

RESEARCH ARTICLE

Proteome and phosphoproteome of Africanized and European honeybee venoms

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Honey bee venom toxins trigger immunological, physiological, and neurological responses within victims. The high occurrence of bee attacks involving potentially fatal toxic and allergic reactions in humans and the prospect of developing novel pharmaceuticals make honey bee venom an attractive target for proteomic studies. Using label-free quantification, we compared the proteome and phosphoproteome of the venom of Africanized honeybees with that of two European subspecies, namely *Apis mellifera ligustica* and *A. m. carnica*. From the total of 51 proteins, 42 were common to all three subspecies. Remarkably, the toxins melittin and icarapin were phosphorylated. In all venoms, icarapin was phosphorylated at the ²⁰⁵Ser residue, which is located in close proximity to its known antigenic site. Melittin, the major toxin of honeybee venoms, was phosphorylated in all venoms at the ¹⁰Thr and ¹⁸Ser residues. ¹⁸Ser phosphorylated melittin—the major of its two phosphorylated forms—was less toxic compared to the native peptide.

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1 Introduction

The infamous “killer” bee, named Africanized honeybee (AHB) resulted from experimental breeding between African bees (*Apis mellifera scutellata*) and European drones. The accidental escape of this aggressive and successful strain led to widespread hybridization with local populations of previously

introduced European honey bees [1]. AHBs spread rapidly through continued outcrossing, from Brazil up through South America and into Central and even North America. Since acquiring African traits, the aggressive behavior of honey bees has become a major concern for agriculture and public health [2].

Honeybee venom (HBV) contains vasoactive and hemolytic substances such as histamine, dopamine, serotonin, melittin, and phospholipases, which are responsible for the physiological effects reported with massive bee stings [3, 4]. Very recently, venomomics has emerged as an important scientific discipline complementing drug discovery efforts and contributing to better understanding of envenomation processes [5]. Graaf et al. reviewed recent progress in the

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Abbreviations: AHBV, Africanized honeybee venom; FDR, false discovery rate; HBV, honeybee venom; LF, label-free; MRJP, major royal jelly protein

Colour Online: See the article online to view Figs. 1 and 2 in colour.

venomics of Hymenoptera and its impact on understanding the molecular mechanisms of bee-, wasp-, and ant-related allergies [6]. Concerning HBV, the first MS-based study by Peiren et al. [7] combining 2DE and MALDI-TOF/TOF protein identification, revealed nine proteins in the venom of *A. m. carnica*. Among these nine proteins, six had previously been described [8–11], while other three were novel [7, 12]. Currently, 12 proteins from *A. mellifera* venom are recognized as allergenic by the International Union of Immunology Societies (ALLERGEN NOMENCLATURE IUIS – Allergen Nomenclature Sub-Committee Home, <http://www.allergen.org>).

Usually venomics efforts were focused on the analysis of protein composition, while the PTMs of its major acting components and their physiological significance remained elusive. 2DE technique has the advantage of visualizing modified proteins, which can appear as multiple pI or MW variants according to their modification states. Conversely, it excludes proteins of low abundance, high hydrophobicity, extreme pI (<4 or >10) and molecular mass (M_r) (<15 kDa or >200 kDa) [13]. In contrast, the LC-MS/MS based shotgun strategy is largely unbiased and overcomes the aforementioned limitations, making it a valuable tool in venomics [14].

The peptide melittin is the major toxin found in bee venoms that constitutes ~50% of its dry weight [15]. It disrupts the phospholipid bilayer of membranes of red blood cells, white blood cells, platelets, and endothelial cells [2, 16]. In conjunction with another major component of the venom, phospholipase A2, melittin triggers cell membrane breakdown by degrading the phospholipids and leading to vascular endothelium damage and inflammatory response activation [17, 18]. Numerous studies have contributed significantly to our understanding of its chemical and structural properties, cellular activities, and its interaction with the lipid bilayer [19, 20]. Modern analytical methods enabled the identification of PTM proteins in bee venoms. Very recently, Blank et al. have characterized two glycosylated HBV allergens, MRJP 8 and MRJP 9 (where MRJP is major royal jelly protein) [21], and Zhanq et al. identified phosphorylated forms of MRJP 1, MRJP 2, and apolipoprotein III [22]. In addition, previous studies suggested that PTMs may affect the IgE binding capacity [23] or influence proteins antigenicity and immunogenicity [24–27]. However, no phosphorylation of melittin or other bee venom toxins was reported.

Because of the low stoichiometry and dynamic regulation of proteins phosphorylation [28] highly sensitive methods for isolation, detection, and quantification of phosphorylation sites are required. Extracted ion chromatogram based label-free (LF) quantification provides a valuable platform for comparative proteomic studies [29]. LF quantification involves the integration of all MS1 peptide signals across their chromatographic elution time and the subsequent alignment of these integrated values across all acquisitions, taking into account potential retention time drifts. The relative quantification arises from the between-acquisition comparison of the integrated values for each identified peptide. Due to the

replacement of stable isotopes labeling steps with advanced informatics, LF quantification essentially allows for the comparison of any number of samples of any biological origin. Another benefit is that MS/MS identifications may be shared among aligned MS1 quantitative traces, allowing the identification and quantification of low abundance species, such as phosphopeptides, whose MS/MS fragmentation may not have been triggered in all samples due to the nature of data-dependent acquisition.

Considering the frequency of attacks by AHB and the previously reported notion that knowledge of the compositional differences of honey bee venoms may aid in the development of a more potent antivenom generic to all subspecies [30, 31], we report here a comparative study of the venoms of two European *A. mellifera* subspecies, *A. m. carnica* and *A. m. ligustica*, and the hybrid AHB. We identified a total of 51 proteins, many of which were previously unreported. Also, our identification of two phosphoproteins, melittin and icarapin, is the first report on bee toxins phosphorylation and its impact on their toxicological properties.

2 Materials and methods

All the toxicological tests performed using animals were approved by the Ethics Committee of the Medicine Faculty of University of São Paulo, protocol 351/10.

2.1 Chemicals

Ammonium bicarbonate, DTT, iodoacetamide, sodium chloride, calcium chloride, formic acid, ACN, Triton X-100, 4-nitrophenyl *N*-acetyl- β -D-glucosaminide, sodium citrate tribasic dihydrate were from Sigma-Aldrich (Munich, Germany). Phosphatase inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany) and porcine trypsin from Promega (Mannheim, Germany); alkaline phosphatase calf intestinal (CIP) from New England BioLabs (Ipswich, MA). C18 spin columns were obtained from Harvard Apparatus (March-Hugstetten, Germany); melittin phosphorylated at ^{18}Ser was synthesized by Peptide Specialty Laboratories (Heidelberg, Germany).

2.2 Venom collection

Africanized *A. mellifera* bees from the Apiary of University of State of São Paulo (UNESP, Rio Claro) were captured and immobilized by quick freezing at -20°C . Each individual animal was dissected and the sting apparatus and the venom reservoirs were removed using tweezers. The venom sacs were lyophilized and stored at -20°C . Samples of venom were manually extracted by squeezing the venom reservoirs against the vials and solubilizing their content in water. *Apis mellifera carnica* venom was kindly donated by a private apiary

in Bulgaria. Venom from *A. m. ligustica* was purchased from Sigma-Aldrich.

2.3 Sample preparation and in-solution digestion

Fresh HBV samples were solubilized in 10 mM ammonium bicarbonate pH 8.0, containing the phosphatase inhibitor cocktail. Protein content was quantified using BCA assay [32]. Disulfide bonds were reduced by adding 10 mM DTT and incubating the solution at 56°C for 30 min. Proteins were further alkylated with 55 mM iodoacetamide at room temperature in the dark for 30 min and digested with porcine trypsin at enzyme to substrate ratio of 1:20 w/w at 37°C for 18 h. Digests were cleaned using C18 microspin columns with 10 µm particle size and 300 Å pore size (Harvard Apparatus, Holliston, MA); collected peptides dried down in a vacuum centrifuge and stored at –20°C until LC-MS/MS analysis.

2.4 LC-MS/MS analyses

Tryptic digests were redissolved in 18 µL of 5% formic acid and 5 µL were injected into a Dionex Ultimate 3000 nano-HPLC system. The system was equipped with 300 µm id × 5 mm trap column and 75 µm × 15 cm analytical column (Acclaim PepMap100 C18, 5 µm/100 Å and 3 µm/100 Å, respectively, both from Dionex (Idstein, Germany)). Solvent A was 2% v/v ACN in aqueous 0.1% v/v formic acid; solvent B was 60% v/v ACN in aqueous 0.1% v/v formic acid. Samples were analyzed by the linear gradient of 6–58% of solvent B delivered in 360 min at the flow rate of 200 nL/min. MS and CID MS/MS spectra were acquired on a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The FT survey scans were acquired from m/z 380–1200 with the target mass resolution of 60,000 (full width at half maximum) at m/z 400. Automated gain control target ion count was set to 1×10^{-6} ions for full FTMS scans and a maximal fill time of 500 ms. MS/MS spectra were acquired at the ion trap using a normalized collision energy of 35%, dynamic exclusion time of 25 s, isolation width of m/z 3.0, and recorded in centroid mode. The data-dependent acquisition cycle consisted of six LTQ CID MS/MS per FT survey scan with the fragmentation threshold of 2000 ion counts; singly charged and charge-unassigned precursor ions were excluded from fragmentation. Lock mass was set to the singly charged dodecamethylcyclohexasiloxane ion ($\text{Si}(\text{CH}_3)_2\text{O}_6$; $m/z = 445.120025$) [33].

2.5 Database searching and protein identification

The peak lists of MS² spectra were generated from .raw files using the Trans-Proteomic Pipeline v.4.3.1 [34]; Mascot v. 2.2.2 (Matrix Science, London, UK) was used for peptide identification by searching against a database compiled from *A. mellifera* entries extracted from NCBI database (release February 23, 2012) to which sequences of human keratins and

porcine trypsin were added. The database included a variable “X” in the 15th position of the full melittin sequence, thus allowing the substitution with any amino acid. Search settings were: precursor mass tolerance of 5 ppm; fragment mass tolerance of 0.6 Da; fixed modification: carbamidomethyl (Cys); variable modifications: acetyl (protein N-terminus), oxidation (Met), phosphorylation (Ser, Thr, Tyr); up to two missed cleavages were allowed. The peptide identifications were filtered at 5% false discovery rate (FDR) according to the Mascot decoy database search strategy and protein identifications were accepted with a minimum of two peptide sequences matched. The software Trans Proteomic Pipeline v. 4.6.1 was used to assess the protein probability with a minimum threshold of 95%. To validate phosphorylation sites identification, the fragment ion annotations were verified manually for each matched MS/MS spectrum. The full list of matched peptides and identified and quantified proteins are provided, respectively, in Supporting Information Tables 1 and 2 that are compiled according to MIAPE (<http://www.psdev.info/node/91>) guidelines.

2.6 LF quantification and data analysis

Quantitative analysis and inferential statistics were carried out as previously described in Vasilj et al. [29] under the same software settings. Briefly, feature extraction and alignment were performed using a customized version of SuperHirn v.0.03 [35]. Features were normalized to the TIC of identified peptides and log2 transformed. The peptide-level ANOVA and Benjamini and Hochberg [36] multiple test correction were performed with DanteR [37]. The cutoff of 5% was used for the quantitative FDR.

2.7 Phosphopeptide synthesis and quality control

Melittin $\text{NH}_2\text{-GIGAVLKVLTTGLPALI-}^{\text{P}}\text{S-WIKRKRQQ-CO}_2\text{H}$ was synthesized with 95% of purity. A phosphopeptide aliquot was treated with alkaline phosphatase (1.0 unit/µg protein) overnight at 37°C and cleavage completeness was assessed by MS by direct infusion of the digest.

2.8 Hemolytic activity assay

One percent v/v suspension of Wistar rats red blood cells was prepared in aqueous 0.85% sodium chloride and 10 mM calcium chloride and incubated with a range of peptide concentrations from 0.5 to 0.000244 µg/µL at room temperature for 90 min. Lysis of cells was assessed by measuring optical density at $\lambda = 540$ nm using a plate reader. Negative control was 1% v/v cell suspension in the same buffer; positive control was 1% v/v cell suspension lysed by 1% v/v Triton X-100. The percentage of hemolysis was calculated as: % hemolysis = $(A - A_0) / (A_x - A_0) \times 100$, where A is the optical density of red blood cells suspension lysed by melittin; A_0 and A_x are optical densities of negative and positive

controls, respectively. Results are expressed as means $\pm$ SD of triplicates.

2.9 Mast cells degranulating activity assay

Degranulation was determined by measuring the release of granule marker β -hexosaminidase from rat peritoneal mast cells as described by Hide et al. [38]. Briefly, well plates containing cells were loaded with mellitin in the concentration range of 0.1–0.0011 $\mu\text{g}/\mu\text{L}$ and incubated for 15 min at 37°C. Fifty microliters aliquots of a solution containing the substrate 1 mM 4-nitrophenyl *N*-acetyl- β -D-glucosaminide and 0.2 M sodium citrate, pH 4.5, were added to 50 μL aliquots of the cell supernatant and incubated for 6 h at 37°C. The reaction was stopped by adding 150 μL of 0.2 M Tris buffer with pH 7.9 and optical density was measured at $\lambda = 405$ nm. The activity was expressed as the percentage of the total β -hexosaminidase released from mast cells lysed by 0.1% v/v Triton X-100. Results are expressed as means $\pm$ SD of triplicates.

2.10 Lactate dehydrogenase assay

Mast cells were loaded into well plates containing mellitin in the concentration range of 0.1–0.0011 $\mu\text{g}/\mu\text{L}$ and incubated for 15 min at 37°C. The stimulated lactate dehydrogenase release was measured from 20 μL supernatant aliquots from each well plate position after 5 min centrifugation at 1000 rpm. The activity was determined by lactate dehydrogenase assay kit from BioTecnica (Varginha, Brazil) according to the manufacturer instructions and expressed in arbitrary enzyme activity units per liter.

2.11 Chemotaxis assay

Chemotaxis was assayed in a chamber from Neuro Probe (Gaithersburg, MD) using polymorphonucleated leukocytes obtained by subcutaneous inflammatory induction in Wistar rats. The upper chamber was filled with a polymorphonucleated leukocyte suspension ($\sim 2 \times 10^4$ cell/mL in 0.9% v/v sodium chloride solution) and the lower chamber was filled with physiological saline solution containing the peptides with a range of concentrations from 0.1 to 0.0011 $\mu\text{g}/\mu\text{L}$. Polycarbonate membrane with pore diameter of 10 μm was placed between both chambers. After 60 min incubation at 37°C, the migrated cells in the lower chamber were counted.

3 Results and discussion

3.1 Proteomic composition of the venoms

Successful antivenom treatment of sting victims depends on several factors including the subspecies of the inflicting bees,

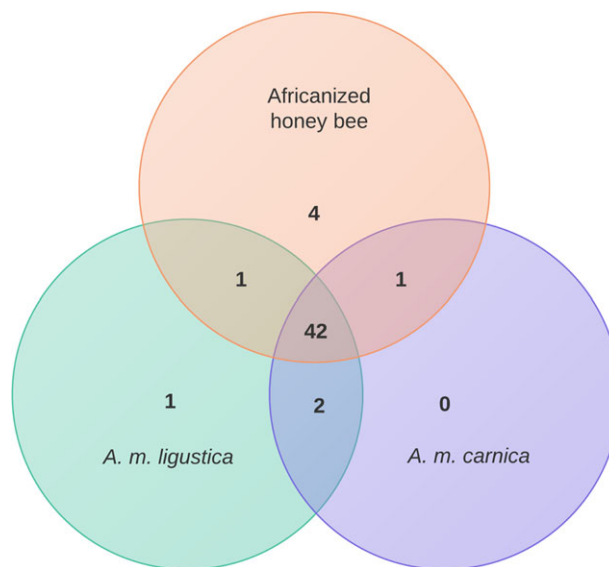


Figure 1. Distribution of proteins identified from Africanized and European honey bee venoms. The Venn diagram shows a total of 42 proteins shared between all three venoms. Also, four proteins were found exclusively in Africanized venom, zero in the venom of *Apis mellifera carnica*, and one in the venom of *A. m. ligustica*.

which is typically unknown at treatment time. It is known that, within the same subspecies, venom composition varies over the course of the year and is also a factor of individual age [39]. Thus, it is very likely that there may also be significant compositional differences on the subspecies level. Knowledge of the molecular differences between venoms influences antivenom production processes and may lead to the development of more potent and generic antivenoms. Here, we characterized for the first time the intersubspecies compositional differences between the venoms of AHB, which are notorious for massive human attacks, as well as two common European subspecies, *A. m. ligustica* and *A. m. carnica*, which are omnipresent in the same habitats.

For analyzing protein mixtures containing no detergents and having low to medium compositional complexity, in-solution digestion with enzymes may be advantageous compared to in-gel digestion due to expectantly higher peptide recovery [40]. To dissect the HBV proteomes we digested them in-solution with trypsin and analyzed the digests using 360 min LC gradients. Duplicate runs were performed for all samples.

By analyzing 30 μg of total protein content from each venom and considering peptides encompassed by a 5% peptide-level FDR, a minimum of two peptides per protein, and a Protein Prophet probability of $>95\%$, we identified in total 51 proteins with 42 proteins common to all venoms (Fig. 1).

The HBV proteins (Table 1) were classified into different groups, according to Gene Ontology criteria (Supporting Information Fig. 1), while ca. 43% were having unknown function. Nevertheless, many of the previously uncharacterized

Table 1. Proteins identified in venoms from three *Apis mellifera* subspecies

gi accession	Protein ^{a)}	Number of matched peptides			Fold change ^{b)}	
		A	C	L	C/A	L/A
229892214	HSP cognate 3	4	1	2	−6.5**	
58585116	Venom serine protease 34 precursor	1	15	19	4.7***	12.8**
94400907	Allergen Api m 6 precursor	4	3	4	−4.0**	
110751310	ER resident protein 29-like	5	5	10	−3.0***	
328787622	Peptidylglycine alpha-hydroxylating monooxygenase	2	1	3	−2.4**	
328781841	Mast cell degranulating peptide-like	4	4	2	−12.3***	−34.7**
74835477	Venom allergen acid phosphatase	5	9	21	1.8**	20.0***
5627	Phospholipase A-2	33	29	27	−1.7***	
66511507	Endochitinase isoform 2	3	5	5	−1.7**	
328779578	Lysozyme c-1	5	7	6	−1.4**	
187281543	Venom dipeptidyl peptidase 4 precursor	4	4	16		5.1**
58585142	Major royal jelly protein 3 precursor	14	2	1		
221325614	C1q-like venom protein precursor	5	4	3		
110756431	Hypothetical protein LOC725074	3	3	3		
58585104	Vitellogenin precursor	0	0	20		
66507455	Venom protease	4	5	6		
328787755	Hypothetical protein LOC100577054	4	5	5		
226533687	Venom serine carboxypeptidase precursor	6	5	4		
328782499	Proactivator polypeptide isoform 1	14	21	25		
58585154	Melittin precursor	6	4	4		
187281550	Venom carboxylesterase-6 precursor	22	19	18		
58585070	Major royal jelly protein 8 precursor	16	17	16		
110758297	Phospholipase A2-like	8	10	9		
328789222	Nucleobindin-2-like	2	6	3		
66514614	Chitinase-like protein Idgf4-like	1	4	4		
58585182	Hyaluronidase precursor	33	38	33		
328790726	Venom acid phosphatase Acph-1-like	4	8	5		
58585086	Transferrin 1 precursor	6	1	17		
67010041	Major royal jelly protein 9 precursor	20	24	20		
58585108	Major royal jelly protein 2 precursor	18	2	1		
66500170	Neutral alpha-glucosidase AB-like isoform 2	1	0	5		
335892796	Peptidyl-prolyl cis-trans isomerase B precursor	8	8	10		
94158822	Odorant binding protein 14 precursor	5	3	6		
328789531	Hypothetical protein LOC408666	18	20	20		
60115688	Icarapin-like precursor	7	7	9		
328788558	Putative cysteine proteinase CG12163-like	6	9	11		

Table 1. Continued

gi accession	Protein ^{a)}	Number of matched peptides			Fold change ^{b)}	
		A	C	L	C/A	L/A
58585138	Major royal jelly protein 5 precursor	11	0	0		
58585164	Alpha-glucosidase precursor	12	0	0		
328780886	Apolipophorins-like	0	0	6		
328785691	Carboxypeptidase D-like	0	1	3		
328790510	dnaJ homolog subfamily B member 11-like	1	2	1		
328787985	Multiple coagulation factor deficiency protein 2 homolog	2	1	0		
328783471	Hypothetical protein LOC725114 isoform 1	2	0	0		
110748908	A disintegrin and metalloproteinase with thrombospondin motifs 3-like	3	2	2		
48101366	Venom serine protease 34	0	0	5		
58585180	Secapin preproprotein	3	3	2		
328783352	Chymotrypsin inhibitor	2	2	2		
66560290	Lysosomal aspartic protease	2	3	0		
328778095	Lysosomal Pro-X carboxypeptidase-like	0	1	2		
58585166	Apamin preproprotein	1	1	1		
58585098	Major royal jelly protein 1	6	0	0		

Notation A, C, and L corresponds to *A. m. Africanized*, *A. m. carnica*, and *A. m. ligustica*, respectively. Label-free quantitative data are expressed as fold changes with respect to the sample A; “-” symbol indicates that for clarity the value is reversed (e.g. A/C); having no value indicates no change according to the 5% statistical significance cutoff.

a) All proteins are reported with a minimum of 95% protein probability according to the Trans-Proteomic Pipeline.

b) Statistical significance is represented by **p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001.

proteins could be classified as bona fide venom components by having similar roles in other animal venoms, especially social *Hymenoptera* insects [41]. Overall, the distribution of proteins between Gene Ontology categories was similar for all three venoms. Among the 12 well-known HBV allergens, 11 are shared by all the analyzed venoms. We identified many novel proteins with a likely role in the envenoming process and/or venom self-protection. The presence of disintegrin and metalloproteinase may be related with the occurrence of myonecrosis, skin damage, edema, among other inflammatory reactions [42]. Cysteine protease CG12163-like is a notable candidate for further and more targeted investigation because a functionally similar protein, a bee venom serine protease, is a recognized allergen [43]. CREG1 (where CREG is cellular repressor of E1A-stimulated genes) protein is related to factors inhibiting cellular growth and differentiation [44]. Six MRJPs were identified in our study. Although homologous with the yellow-like proteins, which are common in arthropods, the MRJPs have been uniquely identified within the *Apis* genus [45]. They constitute ~90% of total proteins of royal jelly [46], the substance produced, secreted, and fed to developing larvae by nurse bees. A recent shotgun proteomics study encompassed the proteomes of royal jelly and derivation glands [47]. Besides the expression of these proteins in hypopharyngeal glands, MRJP1 and MRJP2 were also found in the brains of worker bees [48]. Notably, MRJP

8 and 9 were recently recognized by the International Union of Immunological Societies as potential allergens [21]. Here, MRJPs 1, 2, 3, and 5 were for the first time identified in HBV. Considering all unique proteins found for each sample, two of four unique proteins for AHB venom (AHBV) represent new venom proteins, MRJP 1 and MRJP 5. Moreover, vitellogenin is another protein encountered only in *A. m. ligustica* venom. Vitellogenin, common yolk precursor protein of oviparous taxa, acts as an antioxidant to promote longevity in queen bees [49, 50] and has been recently included into the list of honeybee allergens (ALLERGEN NOMENCLATURE IUIS – Allergen Nomenclature Sub-Committee Home, <http://www.allergen.org>).

The LF quantification revealed that the abundances of ten and four proteins were significantly changing when comparing *A. m. carnica* and *A. m. ligustica* venoms to AHBV, respectively (Table 1). We note that the normalization of LC-MS/MS runs to the total intensity of all matched peptides, while necessary to resolve technical variability in sample concentrations, may lead to quantitative results that do not necessarily follow the trend suggested by changes in spectral counting or sequence coverage for any particular protein (Table 1). The analysis of a protein with lower total intensity will generally result in fewer matched peptides. While the normalization will adjust their total intensity, the number of matched peptides remains the same. However, within series of samples with

Table 2. Variability of melittin sequences in HBV revealed by LC-MS/MS

Calculated mass (Da)	Experimental <i>m/z</i>	Sequence ^{a)}	Mass deviation, ppm	Normalized XIC ^{b)} intensity (%)		
				A	C	L
1496.8967	749.4569	VLTTGLPGLISWIK	1.75	4.11×10^0	5.97×10^{-3}	4.27×10^{-3}
1526.9072	764.4594	VLTTGLP SL ISWIK	−1.92	7.62×10^{-1}	5.52×10^{-1}	7.62×10^{-1}
1536.9279	769.4714	VLTTGLPPLISWIK	−0.31	1.04×10^{-1}	1.07×10^{-1}	1.32×10^{-1}
1540.9229	771.4675	VLTTGLPTLISWIK	−1.53	1.48×10^{-2}	1.29×10^{-2}	1.94×10^{-2}
1552.9592	777.4858	VLTTGLPILISWIK	−1.44	4.33×10^{-4}	2.85×10^{-4}	2.32×10^{-4}
1553.9181	777.9885	VLTTGLPNLISWIK	2.80	1.23×10^{-1}	3.51×10^{-4}	1.78×10^{-4}
1554.9021	778.4582	VLTTGLPDLISWIK	−0.19	5.11×10^{-2}	3.63×10^{-2}	3.70×10^{-2}
1567.9338	784.9763	VLTTGLPQLISWIK	0.97	6.96	8.25	6.73
1568.9178	785.4689	VLTTGLPELISWIK	3.46	1.75×10^{-2}	2.64×10^{-2}	1.06×10^{-2}
1599.9058	800.9584	VLTTGLPCLISWIK	0.62	5.01×10^{-3}	5.22×10^{-3}	7.93×10^{-3}
1625.9545	813.9816	VLTTGLPWLISWIK	−3.26	1.72×10^{-5}	5.63×10^{-5}	0.00
1510.9123	756.4638	VLTTGLPALISWIK	0.51	87.9	91.0	92.3
1590.8786	796.4443	VL p -TTGLPALISWIK	−0.60	4.53×10^{-4}	5.71×10^{-5}	1.65×10^{-5}
1590.8786	796.4461	VLTTGLPALI p -SWIK	−2.83	8.56×10^{-4}	7.63×10^{-5}	4.67×10^{-5}

XIC (extracted ion chromatogram) signals were normalized to the sum of intensities of all sequences in each respective sample to highlight the within-sample sequence variability.

a) The fragment of 8–21 of full melittin sequence. Identified sequences only differed at 15 position (in bold) of the full sequence; phosphorylated residues are designated as *p*-S and *p*-T. Amino acids substitutions were confirmed by manual inspection of CID MS/MS spectra acquired from *A. m.* Africanized venom.

b) XIC intensities of monoisotopic precursors were calculated from *A. m.* Africanized venom sample and normalized to the sum of all intensities to show the relative abundance.

high compositional similarity, variations of the common TIC normalization are generally thought to be the best approach to ensure fair quantitative representation of each sample prior to the statistical sample-to-sample comparisons [51].

Among those ten proteins, five are well-known toxins and two were enriched in *A. m. carnica* venom (venom serine protease 34 and allergen Api m6). When comparing *A. m. ligustica* and AHBV, among four significantly changing proteins, three allergens were enriched in the European bee venom (venom serine protease 34, venom allergen acid phosphatase, and venom dipeptidyl peptidase 4). Therefore, notable differences in allergenic proteins could be an important factor for HBV immunotherapy and using specific venoms could lead to better protection of allergic people. Conversely, only 11 of the 51 proteins are significantly changing between all the samples and nine of these are enriched in AHBV. The abundance of major toxin melittin is unchanging, and the second major toxin phospholipase A2 and mast cell degranulating peptide are more abundant in AHBV. Considering these results in terms of the neutralization of toxic effects, it is highly likely that an antivenom specific for AHBV should be efficient for victims of any subspecies.

Despite the unchanged content of melittin in all three venoms, we searched for other subspecies-specific differences of this major toxin including PTMs and sequence polymorphisms. In this direction, 12 polymorphic sequences of melittin were identified in AHBV and *A. m. carnica* venom and 11 sequences in *A. m. ligustica*, all differing by the amino acid in the 15th residue of the full sequence (Table 2). We performed extracted ion chromatogram quantification to unravel the extent of its polymorphism in each venom sample, although we

could not cover the full melittin sequence because the analyses lacked corresponding N- and C-terminal peptides. The original and only previously known sequence, with an alanine residue in the variable position (¹⁵Ala), was the most abundant for all bee species. It was followed by the three next most abundant peptides, in which the alanine residue was replaced by glutamine, serine, and proline. The polymorphic peptide abundances were very similar across all samples for all sequences except two, (¹⁵Gly) and (¹⁵Asn), which were noticeably higher in AHBV. The observed polymorphism may be related to the mating behavior of the honeybee queen, which copulates with more than a hundred drones during the nuptial flight, accumulating individual packages of sperms giving rise to female worker bees offspring [52].

3.2 Identification of phosphorylated toxins

Among all the identifications, we discovered the phosphorylation of melittin, the major venom toxin, and icarapin, also a known toxin and allergen. The phosphorylated melittin was found in all bee venoms (Supporting Information Fig. 2).

Interestingly, among the 12 polymorphic sequences of melittin only one peptide was phosphorylated, which is the most abundant and previously characterized sequence version having alanine residue in the variable position (Table 2). Two monophosphorylated forms were identified, with the phosphate group located at ¹⁰Thr and ¹⁸Ser positions of the full melittin sequence. Figure 2 presents the manually annotated MS/MS spectra of tryptic peptide ⁸VLTTGLPALISWIK²¹ phosphorylated at the ¹⁸Ser position

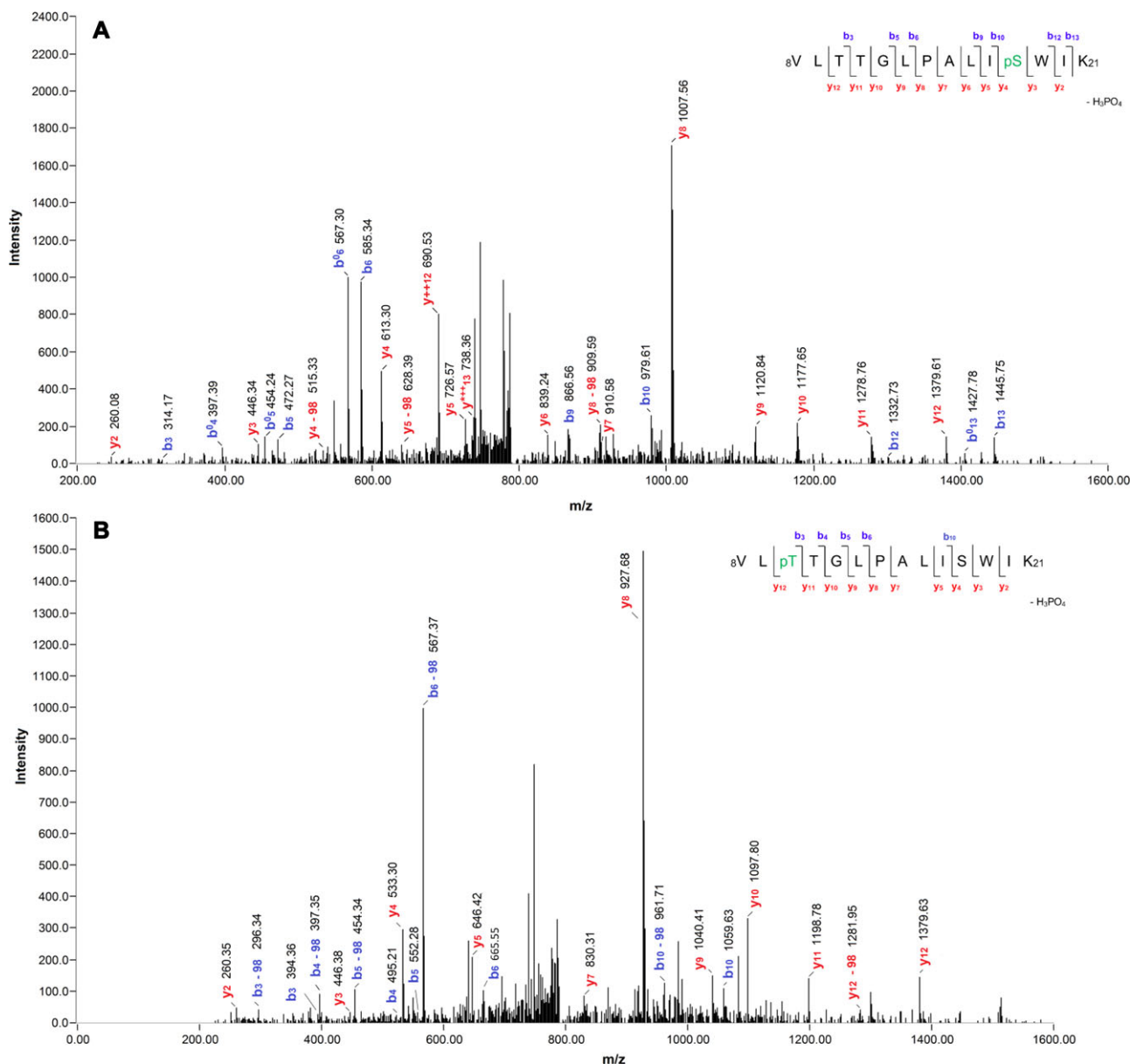


Figure 2. CID MS/MS spectra of the tryptic peptide ⁸VLTGLPALISWIK²¹ phosphorylated at ¹⁸S (panel A) and ¹⁰T (panel B).

([M+2H]²⁺; *m/z* 796.4461) (Fig. 2A) and the ¹⁰Thr position ([M+2H]²⁺; *m/z* 796.4443) (Fig. 2B). Abundant y₈ fragment (*m/z* 1007.5) with N-terminal proline undergoes neutral loss of the phosphate group, among the many other phosphate-lost fragment ions, unequivocally locking the modification at ¹⁸Ser. Conversely, the MS/MS spectrum of the alternative form showed no y-ions, while neutral loss fragments from b₃ and y₁₂ ions localized the phosphorylation site at ¹⁰T.

Another novel phosphorylated protein identified was icarapin. It is a 24 kDa protein described as a new HBV allergen [12]. The phosphorylated tryptic peptide ²⁰²SVEpSVEDFDNEIPK²¹⁵ was found in all three venoms.

Although phosphorylated peptides were clearly detectable and identifiable, the abundance ratio between phosphorylated and native forms was below 0.01%. However, it remains unclear if it is representative of their true physiological levels.

It was previously demonstrated that PTMs affect the specific recognition of antigenic determinants, leading to a different elicitation of an immune response [25, 27, 53]. Particularly, the phosphorylation site is an important allergenic epitope, and its alteration affects the allergenicity of the proteins [23, 54, 55], hence it is conceivable that phosphorylated mellitin and icarapin might also exert the allergenic response different from the native peptide.

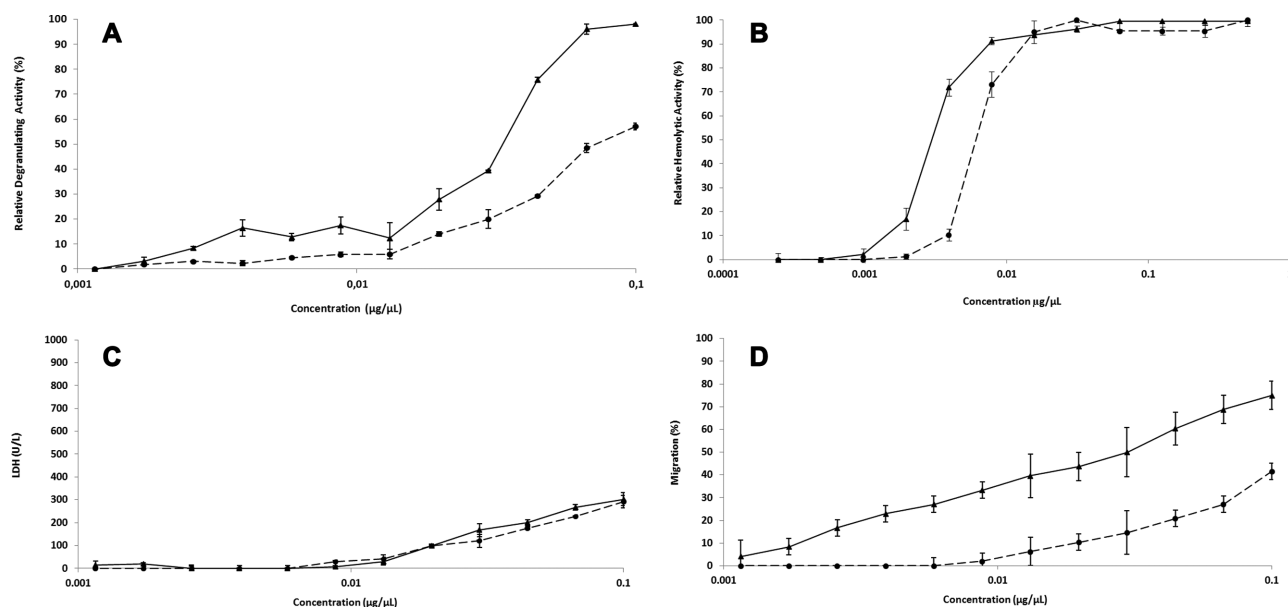


Figure 3. Toxicological properties of native melittin (▲) and ¹⁸S phosphorylated melittin (●) tested using rat cells. (A) Mast cells degranulating activity; (B) hemolytic activity tested by red blood cell lysis. The activity (%) was calculated considering cell lysis by 0.1% v/v Triton X-100 as 100% and by saline as 0%; (C) stimulated release of lactate dehydrogenase (LDH) from mast cells; (D) melittin-induced chemotactic activity. It is expressed as percentage of polymorphonuclear leucocytes migration measured by manual cell counting. In all experiments data points represent the mean ± SD of triplicates ($n = 3$).

3.3 Toxicological properties of phosphorylated melittin

We further compared the toxicity of the phosphorylated/unphosphorylated forms of the most prominent phosphorylated ¹⁸Ser position of the major venom toxin, melittin.

Our tests encompassed three key aspects of its known toxicological activity: its ability to lyse erythrocyte membranes, its signaling capability on the membrane surface of other cells, as well as its stimulation of inflammatory cells (Fig. 3). Due to the occurrence of *p*-¹⁸S-melittin in all HBVs and because of its ca. twofold higher abundance compared to *p*-¹⁰T-melittin (Table 2) we used it for toxicological tests.

We examined the peptide cytotoxicity using the mast cell degranulating assay (Fig. 3A) and observed dose-dependent stimulation of degranulation by both phosphorylated and native melittin peptides, while the former was twofold less active. To assay the hemolytic activity we monitored the lysis of rat erythrocytes. We observed that melittin phosphorylation decreased the hemolytic activity by threefold, as judged by the ratio of concentrations required for 50% hemolysis (Fig. 3B). The lytic activity of melittin peptides on mast cells was assayed by monitoring co-release of lactate dehydrogenase (Fig. 3C). Here, the activities of phosphorylated and native peptides were similar. Next, chemotaxis was assessed using rat polymorphonuclear leucocytes, whose migration was measured by manual cell counting (Fig. 3D). Although chemotactic response was induced by both forms of melittin, native peptide

reached 50% of cell migration at tenfold lower concentration than its phosphorylated form.

Taken together, these results demonstrate that the ¹⁸S phosphorylation reduced—yet, not abrogated completely—the melittin toxicity in all common tests. This finding is in line with previous observations that linked sequence isoforms of melittin to a reduction in hemolytic activity [56]. This indicates that any variation to the primary form of melittin, be it PTM or sequence polymorphism, will likely reduce toxicity. We note, however, that reduced toxicity does not necessarily indicate it might be less potent in antimicrobial or antibacterial tests.

4 Concluding remarks

This work reports in-depth characterization of venoms from three *A. mellifera* honeybee subspecies (Africanized, *A. m. carnica* and *A. m. ligustica*) by shotgun LC-MS/MS analysis. By applying long (6 h) LC gradients and high-resolution MS, we identified 51 proteins, many of which were not previously described as venom components. From these 51 proteins, 42 were shared among all three venoms, including all toxic proteins. Considering the quantitative changes of several known allergens, our results indicate that venom choice may influence immunotherapeutic outcomes within allergic patients. Conversely, due to the overall qualitative and quantitative similarity of the venoms and the quantitative similarity of major toxins, it is highly likely that antivenoms specific for AHBV

should be fully protective in the treatment of toxic reactions from other European honey bee subspecies.

Importantly, we discovered that at least two major toxins—icarapin and melittin—were phosphorylated. Basic toxicological tests revealed that phosphorylation reduced melittin activity compared to the native peptide. Our findings, however, underscore the need to revise basic venomomics strategies by emphasizing the characterization of PTMs and the variability of molecular forms of major toxins.

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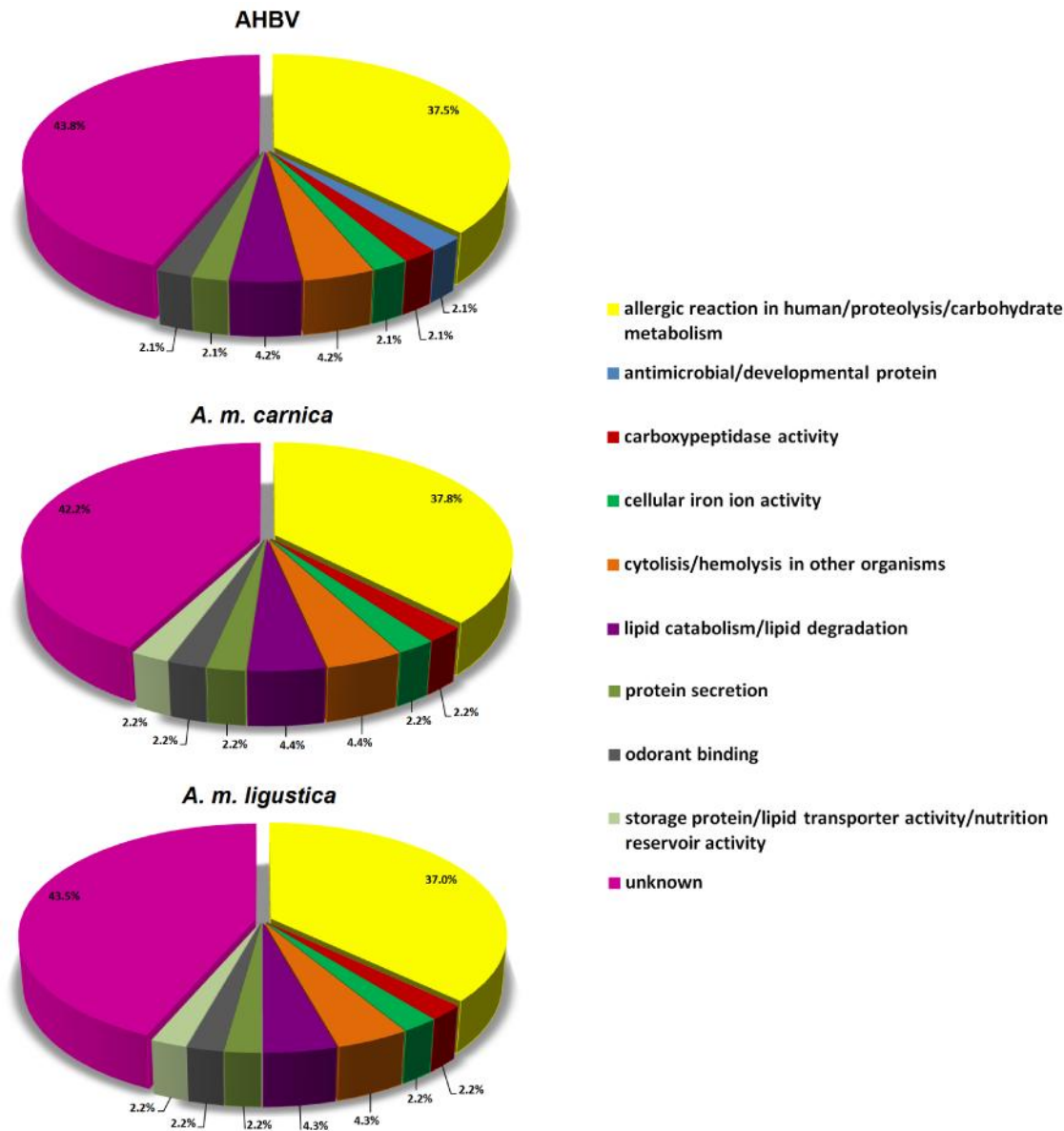
The authors have declared no conflict of interest.

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Supplementary Figure S1: Gene Ontology distribution of proteins from the three different honey bee venoms analyzed. Percentages are calculated as the total number of proteins in a specific GO category divided by the total number of identified proteins.



Supplementary Figure S2: Extracted-Ion Chromatogram (XIC) of the two monophosphorylated melittin peptides, $^8\text{VLTGLPALIpSWIK}^{21}$ (RT: 283.23 min) and $^8\text{VLpTTGLPALISWIK}^{21}$ (RT: 294.21 min).

