

Biochemical variability of venoms from individual European and Africanized honeybees (*Apis mellifera*)

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To study biochemical differences between venom from individual honeybees, venom sacs from 103 European (EU) bees and 92 Africanized bees representing 12 different colonies were dissected, and the dry weight (DW) of venom from each bee was determined. Venom from each of these bees was studied with isoelectric focusing and functional assays for phospholipase A₂ and melittin. Phospholipase concentrations in individual EU bee venoms varied between 1.8% and 27.4% (wt/wt). The melittin concentration in EU bee venom varied less and, on the average, was found to be much lower than previously reported. There was an eightfold to ninefold difference between lowest and highest venom sac DW contents, suggesting the possibility of highly variable venom delivery from bee stings. One EU bee contained >300 µg of venom, three times the recommended maintenance dose for venom immunotherapy. Isoelectric focusing also demonstrated large differences between individual bees, with respect to major and minor components of their venoms. Africanized bees contained significantly less venom but more phospholipase than did EU bees. Bee venoms from different colonies differed in their DW content and in their concentrations of phospholipase and melittin. The results are relevant to the uncertainty of responses from sting challenges and field stings in allergic patients and massive stinging attacks on normal subjects. (*J ALLERGY CLIN IMMUNOL* 1992;90:59-65.)

Key words: Venom, honeybee, melittin, phospholipase, Africanized, biologic variability, isoelectric focusing

The severity of systemic reactions to honeybee stings does not correlate well with the degree of sensitivity of the allergic patient in skin testing with venom, or with serum levels of venom-specific antibodies of any class.^{1, 2} This imperfect relationship between sting reactions and tests with pooled venom could be explained, in part, by variability of the amount of venom delivered by a sting or by variability of the content of one or more antigenic components of the venom from individual bees.

The purpose of this study was to determine the

Abbreviations used

PLA ₂ :	Phospholipase A ₂
AF:	Africanized
EU:	European
IEF:	Isoelectric focusing
DW:	Dry weight
%(wt/wt):	Percent of DW
%(vol/vol):	Volumetric percent
CV:	Coefficient of variation

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variability of venom sacs from individual bees with respect to venom sac DW content, and concentrations of PLA₂ and melittin. In addition, venom was studied by IEF to extend our previous observations of electrophoretic differences between venoms of individual bees³ and to compare specific activity data with IEF patterns in polyacrylamide gels. The recent arrival of AF bees (*Apis mellifera scutellata*) in Texas and the increased risk of multiple stings (which are usually inflicted by a single colony) suggest the need to study also differences between colonies of bees.

MATERIAL AND METHODS

Collection of bees, venom isolation, and DW determinations

EU bees (*Apis mellifera mellifera* and *A. mellifera ligustica*) were captured with an insect net outside hive entrances of five colonies near Tucson, Ariz. The captured bees were cooled immediately on ice, frozen, and stored at -20°C until they were dissected. The colonies chosen were at least five miles apart, and bees from each colony were stored separately.

AF bees were captured near the entrances of seven colonies in Guanacaste, Costa Rica, with an insect net. Participants in the collection procedure wore protective clothing. The bee colonies were feral and were no closer together than five miles. The area sampled was several hundred square miles. These bees were also cooled immediately and frozen at -20°C before transportation to Tucson for dissection.

Venom sacs were dissected from 30 bees from each colony, as described previously.^{3,4} Each sac was completely emptied and rinsed into three drops of distilled water on a small piece of preweighed aluminum foil. The venom specimens were then lyophilized and reweighed with a Cahn microbalance to estimate DW of the contents of each venom sac. The samples were then stored in airtight containers in the dark at room temperature.

PLA₂ Assay

PLA₂ was assayed by measurement of zones of clearing around wells punched in agarose gels containing egg yolk.⁵ Egg yolk, 2.5 ml, and 7.5 ml of 0.15 mol/L of NaCl were mixed and centrifuged for 10 minutes at 325 g. The supernatant was added to a final concentration of 2% (vol/vol) in a 50° C solution of 50 mmol/L of Tris HCl (pH 8.0), 0.6% agarose, and 0.1 mmol/L of CaCl₂. The warm agarose was poured into flat-bottomed plastic plates to a depth of 2 mm, and wells, 4 mm in diameter, were punched into the gels 18 mm apart. Venom pooled from 1000 bees from a single colony for use as a standard was assayed for PLA₂ by testing in eight amounts varying from 2 to 30 ng per well in parallel with the same amounts of purified PLA₂ (Sigma Chemical Co., St. Louis, Mo.). The dry pooled venom, venoms from each bee, and the PLA₂ were reconstituted to 1 mg/ml with distilled water into siliconized glassware with siliconized Hamilton syringes. After application to the wells, the samples were allowed to diffuse for 24 hours in a humid chamber at room temperature. Zones of clearing were measured with a calibrating viewer (model 2743, Transdyne General Corp., Ann Arbor, Mich.).

The relationships between the diameters of the zones of clearing and the logarithm of the amounts of PLA₂ or venom were analyzed by linear regression. The mean diameters of the PLA₂ zones were calculated, and the amounts of PLA₂ and standard venom corresponding to that mean diameter were then determined. The PLA₂ concentration in the standard venom was calculated from $([\text{PLA}_2] \div [\text{standard venom}]) \times 100$. PLA₂ concentrations in the individual ven-

oms were determined by assay of 2, 4, 8, and 12 ng of individual bee venoms against the same amounts of standard venom. The mean diameter of the standard venom zones and the amounts of individual venom and standard venom corresponding to that mean diameter were then determined. The unknown PLA₂ concentrations were calculated from $([\text{individual bee venom}] \div [\text{standard venom}]) \times [\text{PLA}_2]_{\text{standard venom}}$. The reproducibility of the assay for 16 determinations of 5 ng of PLA₂, revealing an average zone diameter of 11.8 mm, was indicated by an SEM of 0.13 mm and a CV of 4.2%. In the assay of purified PLA₂, melittin added to PLA₂ in a fivefold excess did not affect the results, and no band at the isoelectric point of melittin was visible when PLA₂ was analyzed in IEF gels and stained with Coomassie blue and a silver stain.

Melittin assay

The concentration of melittin in venom was determined by measurement of the diameter of zones of hemolysis surrounding wells punched into a blood-agarose plate. Human red blood cells from citrated human blood collected within 1 week were washed three times by mixing gently with a buffer containing 25 mmol/L of Tris, 120 mmol/L of NaCl, and 5 mmol/L of KCl (pH 7.68). Blood-agarose gels were prepared in the same buffer containing 0.6% agarose and 0.25% (vol/vol) washed red blood cells. In preliminary studies, addition of ethylenediaminetetraacetic acid to the gel mixture did not substantially change the hemolysis obtained from mixtures of melittin and PLA₂ in proportions normally found in bee venom. Therefore, ethylenediaminetetraacetic acid was not included in the assay gels. The gels were poured into plates, and wells were punched as for the PLA₂ assay.

It was found that PLA₂ could inhibit or augment the hemolysis induced by melittin, depending on the experimental conditions. With a diffusion time of 4 hours, this interaction was at a minimum and was slightly inhibitory. Therefore, the diameters of zones of hemolysis were measured after 4 hours of diffusion at 23° C in a dark humid chamber. To test the possibility that PLA₂ could have a dose-dependent effect on the assay for melittin, pure synthetic melittin was prepared in the Macromolecular Structure Facility, University of Arizona, with a 431A automated peptide synthesizer (Applied Biosystems, Inc., Foster City, Calif.) with fluorenylmethoxycarbonyl Fmoc chemistry, and was purified by reverse-phase high-performance liquid chromatography. The average diameter of the zones of hemolysis for 4 µg of pure synthetic melittin was 12.4 mm, and for 4 µg of native melittin (Sigma Chemical Co., catalog No., M 2272), 11.9 mm. PLA₂ was then added to 4 µg of these two melittin preparations in amounts between 0.05 and 2 µg, extending beyond the range of ratios of PLA₂ to melittin expected in whole bee venom. As illustrated in Fig. 1, PLA₂ added to synthetic melittin in ratios $>0.025:1$ did not further inhibit hemolysis, and PLA₂ added to native melittin in ratios of 0.0125:1 did not further inhibit hemolysis. Addition of PLA₂ inhibited hemolysis from native melittin less than hemolysis from synthetic melittin, and there was maximal inhibition of both melittin preparations

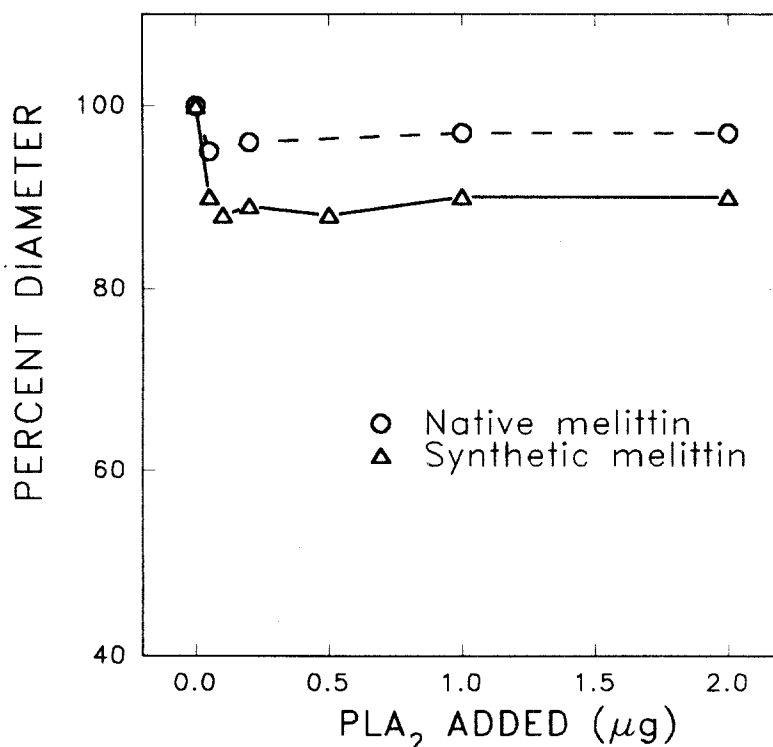


FIG. 1. Inhibition of melittin-induced hemolysis by PLA₂. PLA₂ was added to 4 μg amounts of native melittin (Sigma Chemical Co., catalog No. M 2272) or pure synthetic melittin produced by a peptide synthesizer. Mean uninhibited diameter of hemolysis for native melittin was 11.9 mm; for synthetic melittin, 12.4 mm, explaining why the inhibition curve for synthetic melittin lies below that for native melittin.

by 0.025:1 PLA₂/melittin ratios. This finding was found to be consistent with approximately 1% contamination of the native product with PLA₂. Since the maximum inhibition of hemolysis by addition of PLA₂ to native melittin was 3%, it was possible to determine the true concentration of melittin in the standard reference venom and the venoms from individual bees with a correction factor of 0.97.

The same venom pool used for the PLA₂ assay was used for standards in the melittin assay. The venom pool was standardized by testing eight amounts of melittin (Sigma Chemical Co.): 1, 2, 4, 6, 8, 10, 12, and 14 μg per well together with eight amounts (between 2 and 28 μg) of the pooled venom. Melittin and venoms were reconstituted as described for the PLA₂ assay. The melittin concentration in the standard venom pool was determined from regression analysis as in the PLA₂ assay: $([\text{melittin}] \div [\text{standard venom}]) \times 100 \times 0.97$, at the average diameter of the melittin zones. The melittin concentration in venom from individual bees was then determined by assay of 2, 4, 8, and 14 μg of individual bee venoms against 2, 4, 8, and 14 μg of standard venom. The mean diameter of the standard venom zones, and the amounts of individual venom and standard venom corresponding to that mean diameter, were then determined. The melittin concentration was calculated from $([\text{individual bee venom}] \div [\text{standard venom}]) \times [\text{melittin}]_{\text{standard venom}}$. The reproducibility of the assay, as-

essed by 16 determinations of 4 μg of melittin, eliciting an average zone diameter of 11.9 mm, was indicated by an SEM of 0.075 mm and a CV of 2.5%.

IEF

Bee venom was applied to 5 mm wide wicks for placement on thin-layer IEF polyacrylamide gels as previously described.⁶ Each gel was loaded with 100 μg of venom samples in 8 μl of H₂O. PLA₂ and melittin standards were tested in parallel with the venom samples from individual bees. The gels were stained with Coomassie blue, photographed, and counterstained with a silver staining method.¹ Unlike most other proteins, both melittin and PLA₂ stained more strongly with Coomassie blue than with the silver stain.

Statistical analysis

The reliability of the PLA₂ and melittin assays was tested as follows: If the ratio of the slopes of the regression lines for the individual venom and the standard venom in either assay fell outside the range of 0.8 to 1.2, data on all three assays (DW, PLA₂ and melittin) for that bee were discarded. This problem was encountered with so many bees in two of the seven Af bee colonies that the remaining numbers of bees in the two colonies were too small for statistical analysis. Therefore, only five of the seven AF bee colonies were

TABLE I. DW, PLA₂, and melittin concentrations of venom sac contents of individual EU bees (n = 103) and AF bees (n = 92)

	Bee type*	Mean	SD	CV	Range	95% CI on mean
DW† (μg)	EU	134.1	53.5	39.9	38-330	123.8-144.4
	AF	98.2	35.9	35.9	34-200	90.9-105.5
PLA ₂ ‡ (% wt/wt)	EU	11.1	5.2	47.1	1.8-27.4	10.1-12.1
	AF	18.1	4.9	27.1	5.1-31.0	17.1-19.1
Melittin§ (% wt/wt)	EU	29.6	4.6	15.4	12.3-40.2	28.7-30.5
	AF	25.5	6.4	25.5	5.4-39.3	24.3-26.8

CI, Confidence interval.

*Differences between EU and AF bee venom were significant.

† $p < 0.0001$ for dry weight.‡ $p < 0.0001$ for melittin.§ $p < 0.0001$ for PLA₂ concentrations.

analyzed. With the above criteria for slope ratios, data were analyzed from 103 of 150 EU bees and 92 of 210 AF bees. For all three assays (DW, PLA₂ assay, and melittin), differences between individual bees and differences between pooled venoms from different colonies were compared with Student's *t* tests and discriminant analysis programs in the SPSS Release 4.0 (SPSS, Inc., Chicago, Ill.) on a VAX/VMS mainframe computer. The CV of results of assays on individual bee-venom samples was calculated from $([SD \div \text{mean}] \times 100)$. To correlate IEF results with PLA₂ and melittin assay results, PLA₂ and melittin bands were graded into six intensity levels and analyzed by Spearman's rank correlation.

RESULTS

Evaluation of collection method

To test the possibility that bees could lose some of their venom when they were captured with an insect net, a new insect net in which 200 EU bees were caught was extracted and tested for venom. Before bee collection, the net was exhaustively washed to totally remove substances that were found to cause false-positive results in the PLA₂ assay. The bees were removed from the net, which was then extracted with 60 ml of deionized water for 10 minutes, lyophilized, and reconstituted to 1 ml with deionized water. The net extract was found to contain 1.05 mg of venom as determined by the egg yolk assay for PLA₂, with pooled-venom standards. Therefore, the average deposition of venom in the insect net was 5.25 μg per bee, which is <4% of the average venom sac content of EU bees.

Variability of venom from individual bees

The determinations of DW, PLA₂ concentration, and melittin concentration in venom from individual venom sacs indicated a considerable degree of variability, for example, the CV for the PLA₂ concentration was 47.1% in EU bee venoms and 27.1% in AF

bee venoms. As presented in Table I, there was a wide range of values for all three parameters in both EU and AF bees. Several EU venom sacs contained >250 μg DW of venom; of which one sac contained >300 μg. IEF also demonstrated a high degree of variability among venoms from individual bees (Fig. 2). Intensity of bands corresponding to PLA₂ and melittin correlated with their corresponding assay results (for melittin, $r_s = 0.47$ and $p < 0.001$; for PLA₂, $r_s = 0.41$ and $p < 0.001$).

Differences between colonies

There were significant differences in venom DW, PLA₂, and melittin concentrations in venom from different colonies (Tables II to IV). Discriminant analysis demonstrated that intercolony differences in PLA₂ concentration and melittin concentration were largely determined by their type, EU or AF. In general, the intercolony CVs were less than the CVs of individual bees. With the exception of one colony of EU bees that had a high average PLA₂ concentration in their venom, venom PLA₂ concentrations in EU bee colonies did not differ from each other at the 0.05 level.

Differences between EU and AF bee venoms

Venom from AF bees had a significantly lower concentration of melittin as compared with venom from EU bees (Table I). AF bees also had a significantly lower DW of venom sac contents, indicating that the total amount of melittin in venom sacs of AF bees was much less than in the sacs of EU bees (Tables I and IV). However, AF bee venom had a significantly higher PLA₂ concentration than did EU bee venom (Tables I and III). IEF patterns of most individual AF bee venoms agreed with the assay results, demonstrating weaker staining of melittin bands and stronger staining of PLA₂ bands, as compared with EU bee venom analyses (Fig. 2).

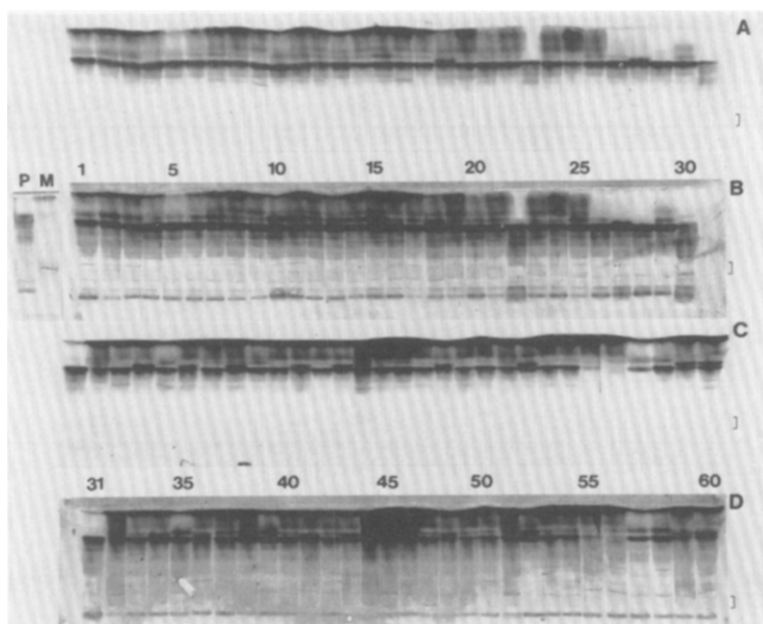


FIG. 2. IEF patterns of venoms from individual bees. Wicks, each containing 100 μ g of venom from a single bee, were applied to each *lane* on IEF gels, in positions indicated by *brackets*, where a sample application artifact is observed. *Gels A* and *C* were stained by Coomassie blue G-250. Same two gels were then counterstained by a silver stain method (*B* and *D*). AF venoms were applied to lanes 1 through 31; EU venoms, applied to lanes 32 through 60. Intensity of major band for PLA₂ tended to be stronger in AF than in EU bee venoms. Some inconsistencies between IEF patterns and functional assays were found, e.g., melittin content of venoms analyzed in *lanes 5, 20, 22, and 26* were 28.7, 27.1, 25.1, and 23.5, respectively. In these venoms, acidic bands were stronger, suggesting that acidic proteins may potentiate hemolysis; *P*, PLA₂ standard; *M*, melittin standard. Gels oriented with anode, *bottom*. *Numbers* refer to lanes in the gels above and below the numbers.

TABLE II. Comparison between mean DWs of venom from individual bees from different colonies

Mean	No.	Colony	E1	A2	A3	A4	A5	E5	A1	E4	E3	E2
78.1	9	E1										
82.2	21	A2										
97.5	24	A3										
97.8	18	A4										
102.2	19	A5	*	*								
118.9	27	E5	*	*								
126.2	10	A1	*	*								
141.7	26	E4	*	*	*	*	*					
151.5	21	E3	*	*	*	*	*	*				
151.8	20	E2	*	*	*	*	*	*				

A1-A5, Five colonies of AF bees; E1-E5, five colonies of EU bees.

CV of means among AF bee colonies, 15.7%; CV of means among EU bee colonies, 24.25%.

*Pairs of colonies significantly different at the 0.05 level (multiple range test, least significant difference procedure).

DISCUSSION

As expected, this study identified differences between the amount and composition of venoms from individual bees. It is known that the age of the bee affects the content of PLA₂ in venom,⁷ but this study

was confined to adult, mature, colony defender or forager bees. Therefore, the considerable variation of venoms from individual bees found is relevant to the common stinging behavior of bees.

The possibility that the variation found in the DW

TABLE III. Comparison between mean PLA₂ concentrations in venom from individual bees from different colonies

Mean	No.	Colony	E3	E2	E5	E4	A4	A3	A5	A2	E1	A1
8.8	21	E3										
9.9	20	E2										
10.9	27	E5										
11.2	26	E4										
17.3	18	A4										
17.7	24	A3	*	*	*	*						
17.8	19	A5	*	*	*	*						
17.8	21	A2	*	*	*	*						
19.5	9	E1	*	*	*	*						
21.3	10	A1	*	*	*	*	*	*				

CV of means among AF bee colonies, 9.0%; CV of means among EU bee colonies, 35.36%.

*See footnote to Table II.

TABLE IV. Comparison between mean melittin concentrations in venom from individual bees from different colonies

Mean	No.	Colony	A5	A4	A2	A1	E3	E4	E2	A3	E5	E1
23.0	19	A5										
23.9	18	A4										
26.0	21	A2										
26.5	10	A1										
28.9	21	E3	*	*								
29.3	26	E4	*	*	*							
30.3	20	E2	*	*	*							
30.9	24	A3	*	*	*	*						
31.6	27	E5	*	*	*	*						
34.5	9	E1	*	*	*	*	*	*				

CV of means among AF bee colonies, 11.77%; CV of means among EU bee colonies, 7.3%.

*See footnote to Table II.

of venom sac contents of individual mature bees was caused by the net collection technique was excluded by demonstrating that <4% of the average venom sac content of EU bees was deposited on the insect net during collection. The CV of venom DW was much larger (40%). Although it was not possible to repeat this experiment with AF bees, the CV of DWs of AF bees that had been collected for the study was similar to that of EU bees, suggesting that it is unlikely that large numbers of AF bees would have deposited an appreciable amount of venom on the insect netting during their collection in Costa Rica. In addition, it is known that extrusion of appreciable amounts of venom from the sac requires either electrical stimulation of the bee or autotomization (exenteration) of the stinging apparatus after the barbed stinger is firmly embedded in skin or a similar material.

It is not clear why many of the bees studied contained venom that demonstrated PLA₂ or melittin as-

say slopes that differed from the standard. It is possible that there were substances in the venoms of these bees that interfered with the assay. Discarding results from these assays probably reduced the variability of the remaining bees studied, but variability between bee venoms was still a major finding in this study.

The greatest differences between bees were in the DW of venom sac contents and the concentration of PLA₂, particularly for EU bees. Since PLA₂ is the most allergenic of bee-venom components, it could be important if an allergic individual were stung by a bee with a high venom concentration of PLA₂ (30%, wt/wt, in our study) instead of a bee with a low venom concentration of PLA₂ (2%, wt/wt). Information on the variability of venom actually delivered by a natural sting is not available, but the sevenfold difference found between the highest and lowest venom sac DW in EU bees could greatly affect the sting delivery of all venom components. The large bee-to-bee differ-

ences in IEF patterns were not predicted by PLA₂ or melittin assay results, suggesting that other components also varied. It is possible that other substances in bee venom also focus close to the isoelectric point of PLA₂ and melittin. Whether electrophoretic differences were present in other allergenic components, for example, hyaluronidase, or in predominantly toxic peptides, was beyond the scope of this study. The quantitative and qualitative differences between venoms from individual bees suggest that sting-challenge studies with live bees could be unreliable. Similarly, it may not be possible to rely on the history of a reaction to a single bee sting in the field.

In honeybee-venom immunotherapy, the recommended maintenance dose of 100 µg was based, in part, on the assumption that bees contain 50 µg of venom.⁸ This study demonstrated a mean DW of 134 µg and a maximum weight that exceeded 300 µg in EU bee venom sacs. Even when the lack of quantitative knowledge of actual sting delivery is taken into account, it is likely that sting delivery often far exceeds 50 µg. It is known that 100 µg doses of venom provide good protection for most patients after several years of therapy, but our results suggest that tolerance of 100 µg injection doses does not imply that a sting would be tolerated early in the treatment course.

Differences between venoms from different colonies could be important, particularly for AF bees, because of their tendency to sting en masse in defence of their colonies. Some colonies of AF bees were significantly different from each other with respect to venom DW, concentrations of melittin, and PLA₂ in venom. Nevertheless, most of the intercolony variation was small compared with variation among individual bees and was partly explained by differences between type of bee (EU or AF). Further studies of larger numbers of colonies are needed to confirm this impression.

A previously published comparison of pooled EU and pooled AF bee venoms did not demonstrate differences in venom composition.⁹ However, the present study confirmed our previous finding of low venom sac DWs and low concentrations of melittin in AF bee venom³ and is consistent with a study of the smaller venom sacs and lower content of hyaluronidase in venom of AF bees.¹⁰ In addition, our study demonstrated that the total content of PLA₂ in AF bee venom sacs after correction for venom DW is 19.4% higher than that in EU bee venom. Thus, it is likely

that AF bees usually deliver more PLA₂ per sting than do EU bees. This could be significant for allergic individuals who become exposed to risk of stings from AF bees en masse because PLA₂ is known to be the major allergen in honeybee venom.

It is of interest that the average concentration of melittin in EU bee venom was much less than previously published estimates of the concentration of melittin in pooled bee venom.¹¹ One explanation of the discrepancy could be differences between the colonies studied.

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