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Beyond the antipredatory defence: Honey bee venom function as a component of social immunity

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ABSTRACT

The honey bee colonies, with the relevant number of immature brood and adults, and stable, high levels of humidity and temperatures of their nests, result in suitable environments for the development of microorganisms including pathogens. In response, honey bees evolved several adaptations to face the increased risks of epidemic diseases. As the antimicrobial venom peptides of *Apis mellifera* are present both on the cuticle of adult bees and on the nest wax it has been recently suggested that these substances act as a social antiseptic device. Since the use of venom by honey bees in the context of social immunity needs to be more deeply investigated, we extended the study of this potential role of the venom to different species of the genus *Apis* (*A. mellifera*, *Apis dorsata*, *Apis cerana* and *Apis andreniformis)* using MALDI-TOF mass spectrometry techniques. In particular we investigated whether (similarly to *A. mellifera*) the venom is spread over the body cuticle and on the comb wax of these three Asian species. Our results confirm the idea that the venom functions are well beyond the classical stereotype of defence against predators, and suggest that the different nesting biology of these species may be related to the use of the venom in a social immunity context. The presence of antimicrobial peptides on the comb wax of the cavity-dwelling species and on the cuticle of workers of all the studied species represents a good example of "collective immunity" and a component of the "social immunity " respectively.

1. Introduction

The use of weapons as defence against enemies was one of the major factors that favoured the evolution of social life in insects (Andersson, 1984; Starr, 1985). In the eusocial Aculeate Hymenoptera, the stinging apparatus and the venom, originally evolved as devices to paralyse preys, became arms to defend the colony mainly from the attacks of vertebrate predators. More than any other group of social insects, honey bee colonies, with their rich store of honey and pollen, high mass of immature brood and adults, are rewarding targets for many predators. At the same time, another important selective force, directly consequent to the development of a complex sociality, is represented by the necessity of defence against pathogens and parasites (Otvos, 2000). Indeed, the constant and relatively high temperature and the high humidity levels maintained in a bee nest results in a suitable environment for the incubation of microorganisms (protozoa, fungi, bacteria and viruses) which in most cases can be pathogenic. In response, honey bees have evolved several physiological, behavioural, and organisational adaptations to fight the increased risks of epidemic diseases (Wilson-Rich et al., 2009). The strategies honey bee species adopt to reduce these risks include the collection and use of antimicrobial natural substances (such as propolis (Simone-Finstrom and Spivak, 2010)) and glands secretions which are particularly

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important as primary barriers to infection (Zasloff, 2002; Stow et al., 2007). Venom gland, in honey bees as well as in other Hymenoptera, has recently been reported as an important source of antimicrobial substances (Kuhn-Nentwig, 2003). The application of venom on the body surface as a way of protection against pathogens has been suggested for the ants *Solenopsis invicta* (Obin and Vander Meer, 1985) and *Pachycondila goeldii* (Orivel et al., 2001), for the paper wasp *Polistes dominulus* (Turillazzi et al., 2006) and for the honey bee *Apis mellifera* (Baracchi and Turillazzi, 2010). Honey bee venom is composed of a wide spectrum of molecules, ranging from biogenic amines to peptides and proteins, the structure and function of which have been determined in great part (Kreil, 1973; Hoffman, 1996). Three peptides are the major compounds of the venom fraction ranging from 1000 to 4000 Da. Melittin, a basic peptide of 26 residues (~2.8 kDa), accounts for 50% of the venom dry weight (Ownby et al., 1997; de Lima and Brochetto-Braga, 2003) and exhibits an amphipathic structure and lipid membrane permeability. Apamin is a small 18 amino acids (MW ~2.0 kDa) peptide accounting for less than 2% of venom dry weight which presents a neurotoxic action (de Lima and Brochetto-Braga, 2003). MCD peptide comprises 22 amino acid residues (MW ~2.6 kDa) and causes mast cell break down; it accounts for about 2% of the venom dry weight (de Lima and Brochetto-Braga, 2003). Recently we have confirmed that these three molecules at *m/z* 2026.82, 2586.53 and 2845.63 together with many other venom compounds are present also on the cuticle of the workers and on the nest wax of *A. mellifera* (Baracchi and Turillazzi, 2010). As the antiseptic properties of melittin is well documented (Kuhn-Nentwig, 2003) and an antimicrobial action was also suggested for apamin and MCD (Froy and Gurevitz, 1998), we hypothesized that venom peptides act as a colonial antimicrobial protection (Baracchi and Turillazzi, 2010). The wax comb could represent a 'medium'' where the venom is deposited to act as a social antiseptic device. In this case, the use of the venom blend can be considered as a component of the so called social immunity (see Cremer et al., 2007; Wilson-Rich et al., 2009; Cotter and Kilner, 2011) together with other physiological, behavioural and organisational adaptations, such as social fever (Starks et al., 2000), nest construction with antimicrobial material (Simone et al., 2009) and hygienic behaviours against pathogens and parasites (Spivak and Gilliam, 1998a,b; Richard et al., 2008). The use of venom by *A. mellifera* in the context of social immunity, as well as the composition and the role of venom in other species of the genus *Apis*, needs to be more deeply investigated, for example with respect to the ecology and the environmental constraints. For these reasons in the present research we extended the study of the venom peptides across three other species of the genus *Apis*: *Apis dorsata*, *Apis cerana* and *Apis andreniformis*.

Within the nines species of *Apis*, there is a wide variation in the nesting biology; according to their nests features, species are divided into two groups: the cavity-dwelling species (*A. (Apis) cerana*, *A. (Apis) koschevnikovi*, *A. (Apis) mellifera*, *A. (Apis) nigrocincta* and *A. (Apis) nuluensis*) and the open nesting species. The latter are further divided, on the basis of their body size in dwarf honey bees (*A. (Micrapis) florea* and *A. (Micrapis) andreniformis*) and giant honey bees (*A. (Megapis) dorsata* and *A. (Megapis) laboriosa*).

The aim of this study is to evaluate if the environmental constraints (as a consequence of the different nesting biology) and the ecology of the three subgenera of honey bees could affect the composition and the use of the venom in the logic of social immunity. We performed, for the first time, an analytical survey of the medium weight polar substances of the venom of *A. cerana, A. dorsata* and *A. andreniformis* using MALDI-TOF MS. Subsequently, we investigated whether and how, similarly to *A. mellifera*, venom peptides were spread both over the body cuticle and on the wax of the nest of these three Asian species.

2. Materials and methods

2.1. Venom and cuticular compounds collection

During January 2010, 20 newly emerged workers, 30 nurses, 20 guards, 30 foragers and 20 drones were collected and killed by freezing from a colony of *A. cerana* hosted in a wooden small hive at Bukit Katil, Melaka State, Malaysia. We also collected 3 queens from other colonies in the same locality and 40 foragers from a wild hive of *A. cerana* found at Genting Sempah, Pahang State, Malaysia. We then collected and killed by freezing 30 newly emerged workers and 30 adult workers from a wild colony of *A. dorsata* at Bukit Katil, Melaka State, Malaysia, and 30 adult workers from a wild colony of *A. andreniformis* found at Genting Tea Estate, Pahang State, Malaysia. Unfortunately, neither queen nor drones were found in these two latter colonies. Half specimens of each species were dissected and drops of their venom were extracted directly from the tip of the sting with a small capillary glass tube after gently squeezing the venom sac with a glass plate (capillary extraction). A small piece of the capillary containing venom was then placed in a 250 μl glass conical insert with 50 μl of methanol. The other half of the specimens of each species, the queen and the drones of *A. cerana* were singly washed in 200 μl of methanol for 2 min to extract the overall epicuticular polar compounds. During this procedure each bee was held by the tip of its abdomen using a pair of forceps to prevent any possible methanol contamination by the venom. All the extracts were analysed by MALDI-TOF mass spectrometry.

To exclude any possible contamination of the cuticle with the venom during the dissection procedure, we also performed methanol *in situ* micro-extractions on the first gastral tergite of 4 intact workers of *A. cerana* and of 4 intact workers of *A. dorsata*. We directly dispensed 5 μl of methanol on the cuticle pipetting it back at once and then mixing 1 μl of the extract with 1 μl of α-cyano-4-hydroxycinnamic acid (α-CHCA) (10 mg/ml) dissolved in 70/30 Acetonitrile/TFA 0.1% (MALDI matrix) previously deposited on a MALDI plate (Turillazzi et al., 2006). The venom and the cuticle of 30 foragers belonging to three colonies of *A. mellifera ligustica* (Florence, Central Italy) was also extracted and analysed with the same method in order to obtain spectra comparable with those of the Asian species. Finally we collected samples of wax from the

combs of the four species and extracted them in 200 μl of methanol for 2 min before analysing the extracts by MALDI-TOF mass spectrometry.

2.2. Mass spectrometry analyses

2.2.1. MALDI-TOF spectra profiling

Methanol extracts of the venom and of the overall cuticle from each individual bee were analysed using a MALDI-TOF/TOF Ultraflex III (Bruker Daltonics, Bremen, Germany). The instrument was operated in positive ion reflector mode. The accelerating voltage and the Ion Source 2 were set to 25.0 and 21.9 kV, respectively, and the delay time was 20 ns. One μl of the sample was mixed with MALDI matrix (1:1, vol:vol), previously spotted on a stainless steel MALDI target; 800 shots were automatically accumulated for each sample. External calibration was performed with the Bruker Standard Peptide Calibration kit (*m/z* 1000–3500) and the peptidic fraction of the samples was acquired in the range *m/z* 800–4000.

2.2.2. Peptide identification (MALDI-TOF/TOF MS and LC-ESI high resolution MS (LC-ESI/HRMS))

MS/MS spectra of some cuticular peptide ions were recorded on a MALDI-TOF/TOF Ultraflex III mass spectrometer (Bruker Daltonic, Italy) using the LIFT™ method. The sequence of the peptides was deduced by interpreting the MS/MS spectra and by comparing the obtained sequences with those reported in the literature (data not shown). The cuticular extraction samples were submitted also to LC-nano ESI-HRMS analysis by using an Ultimate 3000 HPLC system (Dionex LC Packings, Sunnyvale, CA) coupled to an LTQ Orbitrap mass spectrometer (Thermo Electron Corporation, San Jose, CA). A PepSwift Monolithic PS-DVB column (200 μm I.D. × 5 cm, Dionex LC Packings) was used and it was maintained at a temperature of 45 °C. The gradient solvent system for the micro pump, consisting of 0.1% aqueous formic acid (A) and acetonitrile with 0.1% of formic acid (B), was the following: 0–2 min, 2% B; 2–12 min, 2–50% B; 12–12.5 min, 50–90% B; 12.5–15 min, 90% B; 15–15.5 min, 90-2% B; 15.5–22.5 min, 2% B. The flow rate was maintained at 3 μL/min throughout the analysis. In all cases the injection volume was 10 μL. The nanoESI interface and mass spectrometer parameters were the following: spray voltage 1.25 kV, capillary voltage 40 V, capillary temperature 210 °C, tube lens 115 V. Data were acquired in a range *m/z* 400–2000 with a nominal resolution of 30,000 (at *m/z* 400) in positive ion-mode. Data were processed by using the Xcalibur software with the "Xtract" tool for the calculation of deconvoluted average and monoisotopic molecular weight.

2.3. Statistical analyses

Calibrated spectra were imported into the ClinProTools™ (CPT) software and processed with a procedure described in a previous study on *A. mellifera* (Baracchi and Turillazzi, 2010). The software calculated the areas of the most important peaks that account for statistical differences between the venom of the various groups. These peaks were used by the software to generate a model to classify the spectra and eventually to assign any other new spectrum to a particular group. CPT parameters used for model generation in the various analyses were standardized in order to obtain comparable results: Peak width: 0.01; Smoothing (width: 5 Da, cycles: 1); Average Peak List Calculation (Relative Threshold Base Peak: 0.01, Signal-to-Noise Threshold: 3, Limit Peak Number: false); Area Calculation (Integration Type: end point level); Peak Selection (Use All Peaks: true, Sort Mode: *P*-value tta); Model Generation (Algorithm: Quick Classifier); Cross Validation (Percent Leave Out: 20%, Number of Iterations: 10).

3. Results

3.1. Venom reference spectra

3.1.1. Venom of A. andreniformis, A. cerana, A. dorsata and A. mellifera

The identification of melittin for *A. cerana*, *A. dorsata*, *A. andreniformis* and apamin for *A. andreniformis* was confirmed by sequencing the respective peaks by MALDI-TOF MS/MS analysis and via determination of the exact MW with LTQ Orbitrap analysis (*A. cerana:* Exp. Mw = 2845,75 Da; *A. dorsata*: Exp. Mw = 2846,72 Da and *Apis andreniformins*: melittin Exp. Mw = 2817,72 Da and apamin Exp. Mw, 2073,18 Da). The identification of apamin for *A. cerana* and *A. dorsata* was confirmed by the determination of the exact MW using the LTQ Orbitrap (*A. cerana*: Exp. Mw = 2026,89 Da and *A. dorsata*: Exp. Mw = 2026.83 Da).

The CPT software found 26, 27,27 and 29 main peaks in the venom of *A. cerana, A. dorsata, A. andreniformis* and *A. mellifera* respectively. These compounds have molecular weights in the range 990–3020 Da, 998–3049 Da, 990–3143 Da and 1006–3148 Da respectively (Table 1).

Using the venom spectra of these four species, CPT model recognized 98.33% of workers to their species with a cross validation of 95.7%. In the cross validation test the 100% of both *A. mellifera* and *A. andreniformis,* the 90% and 93% of *A. dorsata* and *A. cerana* respectively were correctly assigned to their group.

3.1.2. Venom of different classes of workers of A. cerana

Fig. 1A reports the representations in Gel View (Facility supplied by the CPT software) of the pure venom of the four classes of workers analysed in *A. cerana*: newly emerged workers ($N = 10$), nurses ($N = 15$), guards ($N = 10$) and foragers ($N = 15$). CPT model recognized correctly only 67.51% of the bees in the assigned class with a cross validation of 65.1% using melittin as the main peak for discrimination (Table 2). However the newly emerged bees were recognized at 100% with respect to all adult bees. In fact, as it can be observed from the Gel View figure, the venom of the newly emerged bees visibly differentiates for the lack of all the most abundant peaks, like apamin and melittin, with respect to those of the other groups. When we compared the venom of the foragers belonging to the two different sampled colonies the CPT model recognized correctly 76.1% of the bees with a cross validation of 65.7%.

Table 1
List of the peaks ranging from 950 Da to 4000 Da identified by CPT in a set of 40, 30, 30 and 30 workers crude venom of *A. cerana, A. dorsata, A. andreniformis* and *A. mellifera* respectively and relative percentages in brackets. As $Z = 1$, value are in equivalent Daltons.

Venom Peaks *(m/z)*			
Apis cerana	*Apis dorsata*	*Apis andreniformis*	*Apis mellifera*
990 (0.001)	998 (0.001)	990 (0.013)	1006 (0.001)
1017 (0.001)	1073 (0.001)	1017 (0.011)	1014 (0.44)
1028 (0.001)	1260 (0.001)	1039 (0.115)	1024 (0.001)
1045 (0.001)	1274 (0.001)	1042 (0.005)	1034 (0.002)
1260 (0.001)	1897 (0.00)	1307 (0.009)	1060 (0.001)
1430 (0.001)	1973 (0.001)	1355 (0.003)	1066 (0.001)
1442 (0.011)	1991 (0.11)	1420 (0.072)	1105 (0.003)
1997 (0.001)	1999 (0.001)	1429 (0.212)	1294 (0.11)
2013 (0.029)	2014 (0.58)	1938 (0.116)	1434 (0.14)
apamin (1.85)	apamin (4.31)	2035 (0.240)	1443 (0.27)
2113 (0.001)	2083 (0.001)	2059 (0.518)	1454 (0.062)
2190 (0.005)	2121 (0.001)	apamin (6.10)	1462 (0.034)
2208 (0.046)	2175 (0.001)	2083 (0.113)	1510 (0.001)
2269 (0.001)	2190 (0.001)	2511 (0.152)	1524 (0.047)
2307 (0.001)	2239 (0.001)	2780 (0.760)	1668 (0.039)
2592 (0.17)	2575 (0.001)	melittin (80.54)	1761 (0.001)
2793 (0.79)	2793 (0.72)	2830 (0.575)	1797 (0.011)
2809 (4.05)	2811 (5.39)	2875 (1.917)	1996 (0.17)
2820 (0.53)	2820 (1.00)	2899 (0.580)	2013 (1.03)
melittin (88.47)	melittin (82.87)	2918 (1.366)	apamin (16.16)
2859 (1.44)	2859 (1.49)	2940 (0.473)	2208 (0.32)
2877 (0.08)	2906 (1.09)	2981 (5.065)	2335 (0.004)
2904 (0.47)	2946 (0.16)	2992 (0.122)	2435 (0.12)
2993 (0.28)	2995 (0.33)	3002 (0.167)	2588 (1.17)
3009 (1.77)	3010 (0.44)	3019 (0.450)	2793 (0.44)
3020 (0.037)	3031 (0.73)	3037 (0.099)	2809 (2.81)
	3049 (0.75)	3143 (0.197)	melittin (76.51)
			2879 (0.001)
			3048 (0.49)

3.1.3. *Venom of different classes of workers of* A. dorsata

Fig. 2A reports the representations in Gel View of the pure venom of the two classes of workers analysed in *A. dorsata*: newly emerged bees ($N = 15$) and older bees ($N = 15$). CPT model recognized correctly 100% of the bees in the assigned class with a cross validation of 96.3% using as main peaks for discrimination melittin, and those at *m/z* 2809 and 2793 (Table 2). In this case, the newly emerged bees were characterized by the lack of all the main peaks with *m/z* higher than 2500 which were present, instead, in the venom of the adult bees.

3.2. *Cuticular extracts reference spectra*

3.2.1. *Cuticular extracts of queens, drones and different age classes of workers of* A. cerana

The relative amount of substances detected by the MALDI MS in the total body methanol extracts of drones was very low with respect to those of the females. Precisely only 3 out of 20 analysed males presented melittin and apamin but only in traces. The main peaks found in the venom (melittin and apamin) were still the most abundant ones in the spectra of the cuticle extracts of the workers while other peaks, ranging from 1000 to 1100 Da, were exclusively present in the cuticle extracts. CPT model recognized 100% of queens and workers with a cross validation of 100% using the peaks at *m/z* 1997, *m/z* 2190 and *m/z* 1196. A CPT model applied to the workers in order to check for differences among newly emerged bees, nurses, guards and foragers did not furnish a good discrimination (56.88% of recognition with 46.51% of cross validation) when using mellitin and peaks at *m/z* 2307 and 2435. However, also in this case, a perfect discrimination was obtained between newly emerged workers, which did not present any peaks in the cuticle extracts, and all the other females (Fig. 1B). When we compared the cuticular extract of the foragers belonging to the two different colonies analysed the CPT model recognized correctly 79.2% of the bees with a cross validation of 68.3%.

3.2.2. *Cuticular extracts of workers of* A. dorsata

CPT model applied to newly emerged bees and adult bees provided a perfect discrimination (100% of recognition with 100% of cross validation) using apamin and the peak at *m/z* 2190 (Table 2).

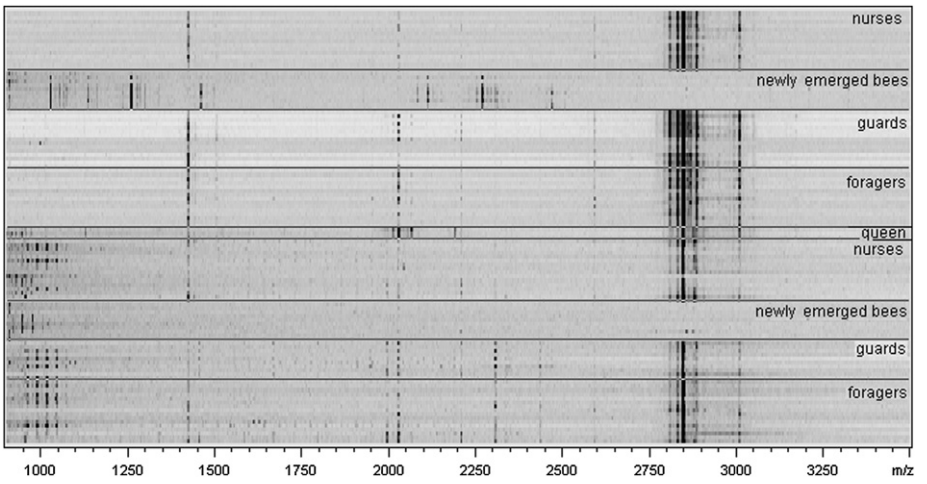

Fig. 1. Gel View Facility supplied by the CPT software in order to choose the most suitable peaks for discrimination of *A. cerana* venom (A) and cuticle (B). The spectral pattern of each individual is shown as a horizontal line with grey intensity standing for the relative abundance of the peaks.

Table 2
CPT model for recognition of 950–4000 Da fraction of the venom of individuals belonging to different groups of bees and the cuticle of *A. cerana* and *A. dorsata*.

	Recognition capability (%)	Cross validation (%)	Principal peaks *m/z*
Apis cerana			
Venom Foragers/guards/nurses/newly emerged bees ($N = 50$)	67.51	65.10	melittin
Venom Adult bees/newly emerged bees ($N = 50$)	100	100	2820 melittin
Cuticular layer Queens/workers ($N = 43$)	100	100	1997 2190 1196
Cuticular layer Foragers/guards/nurses/newly emerged bees ($N = 50$)	56.88	46.51	1997 2307 melittin
Cuticular layer Adult bees/newly emerged bees ($N = 50$)	100	100	2793 2809 melittin 2904
Apis dorsata			
Venom Adult bees/newly emerged bees ($N = 30$)	100	96.3	2793 2809 melitin
Cuticular layer Adult bees/newly emerged bees ($N = 30$)	100	100	apamin 2190

3.3. Comparison among extracts of the cuticle and of the venom

The spectra registered from the cuticle extracts and the venom of workers of each species were imported together into the CPT software which selected 19, 19, 23 and 21 peaks in *A. cerana*, *A. dorsata*, *A. andreniformis* and *A. mellifera* respectively. Considering the threshold parameter of at least 0.1% for the quantity of a given peak, in *A. cerana* and *A. mellifera* 16 out of 19 and 16 out of 21 identified peaks were common to the venom and to the cuticle while in *A. dorsata* only 6 out of 19 peaks identified have been found in the venom and on the cuticle. Twelve out of the 23 identified peaks were common to the venom and to the cuticle in *A. andreniformis*. Three peaks in *A. cerana*, 4 in *A. dorsata* and *A. andreniformis* and 5 in *A. mellifera* workers have been detected only in the cuticle extracts even if these peaks were on average less than 3.0% of the quantities of the entire venom spectrum in all these species. Thirteen peaks in *A. dorsata* and 8 peaks in *A. andreniformis* were present only in the venom spectra and could not be found on the cuticle, while in *A. cerana* and *A. mellifera* all the peaks found in the venom were also found in the cuticle extracts (Figs. 3 and 4). In all the species, melittin was in percentage the most intense peak in the venom whereas in the cuticle extract melittin was almost absent in both *A. dorsata* and *A. andreniformis* which, instead, presented a higher peak intensity for apamin.

The spectra obtained with the *in situ* micro-extractions performed on the first gastral tergite of intact workers of *A. cerana* and *A. dorsata*, even if very feeble, substantially showed the presence of apamin and melittin on the cuticle of the first species and of apamin on the cuticle of the second one, confirming the results obtained on the body extracts.

3.4. Comb wax extracts

A further analysis performed on sample of comb wax of the nests of the four species demonstrated that the presence of venom compounds was an important feature of the hive environment at least in *A. mellifera* and *A. cerana*. Melittin was in percentage the most intense peak in the wax extracts of both these two cavity-dwelling species,

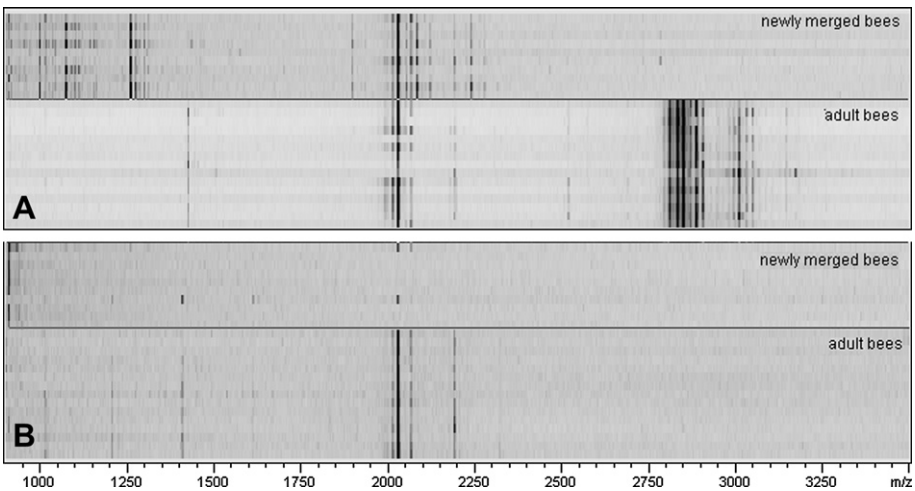

Fig. 2. Gel View Facility supplied by the CPT software in order to choose the most suitable peaks for discrimination of *A. dorsata* venom (A) and cuticle (B). The spectral pattern of each individual is shown as a horizontal line with grey intensity standing for the relative abundance of the peaks.

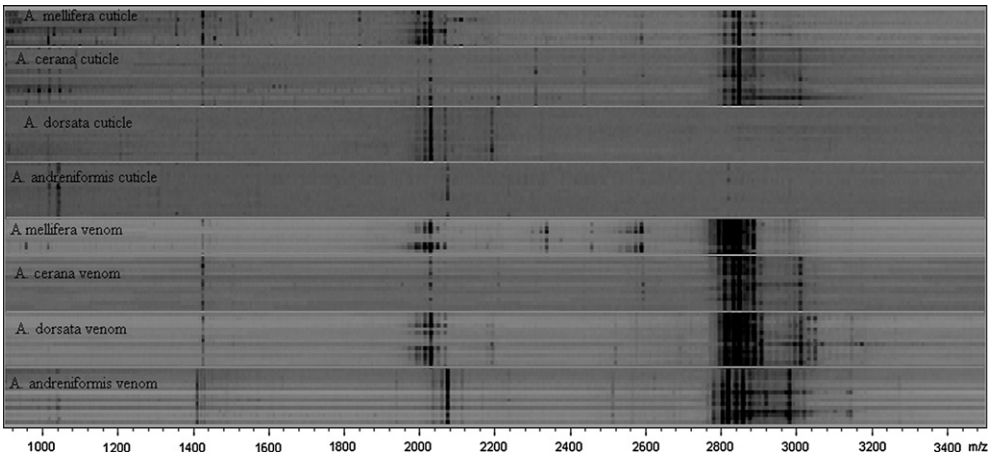

Fig. 3. Gel View Facility supplied by the CPT software when the data for the pure venom extract and for the cuticular extract of the four species were processed together.

while apamin was the second most intense peak only in *A. mellifera* and was present in minor quantities in *A. cerana* (reflecting the venom profile). On the contrary, no melittin nor apamin were found in the wax extracts belonging to the comb of the giant honey bee *A. dorsata* (where melittin was detected only in very low quantity) and of the dwarf honey bee *A. andreniformis*.

4. Discussion and conclusion

The venom of honey bees, as well as these of the other Hymenoptera species, is a complex and multifunctional secretion (Turillazzi, 2006). The present work shows as the medium molecular weight (MW) component of the venom (roughly from 900 Da to 4000 Da) is quite constant through the genus *Apis*. Indeed, in all the studied species the main peaks are invariably melittin and apamin which represent on average the 77–88% and 2–16% of the medium MW venom component respectively. On the other hand, as recently described for *A. mellifera* (Baracchi and Turillazzi, 2010), we confirmed the presence of several minor peaks in all the analysed venoms. Even if at the present we do not know exactly the nature of these compounds, they have a sufficient degree of variance among the species representing also a useful tool for taxonomic studies as previously found for other Hymenoptera (Turillazzi et al., 2007; Baracchi et al., 2009).

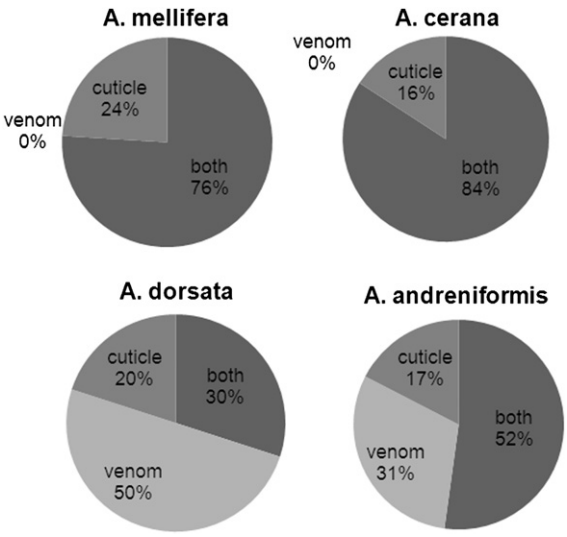

Fig. 4. Percentage of peaks characteristic of the cuticle, of the venom and present in both cuticle and venom.

Even if venom production is also influenced by the seasons (Ferreira et al., 2010), in an interesting paper, Schmidt states that *A. cerana* produces a quantity of venom which is half the quantity of that produced by *A. dorsata* and *A. mellifera* with the same body weight (Schmidt, 1995). However, comparing the 950–4000 Da fraction of the venom of *A. cerana*, *A. dorsata* and *A. mellifera* it is interesting to observe that the first one has the highest percentage of melittin with respect to the two other species. In this respect *A. andreniformis*, the smallest species of all examined in this research has even the highest of all. As melittin is the most toxic of all the compounds of the honey bee venom (Schmidt, 1995), the relative increase of this peptide could represent a compensation for the reduced size of the body and of the venom reservoir, supporting the defence against predators as one of the major driving force acting on venom.

However, as already stressed in the introduction, in the evolution of sociality the defencive function of the venom is coupled with its antimicrobial properties (Froy and Gurevitz, 1998; Kuhn-Nentwig, 2003). In this study we have found that the crude venom spectra belonging to the four species of honey bees show smaller differences with respect to those found in the cuticular extracts.

In particular, *A. mellifera* and *A. cerana*, have the cuticles profiles that are more similar (both for the number and for the relative abundance of the peaks) to the corresponding venom ones with respect to the two other species.

Intriguingly, the major difference occurs between the cavity-dwelling species and the open nesting ones: notwithstanding melittin and apamin are present in the venom of every species they have been found only on the cuticle of *A. cerana* and *A. mellifera* while are almost absent on the cuticle of *A. dorsata* and *A. andreniformis*. Similarly, melittin and apamin are present in the wax of both the two cavity-dwelling species but are not evident in the wax of the combs of the giant honey bee *A. dorsata* and of the dwarf honey bee *A. andreniformis*. This seems to be related to the different ecology and nesting biology of these groups of species, that is, the composition of the venom could be adapted to different micro environmental conditions of the colony in the logic of social immunity. Even if the temperature and probably the humidity of the hive are well regulated in all the *Apis* species, it is likely that inside a cavity the environment is more suitable for the incubation of microorganisms with respect to open space and the cavity nesting species are exposed to higher levels of microbial challenge. Even if we could not make comparative measures such as moisture levels and debris accumulation increvices or open colonies, and the possible variables determining microbial communities are numerous (Atlas, 1984), it is evident that dryness limits both general and pathogenic fungal abundance and richness (Talley et al., 2002).

The presence of venom peptides on the cuticle of all the examined species (even if in a very different amounts) may be explained by the smearing of the venom on the cuticle during self-grooming movements. This agrees with the complete lack of any venom peptides on the cuticle of newly emerged bees. The fact that these peptides are of venom origin is confirmed by the complete absence of them on the cuticle of *A. mellifera* (Baracchi and Turillazzi, 2010) and *A. cerana* drones.

Thus, assuming that venom gland is the only source of some of the cuticular peptides, and that bees are not able to choose which peptides spread selectively on the cuticles, it is possible that differences in cuticular profiles depends on the time these compounds are able to remain on and how often venom is applied on the insect bodies. Thus, by comparing the relative overall amount of peptides both in the venom and in the cuticle extracts of the examined species (see Figs. 3 and 4), it seems that *A. cerana* performs the highest "venom bathing" activity followed by *A. mellifera*. This scenario is supported by the higher rate of grooming activity characteristic of *A. cerana* with respect to *A. mellifera* (Büchler et al., 1992). The considerable amount of cuticular antimicrobial peptides found in *A. cerana* agrees also with the fact that this species shows a higher production of haemolimph antimicrobial peptides, as a consequence of a higher exposure to pathogens and infections (Xu et al., 2009). Our results, in line with those of a previous paper (Baracchi and Turillazzi, 2010), suggest that the venom function is beyond the classical stereotype of defence against predators, and that the wax comb, at least in the cavity-dwelling species, represents a "medium" where the venom is deposited to act as a social antiseptic device, representing a good example of "collective immunity" (*sensu* Cotter and Kilner, 2011). Further, in line with the recent broad definition by Cotter and Kilner, we propose to consider the presence of the major antimicrobial venom peptides (mainly melittin and, maybe, apamin) on the cuticle of the workers as a component of the so called "social immunity". This new definition encompasses the "herd immunity" (Frank, 1998) that arises from investment in personal immunity (i.e antimicrobial peptides spread on insect body), through the consequent reduction in the force of infection felt by neighbouring susceptible individuals (new emerged honey bees, drones and immature brood).

Conflict of interest

The authors declare that there are no conflicts of interest.

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