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# Unraveling the venom proteome of the bumblebee (*Bombus terrestris*) by integrating a combinatorial peptide ligand library approach with FT-ICR MS

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## ABSTRACT

Within the Apidae, the largest family of bees with over 5600 described species, the honeybee is the sole species with a well studied venom proteome. So far, only little research has focused on bumblebee venom. Recently, the genome sequence of the European large earth bumblebee (*Bombus terrestris*) became available and this allowed the first in-depth proteomic analysis of its venom composition. We identified 57 compounds, with 52 of them never described in bumblebee venom. Remarkably, 72% of the detected compounds were found to have a honeybee venom homolog, which reflects the similar defensive function of both venoms and the high degree of homology between both genomes. However, both venoms contain a selection of species-specific toxins, revealing distinct damaging effects that may have evolved in response to species-specific attackers. Further, this study extends the list of potential venom allergens. The availability of both the honeybee and bumblebee venom proteome may help to develop a strategy that solves the current issue of false double sensitivity in allergy diagnosis, which is caused by cross-reactivity between both venoms. A correct diagnosis is important as it is recommended to perform an immunotherapy with venom of the culprit species.

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## 1. Introduction

Several Hymenopteran stinging species are known to cause allergic reactions. As bumblebees are not aggressive, the risk of being stung by a bumblebee is very small. However, due to the use of bumblebees as pollinators of greenhouse plants, the prevalence of bumblebee venom allergy increased, especially in greenhouse workers (Bucher et al., 2001; Hoffman et al., 2001; de Groot, 2006). Furthermore, a significant immunological cross-reactivity

between bumblebee and honeybee venom is caused by the presence of cross-reactive IgE-antibodies which recognize similar protein and carbohydrate epitopes. Hence, concurrent sensitization can be found in many patients (Bilo et al., 2005).

Knowledge of the venom composition of multiple Hymenopteran species may contribute to an improved allergy diagnosis and treatment by immunotherapy. Venom immunotherapy (VIT) is preferably executed using venom of the culprit species, but sometimes the decision which life-saving immunotherapy should be started is difficult to make. Indeed, patients often fail to identify the stinging insect species and the modern whole-venom-based immunodiagnostics not always brings relief due to cross-reactivity or double sensitivity (Bilo et al., 2005).

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Component-resolved diagnosis using differential species-specific venom allergens may solve this issue by the detection of species-specific IgE antibodies in patient's serum (Muller et al., 2009). However, well studied venom proteomes and allergen repertoires are a prerequisite for the development of such diagnostic tools.

Within the Apidae, the largest family of bees with over 5600 described species (Cardinal et al., 2010), the honeybee is the sole species with a well-studied venom protein composition (Van Vaerenbergh et al., 2013b). So far, only few research studies have focused on bumblebee venom. Using enzymatic activity tests, both high abundant proteins [phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and casein hydrolyzing protease] and minor components (hyaluronidase and acid phosphatase) were detected in the venom of the European large earth bumblebee, *Bombus terrestris* (Hoffman et al., 2001). The PLA<sub>2</sub> and casein hydrolyzing protease are recognized by IgE antibodies and are known as the allergens Bom t 1 and Bom t 4, respectively (Hoffman et al., 2001). Recently, a venom serine protease (Qiu et al., 2011) and a Kunitz-type serine protease inhibitor (Qiu et al., 2013) were identified, which affect the victim's hemostatic system via the venom serine protease inhibitor-mediated antifibrinolytic activity and venom serine protease-mediated fibrin(ogen)olytic activities (Qiu et al., 2013). In addition, two venom bombolitinins have been described, which constitute the highest abundant compounds in bumblebee venom [(Qiu et al., 2012a); GenBank: ADY75782.1]. Venom proteomes of few other bumblebee species (*Bombus ignitus*, *Bombus pennsylvanicus*, *Bombus lapidarius*, *Bombus hypocyrtus*, *Bombus terrestris*, *Bombus lucorum*, *Bombus agrorum*, *Bombus terrestris*, *Bombus lucorum*, *Bombus agrorum*) have been investigated, identifying PLA<sub>2</sub>s (Xin et al., 2009; Hoffman et al., 2001), serine proteases (Hoffman et al., 2001; Qiu et al., 2012b; Choo et al., 2010b), bombolitinins (Argiolas and Pisano, 1985; Choo et al., 2010a; Favreau et al., 2006), a mast cell degranulating peptide (Argiolas et al., 1985) and a Kunitz-type serine protease inhibitor (Choo et al., 2012a; Choo et al., 2012b).

Most preceding research relied on single compound-oriented, time-consuming and low sensitive techniques such as bio-assays and chemical sequencing via Edman degradation, identifying only a few, primarily high abundant, compounds. Novel mass spectrometry-based studies often apply bottom-up shotgun approaches. So far, *B. lapidarius* is the only bumblebee species from which MS data on its venom proteins are available. ESI-MS resulted in the detection of 24 compounds and the three major compounds were identified as three bombolitinins using a combination of tandem MS with Edman degradation (Favreau et al., 2006). However, this study was hampered by the lack of a well annotated genome which is a prerequisite for further completing the list of venom proteins (de Graaf et al., 2009), as the mass spectra can then be searched against the available protein predictions. We previously reported that the use of high performing mass spectrometry technologies can result in a comprehensive identification of the venom components, as was shown for the parasitoid wasp *Nasonia vitripennis* and the honeybee *Apis mellifera* (de Graaf et al., 2010; Van Vaerenbergh et al., 2013b). We have demonstrated that additional low abundant compounds were accessed by reducing the dynamic

range of protein concentrations in venom samples by incubation with a combinatorial hexapeptide ligand library (CPLL) (Van Vaerenbergh et al., 2013b). The recent genome publication of the bumblebee *B. terrestris* (Sadd et al., 2015) paved the way for a similar approach for analyzing the venom composition of this species.

The present study explored the worker venom proteome of the large earth bumblebee (*B. terrestris*) by high resolution mass spectrometry analysis (LC-ESI-LTQ-FT-ICR MS/MS). As PAGE separation of *B. terrestris* venom proteins revealed several high abundant venom compounds (de Graaf et al., 2009; Qiu et al., 2011) which may hamper the detection of low abundant compounds in a mass spectrometry experiment, CPLL pretreatment of the venom sample has been conducted. Venom compounds were identified by searching MS/MS spectra against *B. terrestris* genome protein prediction sets (Sadd et al., 2015). Functions of the identified compounds were predicted using bioinformatics.

## 2. Material and methods

### 2.1. Venom collection

Worker bumblebees (*B. terrestris*) were collected from three commercially available nests (Biobest Co., Belgium). Pure venom was collected as was previously described for honeybees (Peiren et al., 2005). Venom of 75 bumblebees was pooled to a protein concentration of 69.54 mg/ml, determined by Bradford protein assay (Thermo Scientific Pierce, Hudson, NH, USA).

### 2.2. Mass spectrometric analysis

Unless otherwise indicated, all experiments were conducted as described in a preceding honeybee venom proteome study (Van Vaerenbergh et al., 2013b). The dynamic range of protein concentrations in the bumblebee venom sample was compressed by a CPLL approach (ProteoMiner protein enrichment small-capacity kit, Bio-Rad Laboratories, Hercules, CA, USA). Approximately 100 µg and 70 µg of the CPLL flow-through and eluted protein fractions, respectively, were separated on a 10% Tris-glycine-SDS-PAGE gel. In addition, 25 µg of CPLL flow-through proteins and 30 µg of CPLL elution proteins were separated on a 16.5% Tris-tricine-SDS-PAGE gel. After Coomassie staining, proteins were in-gel reduced and alkylated. All flow-through and elution protein bands larger than 40 kDa were cut out of the 10% glycine gel, while those smaller than 40 kDa were cut out of the 16.5% tricine gel. Also gel parts without any visible protein bands were excised and analyzed. After in-gel tryptic digestion, tryptic peptides were extracted from the gel and analyzed by LC-ESI-LTQ-FT-ICR MS/MS. MS/MS data were searched using the Mascot v2.3 database search engine (Matrix Science, London, UK) against two protein databases of the newly released Bter\_1.0 genome (Sadd et al., 2015): the Augustus5 ([http://hymenopteragenome.org/beebase/?q=consortium\\_datasets](http://hymenopteragenome.org/beebase/?q=consortium_datasets); database contains 30976 sequences and 34153612 residuals) and the Bter\_1.0 NCBI Refseq database ([ftp.ncbi.nih.gov/genomes/Bombus\\_terrestris/protein/](ftp.ncbi.nih.gov/genomes/Bombus_terrestris/protein/));

database contains 10577 sequences and 5951597 residuals), added with characterized *B. terrestris* venom sequences: phospholipase A<sub>2</sub> (Genbank: P82971.1), serine protease (GenBank: ADY75780.1), bombolitin 1 (GenBank: ADY75781.1) and bombolitin 2 (GenBank: ADY75782.1). To determine false discovery rates (FDR) for the identity threshold, an automatic decoy database search was conducted. Setting the significance threshold at 0.0016 led to an FDR of 0.99% for the Augustus5 search and 0.53% for the Bter\_1.0 NCBI Refseq search. Searches were executed with carbamidomethylation of cysteines as a fixed modification and oxidation of methionines as a variable modification. One tryptic miscleavage was permitted and peptide mass tolerance and MS/MS tolerance were set to 10 ppm and 0.3 Da, respectively. Precursor peptide charge state was set to 2+ and 3+.

### 2.3. Criteria for positive identifications

We defined positive protein identifications as queries detected by at least two unique, bold and red (significant and top ranking) peptides from the Mascot output with an ion score  $\geq 30$ . In addition, the discovery of small peptides was enabled by allowing queries with a sequence coverage higher than 10% due to the detection of only one, bold and red (significant and top ranking) peptide with an ion score  $\geq 30$ . All protein identifications were merged in one list and all double identifiers were removed.

### 2.4. Sequence analysis

Subsequently, a bioinformatics analysis of identified compounds was executed. As not all sequences are correct in prediction datasets, we tried to determine the correct protein sequence for each identification by searching for *B. terrestris* EST data and homologs of well-annotated species (*Drosophila melanogaster*, *Mus musculus* and *Homo sapiens*). *B. terrestris* EST sequences were searched by Blasts of each prediction against the Bter\_1.0 genome scaffolds on Beebase (Munoz-Torres et al., 2011), while homologs were searched against the Uniprot database using the Blast algorithm (The Uniprot Consortium, 2012). A database containing all correct(ed) sequences was constructed and was used for performing bioinformatics analyses: the presence of secretion signal peptides, protein domains, protease and protease inhibitor families and GO-terms, and the existence of similar proteins in exosomes and venoms of other species were determined as previously described (Van Vaerenbergh et al., 2013b).

## 3. Results and discussion

### 3.1. Identification and categorization of venom proteins

This study presents the first in-depth proteomic analysis of the venom of workers of the large earth bumblebee. It revealed 519 unique tryptic peptides (see [Supplementary Tables 1 and 2](#)) providing biological evidence for 57 venom proteins and peptides. Forty-one of the detected proteins show high sequence similarity to one of the compounds previously identified from honeybee venom

([Table 1](#)). The 16 other venom compounds seem to be specific to bumblebee ([Table 2](#)).

#### 3.1.1. Bumblebee venom proteins with similarity to honeybee venom proteins

In a preceding study, honeybee venom compounds were categorized in the groups of putative toxins and venom trace elements by prediction of their biological function and subcellular location (Van Vaerenbergh et al., 2013b). Due to the high sequence similarity between bumblebee and honeybee venom homologs, we hypothesize that they share similar functions and subcellular locations. The identified putative toxins mainly contribute to the defense or social immunity function of the complete venom. They belong to the classes of esterases, proteases, protease inhibitors, carbohydrate-degrading enzymes, growth factors, major royal jelly proteins (MRJP) and antimicrobial peptides. As low abundant compounds are enriched by the CPLL pretreatment ([Fig. 1](#)), a subgroup of identified proteins was found which probably have no function once they are injected into the victim. These venom trace elements possibly execute a local function in the venom duct or reservoir or are released by leakage of the gland tissue. They were listed according to their subcellular location ([Table 1](#)).

Five of the identified toxins were already described in preceding *B. terrestris* venom studies. First, bombolitin 1 is a small 18 amino acid peptide with antimicrobial activity (Qiu et al., 2012a). It is a high abundant venom compound and shows structural and biological properties similar to honeybee venom melittin. In addition, this study presents full sequence data of four bumblebee venom enzymes, which were hitherto only poorly characterized. The first two described components, hyaluronidase and acid phosphatase were only identified by enzymatic activity tests (Hoffman et al., 2001), while proteomic identifications and sequence information were lacking. Additionally, we also provide corrected sequences of the *B. terrestris* venom allergens Bom t 1 (PLA<sub>2</sub>) and Bom t 4 (casein hydrolyzing protease). The PLA<sub>2</sub>-1 (au5.g6472.t1) derived from the genome sequence shows very high sequence identity (91%) to the previously available Bom t 1 sequence [(Hoffman et al., 2001); UniProt: P82971.1]. BeeBase tblastn searches of both sequences against the *B. terrestris* genome returned the same gene as the best Blast hit. However, a higher Blast score was shown with the Augustus5 PLA<sub>2</sub>-1 prediction and also EST data (GenBank: FN616117.1) support the new sequence. Indeed, the Bom t 1 sequence was exclusively determined by chemical sequence analysis using Edman degradation of its proteolytic peptide fragments (Hoffman et al., 2001), which may be prone to errors. Second, for Bom t 4, only the 20 N-terminal amino acid sequence was available until present [(Hoffman et al., 2001); UniProt: P0CH88.1]. We found that this fragment is exclusively present within the serine protease 1 prediction. As such, this FTMS analysis also revealed the complete Bom t 4 sequence.

For some (classes of) venom toxins, differences between honeybee and bumblebee venom were noticed. First, in addition to PLA<sub>2</sub>-1, a second bumblebee venom PLA<sub>2</sub> (PLA<sub>2</sub>-2) was found for the first time. Both PLA<sub>2</sub>s share 61%

**Table 1**

All identified bumblebee venom compounds with sequence similarity to honeybee venom components are shown. Sequences were categorized in a group of putative toxins and venom trace elements, and further according to their putative function and subcellular location. GenBank/Augustus accession numbers (Acc N°) and allergen names (Allergen, derived from <http://www.allergen.org/>) are presented and their finding in preceding *Bombus terrestris* venom proteomic studies (BtV) is marked with "X". In gray, the corresponding honeybee venom sequences with the highest sequence similarity (Sim. in %) and their allergen names can be found.

| Name                                              | Acc. N°      | Allergen | BtV | Apis mellifera venom protein |                                                   |              |          |
|---------------------------------------------------|--------------|----------|-----|------------------------------|---------------------------------------------------|--------------|----------|
|                                                   |              |          |     | Sim. (%)                     | Name                                              | Acc. N°      | Allergen |
| A. Putative toxins                                |              |          |     |                              |                                                   |              |          |
| Esterases                                         |              |          |     |                              |                                                   |              |          |
| Phospholipase A <sub>2</sub> -1                   | au5.g6472.t1 | Bom t 1  | x   | 45                           | Phospholipase A <sub>2</sub> -1                   | gi 58585172  | Api m 1  |
| Phospholipase A <sub>2</sub> -2                   | gi 340723911 |          |     | 48                           | Phospholipase A <sub>2</sub> -1                   | gi 58585172  | Api m 1  |
| Acid phosphatase                                  | au5.g1511.t1 |          | x   | 67                           | Acid phosphatase 1                                | gi 301601654 | Api m 3  |
| Carboxylesterase                                  | gi 340712251 |          |     | 69                           | Carboxylesterase                                  | gi 187281550 | Api m 8  |
| Proteases and peptidases                          |              |          |     |                              |                                                   |              |          |
| Serine protease 1                                 | gi 340713088 | Bom t 4  | x   | 58                           | CLIP serine protease                              | gi 66507455  |          |
| Serine protease 2                                 | gi 340713090 |          |     | 58                           | CLIP serine protease                              | gi 66507455  |          |
| (CLIP) serine protease 3                          | gi 340713092 |          |     | 56                           | CLIP serine protease                              | gi 66507455  |          |
| Serine protease 4                                 | gi 340713094 |          |     | 53                           | CLIP serine protease                              | gi 66507455  |          |
| Serine protease 5                                 | gi 340713099 |          |     | 53                           | CLIP serine protease                              | gi 66507455  |          |
| CUB serine protease                               | gi 340728251 |          |     | 49                           | CUB serine protease 1                             | gi 58585116  | Api m 7  |
| Dipeptidyl peptidase IV                           | gi 340721615 |          |     | 75                           | Dipeptidyl peptidase IV                           | gi 187281543 | Api m 5  |
| Serine carboxypeptidase                           | gi 340723441 |          |     | 80                           | Serine carboxypeptidase                           | gi 226533687 | Api m 9  |
| Prolylcarboxypeptidase                            | gi 340710015 |          |     | 81                           | Prolylcarboxypeptidase                            | gi 328778095 |          |
| Protease inhibitors                               |              |          |     |                              |                                                   |              |          |
| Serpin 1                                          | gi 340721561 |          |     | 61                           | Serpin 1                                          | gi 328793022 |          |
| Serpin 2                                          | gi 340708853 |          |     | 65                           | Serpin 2                                          | gi 328791596 |          |
| Serpin 3                                          | gi 340728533 |          |     | 54                           | Serpin 1                                          | gi 328793022 |          |
| Carbohydrate metabolism                           |              |          |     |                              |                                                   |              |          |
| Hyaluronidase                                     | gi 340724556 |          | x   | 75                           | Hyaluronidase                                     | gi 58585182  | Api m 2  |
| N-sulfoglucosamine sulfohydrolase                 | gi 340721513 |          |     | 89                           | N-sulfoglucosamine sulfohydrolase                 | gi 328793712 |          |
| Growth factors                                    |              |          |     |                              |                                                   |              |          |
| Platelet-derived growth factor                    | gi 340710875 |          |     | 47                           | Platelet-derived growth factor                    | gi 328789531 |          |
| Major royal jelly proteins                        |              |          |     |                              |                                                   |              |          |
| Major royal jelly protein                         | gi 340716434 |          |     | 55                           | MRJP9                                             | gi 67010041  | Api m 11 |
| Peptides                                          |              |          |     |                              |                                                   |              |          |
| Bombolitin 1                                      | gi 325071353 |          | x   | 39                           | Melittin                                          | gi 58585154  | Api m 4  |
| Other toxins                                      |              |          |     |                              |                                                   |              |          |
| Icarapin                                          | gi 340715455 |          |     | 68                           | Icarapin                                          | gi 60115688  | Api m 10 |
| Antigen 5-like protein                            | gi 340727156 |          |     | 49                           | Antigen 5-like                                    | gi 328784851 |          |
| B. Trace elements                                 |              |          |     |                              |                                                   |              |          |
| Secreted proteins                                 |              |          |     |                              |                                                   |              |          |
| Peptidylglycine α-hydroxylating monooxygenase     | gi 340712968 |          |     | 85                           | Peptidylglycine α-hydroxylating monooxygenase     | gi 328787622 |          |
| Cathepsin F                                       | au5.g2529.t1 |          |     | 80                           | Cathepsin F                                       | gi 328788558 |          |
| Lysozyme c-1                                      | gi 340723421 |          |     | 78                           | Lysozyme c-1                                      | gi 328779578 |          |
| Apolipophorins                                    | gi 340717708 |          |     | 69                           | Apolipophorins                                    | gi 328780884 |          |
| Vitellogenin                                      | au5.g1998.t1 |          |     | 51                           | Vitellogenin                                      | gi 58585104  | Api m 12 |
| Endoplasmic reticulum                             |              |          |     |                              |                                                   |              |          |
| Peptidyl-prolyl cis-trans isomerase B             | gi 340716651 |          |     | 90                           | Peptidyl-prolyl cis-trans isomerase B             | gi 335892796 |          |
| Glucosidase 2, subunit α                          | gi 340709031 |          |     | 89                           | Glucosidase 2, subunit α                          | gi 66500170  |          |
| Calreticulin                                      | gi 340729835 |          |     | 93                           | Calreticulin                                      | gi 66545506  |          |
| Golgi complex                                     |              |          |     |                              |                                                   |              |          |
| Glycoprotein 3-α-L-fucosyltransferase A           | gi 340712422 |          |     | 92                           | Glycoprotein 3-α-L-fucosyltransferase A           | gi 328785366 |          |
| α-Mannosidase 2                                   | gi 340729800 |          |     | 97                           | α-Mannosidase 2                                   | gi 66514147  |          |
| Lysosomes                                         |              |          |     |                              |                                                   |              |          |
| Proactivator polypeptide                          | gi 340712613 |          |     | 76                           | Proactivator polypeptide                          | gi 328782499 |          |
| α-L-fucosidase                                    | gi 340716134 |          |     | 78                           | α-L-fucosidase                                    | gi 328793281 |          |
| Glucosylceramidase                                | gi 340729189 |          |     | 62                           | Glucosylceramidase                                | gi 66511554  |          |
| Aspartic protease (cathepsin D)                   | gi 340729556 |          |     | 87                           | Aspartic protease (cathepsin D)                   | gi 66560290  |          |
| Plasma membrane proteins                          |              |          |     |                              |                                                   |              |          |
| Renin receptor                                    | gi 340718491 |          |     | 87                           | Renin receptor                                    | gi 328776891 |          |
| Multiple inositol polyphosphate phosphatase       | gi 340725135 |          |     | 30                           | Multiple inositol polyphosphate phosphatase       | gi 239787860 |          |
| Mitochondrial proteins                            |              |          |     |                              |                                                   |              |          |
| Phospholipid hydroperoxide glutathione peroxidase | gi 340714042 |          |     | 77                           | Phospholipid hydroperoxide glutathione peroxidase | gi 110756698 |          |
| Proteins without signal peptide                   |              |          |     |                              |                                                   |              |          |
| Actin                                             | gi 340711865 |          |     | 97                           | Actin related protein 1                           | gi 297591985 |          |

**Table 2**

The identified bumblebee-specific compounds were categorized in a group of putative toxins and venom trace elements, and further according to their putative function and subcellular location. The presence of a secretion signal peptide (derived by SignalP) and their presence in exosomes (Ex, derived from <http://www.exocarta.org/>) are indicated with "X". In gray, the results are shown of stand alone Blasts against a venom sequence database, which reveals the existence of similar venom proteins and venom gland transcripts (Ev.) of other species (Species). Type of evidence: P = venom protein; T = venom gland transcript.

| Name                                                                | Acc. N°      | SignalP | Ex | Similar venom compound       |     |                            |
|---------------------------------------------------------------------|--------------|---------|----|------------------------------|-----|----------------------------|
|                                                                     |              |         |    | Species                      | Ev. | Ref.                       |
| <b>A. Putative toxins</b>                                           |              |         |    |                              |     |                            |
| Chitinase                                                           | gi 340721438 | x       |    |                              |     |                            |
| Glucose dehydrogenase                                               | gi 340714301 |         |    | <i>Nasonia vitripennis</i>   | P   | (de Graaf et al., 2010)    |
| Plancitoxin                                                         | gi 340711594 | x       |    | <i>Crotalus adamanteus</i>   | T   | (Rokyta et al., 2012)      |
| Serine protease K12H4.7                                             | gi 340715980 | x       |    |                              |     |                            |
| Metalloproteinase inhibitor                                         | gi 340725794 | x       |    |                              |     |                            |
| C3 and PZP-like $\alpha$ -2-macroglobulin domain-containing protein | gi 340712509 | x       | x  | <i>Crotalus adamanteus</i>   | T   | (Rokyta et al., 2012)      |
| <b>B. Trace elements</b>                                            |              |         |    |                              |     |                            |
| <b>Secreted proteins</b>                                            |              |         |    |                              |     |                            |
| Trehalase                                                           | gi 340724978 | x       | x  | <i>Pimpla hypochondriaca</i> | T   | (Parkinson et al., 2003)   |
| Prolyl 3-hydroxylase                                                | gi 340708955 | x       | x  |                              |     |                            |
| <b>Secretory pathway proteins</b>                                   |              |         |    |                              |     |                            |
| Aldose 1-epimerase                                                  | gi 340722542 | x       | x  |                              |     |                            |
| GlutaminyI-peptide cyclotransferase                                 | gi 340716459 | x       | x  | <i>Hottentotta judaicus</i>  | T   | (Morgenstern et al., 2011) |
| $\alpha$ -N-acetylgalactosaminidase                                 | gi 340722731 | x       |    | <i>Crotalus adamanteus</i>   | T   | (Rokyta et al., 2012)      |
| $\beta$ -hexosaminidase subunit $\beta$                             | gi 340718728 | x       |    |                              |     |                            |
| $\beta$ -mannosidase                                                | gi 340713893 | x       |    |                              |     |                            |
| <b>Proteins without a signal peptide</b>                            |              |         |    |                              |     |                            |
| Catalase                                                            | gi 340714922 |         | x  |                              |     |                            |
| <b>Proteins with an unknown function</b>                            |              |         |    |                              |     |                            |
| Unknown function protein 1                                          | gi 340712339 | x       | /  | <i>Microctonus hyperodae</i> | P   | (Crawford et al., 2008)    |
| Unknown function protein 2                                          | gi 340721301 | x       | /  |                              |     |                            |

sequence identity and are arranged in tandem in the genome (Sadd et al., 2015). Therefore, they probably result from a gene duplication event. In contrast, the two group III PLA<sub>2</sub>s found in honeybee venom (Van Vaerenbergh et al., 2013b) share only 45% sequence identity and are positioned on different chromosomes (Honey Bee Genome Sequencing Consortium, 2014). Moreover, both bumblebee PLA<sub>2</sub>s appear to show highest sequence similarity to the honeybee PLA<sub>2</sub>-1 allergen (Table 1).

Second, our proteomic analysis revealed the presence of multiple serine proteases. Five of these, serine proteases 1 to 5, show high mutual similarity and all are similar to the honeybee venom CLIP serine protease (Table 1). Additionally, a sixth serine protease was described to be present in *B. terrestris* venom (Qiu et al., 2011), but remained undetected in our analysis. This may be caused by venom sample variation (different collection method, spatial and/or seasonal venom variation) or due to its removal during

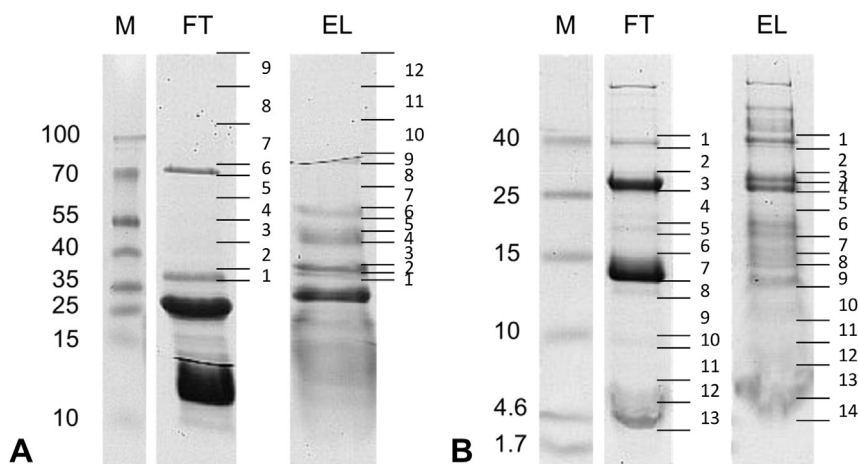


**Fig. 1.** Electrophoretic separation of a combinatorial peptide ligand library (CPLL)-treated bumblebee venom sample. CPLL flow-through (=FT) and elution (=EL) samples are separated on a 10% Tris-glycine-SDS-PAGE gel (A) and a 16.5% Tris-tricine-SDS-PAGE gel (B). The cut-out and analyzed slices are marked by solid lines and are numbered. Tryptic peptides identified in these protein slices are presented in Supplementary Table 2. Molecular weights (kDa) of the marker proteins (M; Thermo Scientific, Rockford, IL, USA) are indicated: A) PageRuler Prestained Protein Ladder; B) Spectra Multicolor Low Range Protein Ladder.

washing procedures of the protein enrichment protocol. The six protease genes appear to be positioned in tandem within the *B. terrestris* genome (Sadd et al., 2015) and may have evolved by gene duplication from a common ancestor. In contrast, the venom CLIP serine protease is the only protease gene present within the syntenic region of the honeybee genome (Honey Bee Genome Sequencing Consortium, 2014). Remarkably, only two of the bumblebee proteases, serine protease 3 and 6, retained the CLIP domain.

Third, compared to honeybee venom, a lower number of acid phosphatases, CUB serine proteases and MRJPs were found within the bumblebee venom. In the honeybee genome, nine MRJP genes reside in a gene cluster, but only MRJP 8 and 9 are detected in the venom (Peiren et al., 2005, 2008; Van Vaerenbergh et al., 2013b). The *B. terrestris* genomic information contains only a single-copy MRJP gene, and our study demonstrates that this compound is one of the venom constituents. Due to its expression by the hypopharyngeal glands, it has been suggested to play a role in food digestion or modification (Kupke et al., 2012). Its presence within honeybee and bumblebee venom may also suggest a toxic function.

Fourth, our analysis revealed a low number of antimicrobial peptides within bumblebee venom. Only bombolitin 1 contains antimicrobial properties (Qiu et al., 2012a). A second *B. terrestris* venom bombolitin is available in GenBank (ADY75782.1) and a higher number of venom bombolitins has been described in other bumblebee species (Favreau et al., 2006; Argiolas and Pisano, 1985). However, no additional bombolitin genes could be found within the *B. terrestris* genome (Sadd et al., 2015). Furthermore, no homologs of the honeybee venom antimicrobial peptides apidaecin (Van Vaerenbergh et al., 2013a) and mast cell degranulating peptide (MCDP) (Van Vaerenbergh et al., 2013b; Baracchi et al., 2013) were found within the venom by mass spectrometric means, although putative homologues genes [apidaecin (GeneID: 100649867); MCDP (GeneID: 100644816 and 100644936)] were found to be present in the genome. In contrast, no traces of the neurotoxic honeybee venom peptides apamin (Baracchi et al., 2013) and tertiapin (Kitamura et al., 2000) were found in the bumblebee genome (Sadd et al., 2015).

Finally, this study found two homologues of proteins described to be present in honeybee venom, which were missing in our honeybee venom study applying an identical proteomic approach (Van Vaerenbergh et al., 2013b). First, the absence of the antigen 5-like (Ag5-like) protein in the venom of summer honeybees was not surprising as it is probably exclusively expressed by the honeybee venom glands during the winter months (Van Vaerenbergh et al., 2013a). Also, high abundant paralogs have been identified in wasp and ant venoms (Van Vaerenbergh et al., 2013a). In contrast to bumblebees and wasps, honeybee workers stay alive during the winter, which may result in differences of the venom composition of summer and winter bees. Unfortunately, the function of the venom Ag5s is unknown, which makes this variation in expression difficult to interpret. Second, vitellogenin is described as a high molecular weight honeybee venom allergen (Blank et al., 2013) and was now also found in bumblebee venom.

### 3.1.2. Bumblebee-specific venom compounds

We also categorized the 16 bumblebee-specific compounds in the groups of putative toxins and venom trace elements (Table 2), which was done by the same approach as described for categorization of the identified honeybee venom compounds (Van Vaerenbergh et al., 2013b). This led to the identification of six putative toxins and eight trace elements. A brief description of their putative functions can be found in Supplementary Table 3. Results of GO-term searches and of searches for venom homologs from other species can be found in Supplementary Tables 4 and 5, respectively.

Both the classical secretory pathway and the exosome-based secretory pathway appeared to be important for protein secretion in the honeybee venom glands (Van Vaerenbergh et al., 2013b). The absence of an N-terminal secretion signal peptide in three of the identified bumblebee venom compounds indicates that their secretion does not involve the classical secretory pathway. While actin and catalase can be secreted by exosomes [(Van Vaerenbergh et al., 2013b) and Table 2], glucose dehydrogenase (GLD) was not found in the current version of ExoCarta, an exosome content database. Also the *N. vitripennis* GLD venom homolog lacks a signal peptide.

At present, the attribution of a function of two compounds is impossible. Basic sequence analysis of unknown function protein 1 (UFP1) has revealed no predicted functional domains or GO-terms. However, it is related to a fruit fly protein (UniProt: Q7KVT8; 42% sequence identity), which is required for its development beyond first instar. Additionally, it shows low similarity (20% sequence identity) to one of the major proteins of the venom reservoir of the parasitoid wasp *Microctonus hyperodae*, which is suggested to be a tyrosine kinase (Crawford et al., 2008). No functional domains, GO-terms or venom homologs were found to elucidate the biological function of UFP2.

### 3.2. Comparison of the honeybee and bumblebee venom composition

Honeybees and bumblebees both belong to the Apidae family but have diverged about 100 million years ago (Stolle et al., 2011). The implementation of an identical proteomic approach (Van Vaerenbergh et al., 2013b) allowed to compare the venom composition of both species. These venoms appear to be similar, as 72% of the detected bumblebee venom compounds proved to have a honeybee venom homolog. Also, a similar number of putative toxins were found (29 bumblebee toxins versus 33 honeybee toxins) and most belong to identical functional classes (Table 1). Moreover, honeybee venom homologs exist for 70% (23/29) of the detected bumblebee toxins. The presence of toxins with similar activities may be explained by the high degree of homology between both genomes (Stolle et al., 2011) and the need for similar defensive actions of both venoms. Honeybee and bumblebee stings cause local tissue damage, which induces death in other insects and pain and inflammation in higher organisms (de Graaf et al., 2009). Their venoms contain typical (Hymenoptera) venom constituents, such as phospholipases A<sub>2</sub>, proteases, acid phosphatases, hyaluronidases, protease inhibitors, growth

factors and MRJPs, which contribute to the venom's toxic actions (Van Vaerenbergh et al., 2013b). Further, these venoms appear to have 18 venom trace elements in common. Some have a relevant function in the venom duct or reservoir for maturation of secretory proteins (e.g. peptidylglycine  $\alpha$ -hydroxylating monooxygenase), while others are secreted by a broad range of tissues as they have an essential function within the extracellular space (e.g. immune system proteins, anti-oxidant enzymes, apolipoprotein) (Van Vaerenbergh et al., 2013b). However, most shared trace elements are typical secretory pathway proteins, which may be unintentionally released due to an inefficient recycling in the venom gland tissue.

Both venoms also contain some species-specific characteristics. The bumblebee venom seems to have a lower complexity due to a smaller number of identified compounds: 57 bumblebee compounds versus 102 honeybee compounds. However, this difference is mainly evoked by a different number of venom trace elements and proteins with an unknown function. Additionally, as mentioned above, variation between both groups of toxins can be noticed and both venoms contain a selection of species-specific toxins, which may point to distinct damaging effects. Therefore, these species may have undergone evolutionary adaptations in response to species-specific attackers. Indeed, although no data have been reported for *B. terrestris*, intraperitoneal injection of venom of the bumblebee *Bombus impatiens* (LD50 = 7.2 mg/kg) in mice showed that it is about half as lethal to vertebrates as honeybee venom (3.5 mg/kg) [28]. Moreover, honeybees and bumblebees differ in their defensive behavior. Compared to honeybees, bumblebees rarely sting humans, which supports the lower activity of bumblebee venom towards vertebrates. Differences in protein abundance among homologs may further increase functional diversity of both venoms. Unfortunately, this parameter information could not be analyzed as it is influenced by the protein enrichment strategy that removes mainly abundant proteins in a non-linear fashion.

The application of venom on the body surface as a way of protection against pathogens has been suggested for multiple Hymenopteran species (Baracchi et al., 2011). Remarkably, in contrast to honeybee venom, only few bumblebee venom antimicrobial peptides were found. However, the social structure of honeybee and bumblebee colonies differs. In contrast to bumblebees which build a new colony every year, honeybees inhabit the hive for multiple years and also regulate the hive temperature during winter months. Additionally, bumblebees form colonies which are much less extensive than those of honeybees. Therefore, bumblebees may be less exposed to pathogens, which may explain the reduced need for a large repertoire of antimicrobial peptides. However, as small peptides are difficult to detect using our approach, future studies are needed to confirm this hypothesis.

### 3.3. Consequences for Hymenoptera venom allergy diagnosis

Although several stinging Apidae species can cause allergic reactions, only the venom of the honeybee has been

properly characterized. This resulted in the identification of 12 allergens (derived from <http://www.allergen.org/>). In contrast, besides the allergens from *B. terrestris*, only two allergens of one other bumblebee species, the North-American bumblebee *B. pennsylvanicus*, have been characterized. Although these allergens from both species are similar, they contain different IgE-reactive epitopes (Hoffman et al., 2001). Also others reported that differences in IgE-binding between venoms of both species exist (Stapel et al., 1998). Further research is needed to determine whether this diversity in venom composition is also true for the other 250 bumblebee species (Williams et al., 2008). At this moment, our high performance approach to analyze the venom composition is applicable to only one other bumblebee species. Besides *B. terrestris*, *B. impatiens* is the only species with an available genome sequence (Sadd et al., 2015).

The unraveled venom proteomes of the honeybee and large earth bumblebee may contribute to an improved Hymenoptera venom allergy diagnosis and treatment. Hymenopteran venoms contain similar proteins and carbohydrates, which results in cross-reactivity. Several studies reported a high degree of cross-reactivity between honeybee and bumblebee venom (Cruz et al., 2012). This is supported by this study with the identification of many homologs with often high sequence identity (Table 1). Moreover, except for Api m 6, all characterized honeybee venom allergens appear to have a bumblebee venom homolog (Table 1), which represent putative bumblebee venom allergens.

Due to the reported high cross-reactivity between both venoms, for many years honeybee venom was used to treat patients sensitized to bumblebee venom (Cruz et al., 2012). However, treatment by VIT in some patients failed (Cruz et al., 2012), indicating the existence of species-specific venom epitopes. Indeed, this study shows that many honeybee- and bumblebee-specific venom compounds can be found. Additionally, in some cases sequence identity between homologs is limited, leading to distinct linear and/or conformational epitopes. For example, very little of the protein surface of the PLA<sub>2</sub> allergens from honeybee (Api m 1) and bumblebee (Bom t 1) venom is conserved (Hoffman et al., 2001). Therefore, further research should characterize the relevant allergens. The use of species-specific venom allergens in component-resolved diagnosis may solve the issue of false double sensitivity in diagnostic tests, which is caused by cross-reactivity. A correct diagnosis is important as it is recommended that VIT is performed using venom of the culprit species.

## 4. Conclusions

This study unraveled the venom proteome of the bumblebee, *B. terrestris*, by integrating a combinatorial peptide ligand library approach with FTMS. In total, 57 venom compounds were found, which could be categorized according to their putative functions. In preceding research, honeybee venom has been analyzed by an identical approach. Many honeybee and bumblebee venom homologs were found, which may be explained by their similar defensive function and the high degree of homology

between both genomes. Besides, this bumblebee species is increasingly used for pollination in greenhouses. Therefore, greenhouse workers are more exposed to bumblebee stings and often develop venom allergy. This study presents a list of potential new venom allergens. The availability of both the honeybee and bumblebee venom proteome may allow to develop a strategy that solves the current issue of false double sensitivity in allergy diagnosis, which is caused by cross-reactivity between both venoms.

## Ethical statement

The authors have no ethical statement to declare.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.toxicon.2013.10.002>.

## Conflict of interest statement

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