outstanding when comparing the two venom collection methodologies (Fig. 1). Therefore, electric was the

methodology selected for the venom analyses comparisons performed through this work.

The monthly venom solutions were normalized to 1 mg mL⁻¹ (dry venom weight) and 20 μ L aliquots were analyzed by reversed-phase HPLC. Background subtracted chromatograms were individually integrated and analyzed at $\lambda = 214$ nm. This wavelength was selected because it represents the condition in which the most peaks were detected, as depicted in Fig. 2A, in a 3D-view of the PDA-scanning chromatogram.

The 12-month superimposed composite image is presented in Fig. 2, panel B. On average, one hundred different peaks could be detected on each sample. However, there are less than 10 major peaks that would serve for quantitative-based comparison; we have chosen the two major peaks clearly identified at 35.67' and 40.38'. The insert in Fig. 2 shows the same twelve chromatograms in a 3D-zoomed style in order to show the myriad of minor peaks present in the venom solution.

There is a clear major peak eluting at 40.38' (Fig. 2B), and a second most intense peak at 35.67' (Fig. 2B). These are consistent peaks through the year and, therefore, were selected for molecular characterization. Mass spectrometric analyses (Fig. 3) and Edman degradation were performed for these peaks and yielded two peptidic sequences, as presented on Table 1. Not surprisingly, the major peak (40.38') corresponds to melittin, the known main component of the bee venom, and the second major peak, to PLA₂.

Next, the peak areas were analyzed. Table 2 presents the monthly variation of the percent area of the 2 major peaks analyzed, a constant peak eluting at RT 32.1' and a random choice of a peak eluting at 15' to serve as controls for the data analyses. The bottom section of this table contains the climatic parameters measured at the hive location, namely: average monthly air temperature, total precipitation rate (mm), mean percent air humidity, and mean daily hours daylight.

As observed in Fig. 2B, there was an apparent significant variation in the areas of the bee venom major peaks, which is presented in detail in Table 2. In order to verify whether this variation was seasonal or related to any climatic

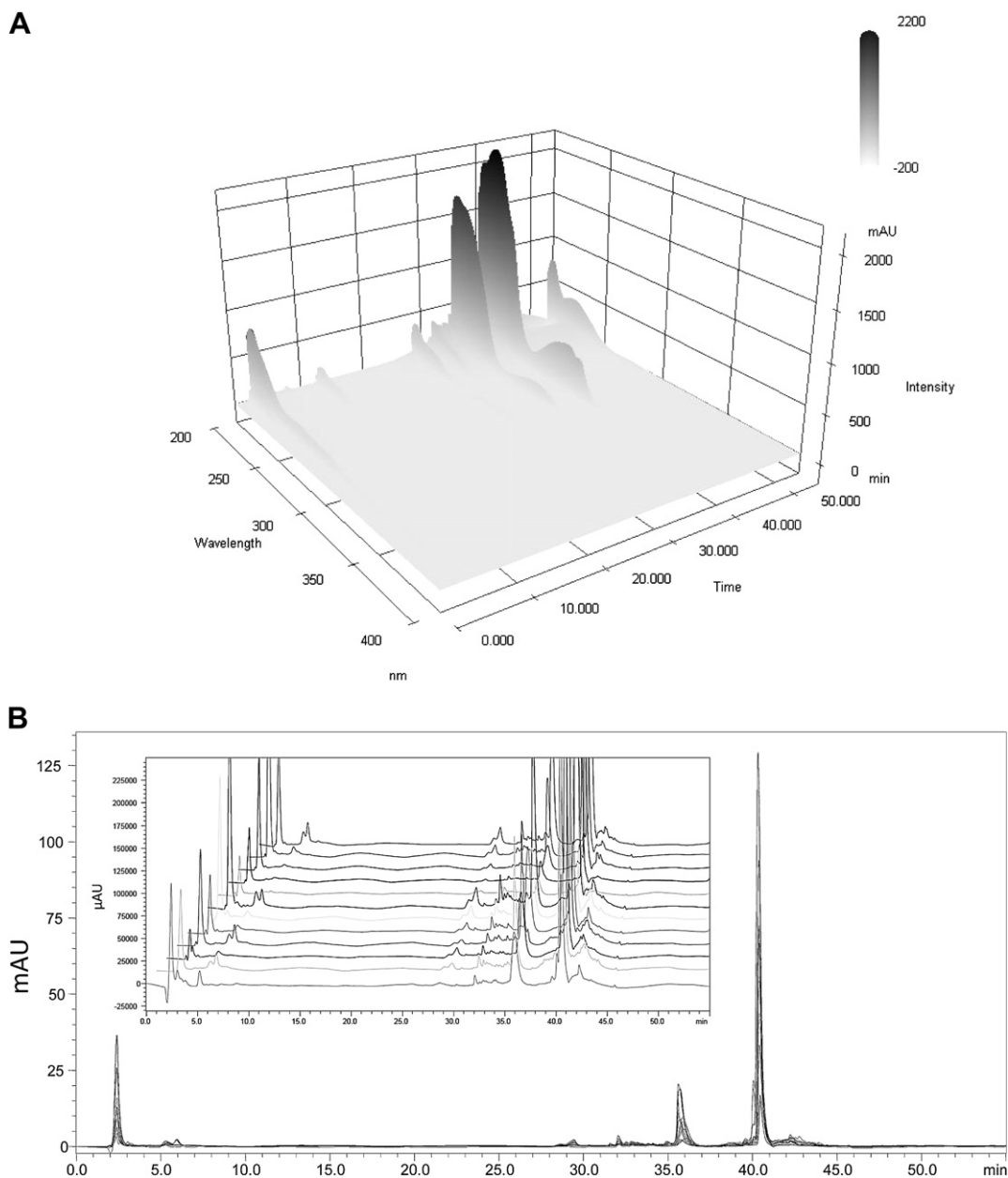


Fig. 2. (A) 3D RP-HPLC profile containing the wavelength scan of *A. mellifera* venom obtained by electrical stimulation. Note the intense absorbance at $\lambda = 214$ and, secondarily, at 280 nm. (B) Superimposition of the twelve monthly RP-HPLC profiles of Africanized honey bee venoms solutions obtained by electrical stimulation, at $\lambda = 214$ nm. Insert: 3D-style depiction of the same data, with greater magnification, starting from October/07 sample (bottom).

parameter, the monthly peak areas of melittin and PLA₂ were plotted as function of the month (Fig. 4, panel A). Due to the data distribution undulating pattern and the fact that half of the values were outside the 1σ range, a periodic-function non-linear regression was performed for these data using $f(x) = BL + A \cdot \sin(F \cdot x + \varphi_0)$, where BL represents the base line, i.e., horizontal displacement, A is the amplitude of the variation, F is the frequency ($t = 2 \cdot \pi / F$, t is the frequency correction for time) and φ_0 the phase shift. Melittin and PLA₂ content in the venom seems to follow a semestral synchronized variation, with frequencies of

5.89 ± 0.65 and 5.28 ± 0.57 months for melittin and PLA₂ respectively (Fig. 4, panel B).

Next, an analysis of the melittin production as a function of the climate was attempted. However, the only climatic parameters that adjusted to a periodic-function non-linear regression were the air temperature ($\square$) and percent relative air humidity (x), as presented in Fig. 5. It seems clear that melittin production ($\bullet$) does not correlate with the analyzed climatic parameters, for the calculated frequencies do not synchronize (8.72 ± 0.53 and 11.42 ± 3.43 , temperature and humidity monthly

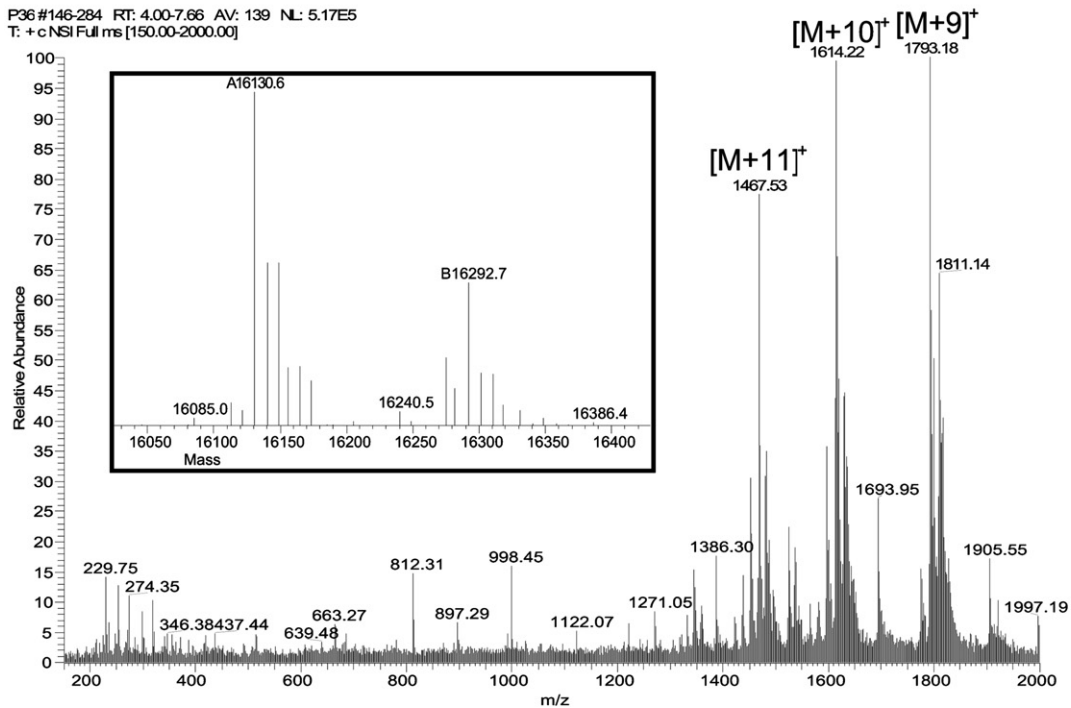
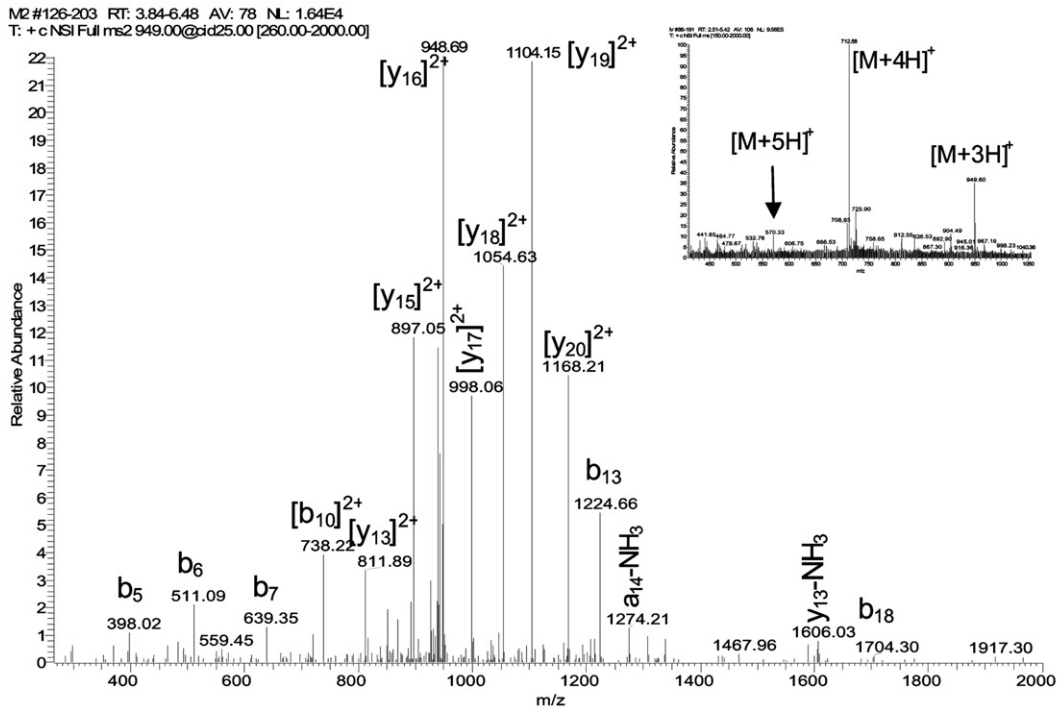


Fig. 3. Mass spectrometric profiles and deconvoluted data of the two compounds selected for investigation in this work. (A) MS^2 profile of the peptide eluting at 40.4' and daughter ion assignment as performed by SEQUEST. Insert: the MS profile of the purified peptide, indicating the ionic envelope. (B) MS profile of the protein eluting at 35.8'. Insert: deconvoluted data.

Table 1Peptidic sequences obtained from *A. mellifera* venom.

RT (min)	Determined sequence	Match (UniProt ref.)
40.38	GIGAVLKVLTTGLPALISWIKRKRQQ–NH ₂	Melittin (P01501)
35.67	IIPGTLWCGHGKSSGPNELGRFKHT...	Phospholipase A2 (P00630)

frequencies, respectively). It is noteworthy to mention that, although the humidity variation frequency could be a harmonic of the melittin production frequency, they are out of phase by almost 4 months ($\Delta\phi_0 = 3.80$ months). This would be the same for PLA₂ production, once its production is synchronized with melittin.

4. Discussion

The remarkable differences observed between the manual and the electrical stimulation venom collections are probably caused by damaged tissue, intestinal contents leakage and other venom duct contents (e.g., contamination). Probably, manual venom collection is subjected to larger intrinsic variations caused more by the collection conditions than the venom composition itself. On the other hand, the electrical stimulation is likely to generate larger amounts of a purer sample, without the need to sacrifice the bees in the collection process. As a consequence, the electrical stimulation venom collection was selected for this work. Fig. 1 contains the superimposition of the two collection methodologies. One should notice the much larger void peak present in the manually stimulated collection, which may represent intestinal contents and other undistinguishable contaminants. Moreover, for the manual stimulation procedure, both melittin and PLA₂ lose their 'major peaks' status (Fig. 1, arrows).

Fig. 2B presents a superimposition of a year of collected venoms. The main profile suits well to depict the high system stability and reproducibility as well as the sample homogeneity and representativeness. However, in terms of visual comparison it lacks clarity. In order to make it clear, a zoomed 3D-style superimposition is presented in the insert, in which one can see that the venom does contain other components than melittin and PLA₂, and that these components do vary as well. However, this study focused on the variation of the two major venom components and their possible correlation to climatic parameters. Further studies are necessary in order to identify more subtle variation present in the other components.

Despite the fact that the AHB typically possess less venom in the reservoir than the EHB (Carniolan and Italian) (Schumacher et al., 1992), they are able to release larger amounts of venom upon extraction (Funari et al., 2001). Moreover, it was observed that the electric produced venom that is cleaner and presents no debris. Fig. 1 points this out accordingly. Both profiles are scaled and one can observe that the manual stimulation not only contains a myriad of peaks eluting in the 25'–45' region, but also more hydrophilic peaks that were not observed in the electric (dashed line). Also noteworthy is the size of the unbound fraction in the manual stimulation. The void peak is approximately 10 times larger than the same peak for

Table 2
Percent chromatographic peaks areas of Africanized honey bee venom analyzed monthly and climatic parameters.

Peak	Monthly percent chromatographic area												Mean $\pm$ SD ^a
	2007						2008						
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	
PLA ₂	18.5 ^b	10.3	9.1	8.4	8.6	13.2	11.9	17.0	8.9	13.1	12.5	14.6	12.2 $\pm$ 3.2
Melittin	55.2	38.0	41.3	23.6	32.9	54.4	43.0	54.2	53.1	40.0	41.8	40.1	43.1 $\pm$ 9.3
CTRL ^c	1.2	0.8	1.0	0.9	1.6	1.3	1.4	1.3	1.0	1.1	1.1	1.0	1.1 $\pm$ 0.2
RND ^d	0.005	–	–	0.497	–	0.097	–	–	–	–	0.112	0.030	0.1 $\pm$ 0.2
Monthly average/totals climatic parameters													
°C ^e	23.9	22.0	22.8	22.5	23.5	23.0	21.6	18.3	17.6	19.1	20.3	20.3	– ⁱ
mm rain ^f	77.7	177.0	180.6	279.3	94.6	60.9	102.8	115.7	30.8	0	104.1	29.9	–
% hum ^g	57.5	67.3	65.4	72.5	68.3	61.1	68.9	54.7	60	40.5	53.3	63.4	–
Ins. hrs. ^h	7.38	6.31	7.81	3.92	5.83	7.34	6.06	7.02	6.6	9.07	7.52	7.78	–

^a Mean $\pm$ standard deviation.^b Gray boxes indicate values outside the 1 σ range.^c CTRL stands for the control peak, e.g., a peak that is constant in area and consistent during the period (RT = 32.10').^d RND stands for a random peak, e.g., an arbitrarily chosen peak monitored during the period (RT = 15.00').^e Temperature in Celsius.^f Total monthly rain, measured in mm.^g Average daily percent humidity of the air.^h Average hours daylight.ⁱ Not detected/not calculated.

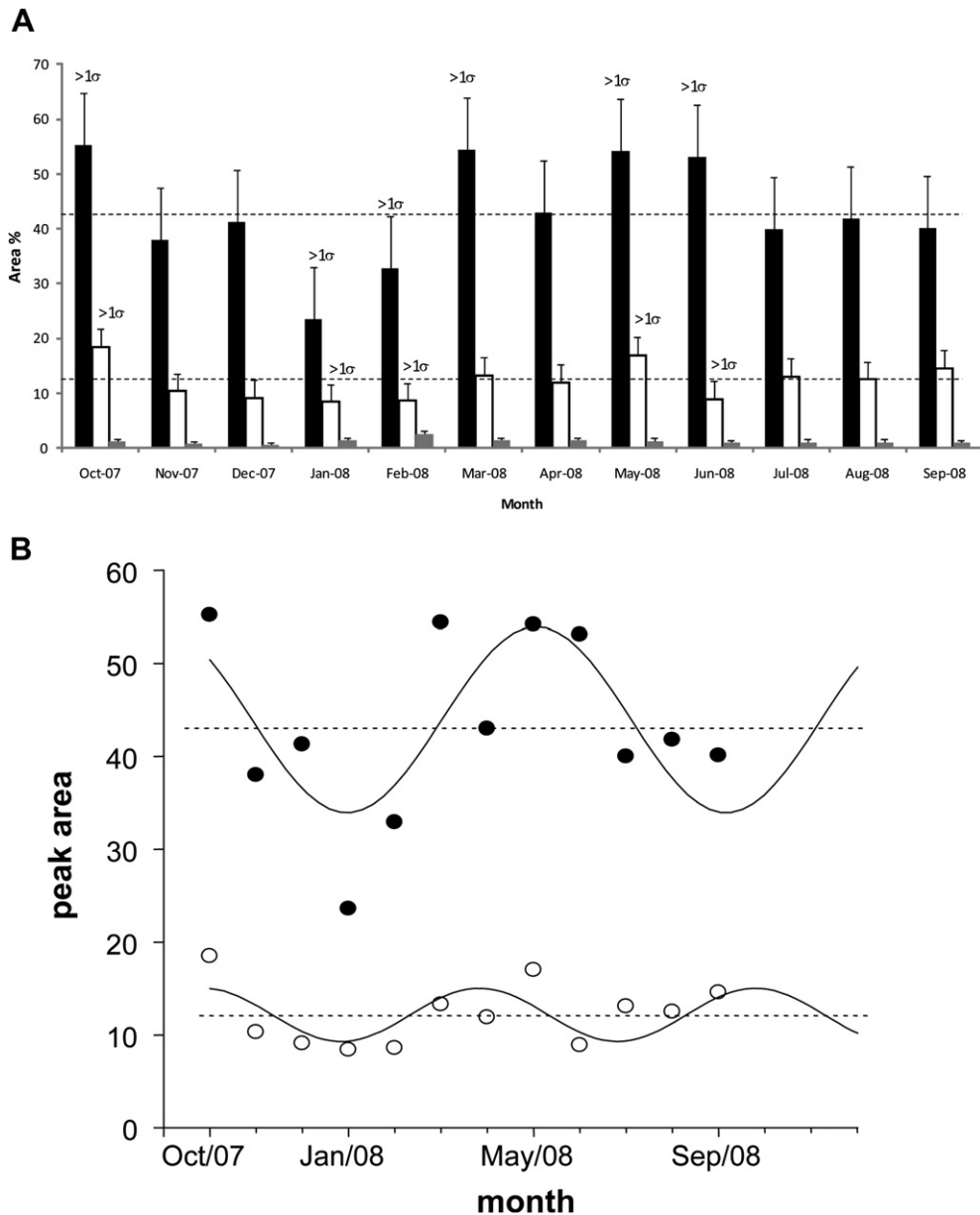


Fig. 4. (A) Normalized monthly percent peak area variation of melittin (black bars); phospholipase A₂ (white bars) and a control peak (32.1 min; gray bar). Variations larger than 1σ are indicated. (B) Data-fitted sinoidal variation of monthly production of melittin (●) and PLA₂ (○). Dashed lines represent the average value.

electric (data not shown, although it can be inferred from the profile). This material may correspond to feces and/or other damaged tissue fragments.

Venom components age (and seasonal, in a lesser degree) variation is not a novelty. In fact, for honey bees, this phenomenon was described almost right after the identification of the venom components themselves. However, in spite of those classical works by Neumann et al. (1953) and Habermann (1972) and more recently, by Owen et al. (1990) and Owen and Pfaff (1995), no current study has been performed, employing contemporary analytical tools such as those depicted here. Mainly, what

has been analyzed are PLA₂ and hyaluronidase production in terms of the bee age (Owen et al., 1990; Owen, 1979). Also, the total amount of protein seems to vary together with the insect age, as studied by Abreu et al. (2000). Melittin production as a function of age and season of the year was analyzed by Owen and Pfaff (1995), but by means of assessing the hemolytic activity of this peptide. More recently, Peiren et al. (2005), performed a proteomic approach to the bee venom by performing 2D gels in which peptides such as melittin cannot be detected.

Finally, the production of specific AHB antivenom should take into account the possible regional variability of

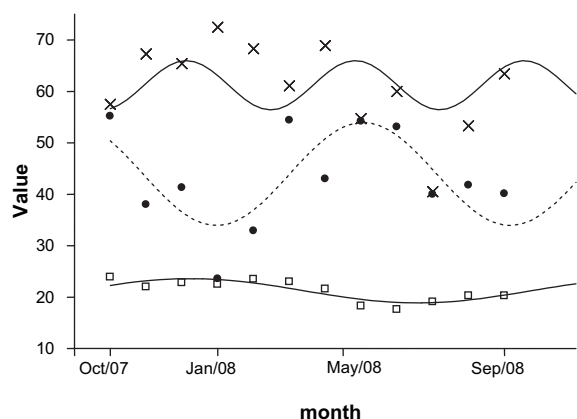


Fig. 5. Data-fitted sinoidal variation of the monthly production of mellitin (●) and humidity (×) and temperature (□) values.

the venom composition due to climatic, seasonal and feeding factors. These variations could be either quantitative or qualitative. The compounds analyzed in this work presented a quantitative variation, but a closer inspection in Fig. 2B can reveal peaks that undergo qualitative variation through the year. The possible qualitative variations still need to be investigated compared with other bees colonies, and other regions. And so does the expected changes in the venom production profile over the year for different hives. All these factors, in addition to the ones studied here in which we describe changes in the proportion of bee venom components as a function of time, need to be thoroughly studied in order to obtain an effective serum-therapy against bee mass-venomation.

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Conflict of interest statement

None declared.

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