Apidologie (2022) 53:16

https://doi.org/10.1007/s13592-022-00928-3

Original article

Honey proteome of the bumblebee *Bombus terrestris*: similarities, differences, and exceptionality compared to honey bee honey as signatures of eusociality evolution

Tomas Erban[1], Elena Shcherbachenko[1,2], Pavel Talacko[3], and Karel Harant[3]

[1] Proteomics and Metabolomics Laboratory, Crop Research Institute, Drnovska 507/73, Prague 6 – Ruzyne 161 06, Czechia
[2] Institute for Environmental Studies, Faculty of Science, Charles University, Benatska 2, 128 01 Prague 2, Czechia
[3] Proteomics Core Facility, Faculty of Science, Charles University, BIOCEV, Prumyslova 595, Vestec 252 42, Czechia

Received 17 September 2021 – Revised 8 March 2022 – Accepted 9 March 2022

Abstract – Although honey bee honey is well studied, information on the characteristics of bumblebee honey is limited. The study goal was to characterize the proteome of *Bombus terrestris* honey and compare it with the known proteome of *Apis mellifera* honey. Honey proteomes could reflect the differences in genetic makeup as well as eusocial organization. Basic characteristics, such as total protein content (0.4 mg/g), pH (4.6), water content (11% lyophilization), and fructose/glucose content (76%) and ratio (1.5), were not unique compared to honey bee honey. Label-free proteomics enabled reliable identification of 107 bumblebee-derived proteins, and this number is greater than that honey bee honey. In contrast, plant-derived proteins are more abundant in bumblebee honey. Approximately 40 homologous proteins in *B. terrestris* and *A. mellifera* honey were identified. Alpha-glucosidase homologous with *A. mellifera* Hbg3 was a major protein. Importantly, MRJPs, alpha-amylase, and glucose oxidase were absent. Yellow-e3-like and carbonic anhydrase are representative bumblebee-specific markers. Together, these differences reflect the lack of royal jelly production in bumblebees. The bumblebee honey proteome is substantially different from that of honey bees. These differences can explain the differences in eusocial organization, especially events connected with different nutrient flows and the lack of food-receiving/storing castes in the annual colonies of bumblebees.

eusociality level / yellow-e3 / BtRJPL / alpha-glucosidase / carbonic anhydrase / alpha-amylase

1. INTRODUCTION

The only extant species in tribe *Apini* are honey bees, which are very important pollinators of wildlife and agricultural crops. Their pollinating services are considered even more important than providing honey and additional bee products (Klein et al. 2007; Hung et al. 2018). For pollination under certain scenarios, different species of bees are more beneficial than honey bees (Goulson 2003). Currently, some pollinator alternatives to managed honey bees are commercially available. Commonly used are commercial bumblebee colonies, especially the buff-tailed bumblebee *Bombus terrestris* Linnaeus, 1758 (de Ruijter 1997; Velthuis and van Doorn 2006; Knapp et al. 2019). *B. terrestris* is the best-known member of tribe *Bombini*, which contemporarily includes only the genus *Bombus* (Dehon et al. 2019). *Apini* are at a higher eusociality level than *Bombini*. An important step for understanding the nature of this difference involves the

Corresponding author: T. Erban, arachnid@centrum.cz
Manuscript editor: Cedric Alaux

comparison of genomes of their representative species (Sadd et al. 2015). Comparison of the genomes indicated cardinal gene differences that provide patterns for understanding the differences in life history, eusociality level and adaptation to environmental pressures between honey bees and bumblebees (Sadd et al. 2015). However, open questions about the molecular nature of the "high" eusociality advances of honey bees compared to bumblebees remain.

Honey bees provide an array of unique bee products, such as beeswax, honey, royal jelly (RJ), venom, propolis and pollen/bee bread, which are utilized by the colony. Humans have learned to utilize these bee products mainly as food nutrients and medicine (Schmidt 1997). An open question is whether we can also utilize natural products of bumblebees similar to honey bees. Despite viable potential, there is gap in the level of knowledge given that apitherapy used for thousands of years relates to products derived from honey bees (Kolayli and Keskin 2020), not bumblebees.

First, we should answer the question of whether bumblebees provide the same products as honey bees. There is one important and substantial difference. Bumblebees do not produce RJ, which is specific to honey bees that use it as food provided by nurse bees for queen and young larvae (Schmitzova et al. 1998; Buttstedt et al. 2014; Altaye et al. 2019). In that relationship, there is a lack of major royal jelly proteins (MRJPs) in bumblebees, and their hypopharyngeal glands (HGs) are reduced compared to honey bees (Albert et al. 2014). Importantly, MRJPs and a number of other proteins occurring in RJ were also identified in the honey proteome of *A. mellifera*, as summarized in a proteomic study (Erban et al. 2019). The proteome of bumblebee honey is unknown; however, it is possible that the major matrix could provide important data about similarities and differences in gland secretions compared to honey bees.

Most people certainly know that honey bees produce honey; however, only a few people are familiar with honey produced by bumblebees. Bumblebee workers store nectar and pollen in wax pots, that is, to some extent similar to honey bees. Indeed, honey pots can be found in bumblebee nests (Sladen 1912; Crane 1972; Konzmann and Lunau 2014; Svanberg and Berggren 2018). Although little is known, bumblebee honey was important before the introduction of apiculture to some countries, and it was also used in various folk religious customs (Svanberg and Berggren 2018). In contrast to honey bees, bumblebees store only a low amount of honey that never exceeds a few ounces, and despite excellent bumblebee honey flavor, it is only rarely used for human consumption (Sladen 1912). Different chemical properties of bumblebee honey might have medical potential, which requires unique compositions to diminish the specific threat of disease. However, there is a complete lack of knowledge in this field. Data on bumblebee honey characteristics are scarce and, to our knowledge, are limited to rough information on moisture and sugar content (Crane 1972).

In addition to the candidate bioactive compounds of plants that can be found in honey (Viteri et al. 2021), interest in the secretions that honey bees provide to honey is increasing. High importance is attributed to proteins that honey bees use to process and/or protect honey. Thus, numerous studies have been devoted to better understand honey bee honey proteins/enzymes and antimicrobial peptides (e.g. Schepartz and Subers 1964; Babacan and Rand 2005, 2007; Won et al. 2008; Sojka et al. 2016; Bucekova et al. 2017; Brudzynski 2020). Proteomic studies that utilized gel-based (e.g. Di Girolamo et al. 2012; Rossano et al. 2012; Chua et al. 2015; Zhang et al. 2019) or gel-free (e.g. Erban et al. 2019, 2021; Bong et al. 2021)) proteomic approaches have provided deep insights into the complexity of this research area. Similar studies aiming to understand bumblebee honey are however lacking. Indeed, an interesting approach involves determining the difference in the honey proteomes between honey bees and bumblebees. This acquired knowledge would potentially feature functional proteome differences that were earlier inferred in the compared annotated genomes of *A. mellifera* and *B. terrestris* (The Honeybee Genome Sequencing Consortium 2006; Sadd et al. 2015). In particular, the key

difference in honey proteomes may be attributed to differences in eusocial behavior connected to food storage. Unlike honey bees, bumblebees are nonperennial and lack a food-receiving/storing caste (Seeley et al. 1996; Noll 2002). Thus, it is possible that different flows of nutrients in perennial and annual colonies (Judd 2011) will affect honey composition. Key signatures reflecting the differences in honey storage could be found in the honey proteomes. Indeed, honey proteins were observed to be very important in *A. mellifera* honey samples. The total protein content in *A. mellifera* honey can differ more than tenfold between honey types, whereas major proteins are provided in a constant ratio (Erban et al. 2019). A different study suggested that worker honey bees may secrete a constant amount of each protein to preserve honey (Lewkowski et al. 2019). Another important characteristic is that some proteins that honey bees provide to honey can be unique to honey samples (Erban et al. 2019). Furthermore, it has been suggested that honey store diversity may be highly adaptive for the "social immunity" of a colony against pathogens (Erler et al. 2014).

In the present study, we performed a comprehensive proteomics analysis of honey from the bumblebee *B. terrestris*. In addition, we provide a comparison with a previously described *A. mellifera* honey proteome. Some additional basic characteristics, such as total protein content, sugars and moisture, of the bumblebee honey samples were measured.

2. MATERIALS AND METHODS

2.1. Biological samples

The biological samples analyzed in this study were honey samples from the bumblebee *B. terrestris*. Commercially available Tripol hives used for pollination consist of three complete bumblebee colonies (Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands). Four Tripol hives (12 colonies overall) were placed in the Crop Research Institute (Prague-Ruzyne, Czechia) at the time of rapeseed *Brassica napus* and apple tree flowering. Notably, each hive/colony was supplied with a sugar water container, which was closed before placing the hives outside (5th May) for the experiment. After 15 days (20th May), the colonies were euthanized using dry ice. For this study, honey stores were manually collected into 50-mL sterile centrifuge tubes. Honey samples per colony were pooled. It was obvious (e.g., coloring) that the bumblebee honey samples were collected outside of the colony. The samples of bumblebee honey were stored in a deep freezer at − 80 °C until use in the proteomic analysis.

2.2. Sample processing for proteomics

Briefly, samples of bumblebee honeys were processed and further analyzed as previously described (Erban et al. 2019). The samples were diluted with 0.2-µm-filtered Nanopure water (Thermo, Waltham, MA, USA) at a ratio of 1 g of honey to 2 mL of H_2O and purified via gel filtration using PD MidiTrap G-25 columns (Cat. No. 28–9180-08, GE Healthcare Life Sciences, Marlborough, MA, USA). The exclusion limit is an M_r of 5000, and proteins with an M_r greater than 5000 should be easily separated from those with an Mr less than 1000. The cleaned samples were lyophilized in PowerDry LL3000 (Thermo), and the total protein content in separate aliquots was determined using Bradford reagent (Cat No. B6916; Sigma–Aldrich).

2.3. 1D-E electrophoresis

The samples of bumblebee honey were tested using one-dimensional gel electrophoresis (1D-E) to assess sample quality and protein purity before nano-LC–MS/MS. Separation was performed using 4 to 12% Tris–glycine SDS–PAGE gels, and the sample buffer contained SDS and dithiothreitol (DTT). Full-Range Amersham Rainbow Marker RPN 800E (GE Healthcare Life Sciences) was used. Electrophoresis was performed at a constant voltage with a Mini-PROTEAN Tetra Cell (Bio–Rad, Shanghai,

China). The samples were stained using PhastGel Blue R 350 Coomassie stain (GE Healthcare Life Sciences). The gel was visualized using the G:BOX documentation system (Syngene, Cambridge, UK). Selected bands were excised and destained by sonication for 30 min in 50% acetonitrile (ACN) and 50 mM ammonium bicarbonate (ABC). Furthermore, the detaining solution was removed, and the gels were dried in ACN. Disulfide bonds were reduced using 10 mm DTT in 100 mM ABC at 60 °C for 30 min. Samples were again dried with ACN, and free cysteine residues were blocked using 55 mM iodoacetamide in 100 mM ABC for 10 min at room temperature in the dark. Samples were dried thoroughly. Gel pieces were covered with digestion buffer (10% ACN, 40 mM ABC and 13 ng/µL trypsin). Proteins were digested overnight at 37 °C. After digestion, 150 µL of 50% ACN with 0.5% formic acid was added, and the samples were sonicated for 30 min. The supernatant with peptides was transferred to a new microcentrifuge tube, and 150 µL of elution solution was added. Samples were again sonicated for 30 min. The solution was removed, combined with the previous solution, and dried using a vacuum concentrator. Dried peptides were reconstituted in 2% ACN with 0.1% trifluoroacetic acid (TFA) and analyzed using nano-LC–MS/MS. Individual raw data were evaluated using the same software and database as noted in the label-free shotgun proteomic experiment (see below); however, carbamidomethyl modification was used instead of MethylThio.

To illustrate the difference in the 1D-E profile in bumblebee honey compared to honey bee honey and royal jelly, representative images that were obtained using the same procedure that was used for bumblebee honey were selected (see above). Polyfloral honey and RJ samples were from Czechia.

2.4. Label-free nano-LC–MS/MS analysis

The samples were processed for mass spectrometry analysis as previously described (Erban et al. 2019). Trypsin digests were subjected to analysis using nano-LC–MS/MS employing an Orbitrap Fusion Tribrid mass spectrometer (Thermo). The raw Thermo mass spectrometry data were evaluated with MaxQuant v1.6.17.0 software using label-free quantification (LFQ) algorithms that require MS/MS identifications of peptides (Cox and Mann 2008; Cox et al. 2014). The key criteria were as follows: a false FDR of 0.01 for proteins and peptides; minimum length of 7 amino acids; MethylThio was the fixed modification; and N-terminal protein acetylation and methionine oxidation were variable modifications. The common contaminants supplied by MaxQuant were included in the search. However, different levels of databases were used in various searches. The database search considered that the honey was produced by *B. terrestris* and that the dominant flowering plants at the time of the experiment were rapeseed and apple trees. By performing preliminary data searches, we found that our previously selected non-honey bee markers in *A. mellifera* honey (Erban et al. 2021) were also useful for the search of bumblebee honey in this study. Thus, the following protein databases from the NCBI repository were used: (i) *B. terrestris* — 22,118 RefSeq sequences (downloaded on 11/10/2020); (ii) *Brassica napus* — 123,490 RefSeq sequences (downloaded on 01/03/2021) and *Malus domestica* — 52,057 RefSeq sequences (downloaded on 01/03/2021); and the (iv) combined database used in our previous study (Erban et al. 2021) that consisted of plant-related marker databases (cupin, germin, berberine, nectarin, Amb_all, beta-D-xylosidase, GAPDH, MetE, nsLTP, and PL-6) and aphid- and plant-related sequences. The data were further evaluated using Perseus v1.6.14.0 software (Tyanova et al. 2016).

In the data evaluation, protein hits were verified individually using Blastp (Altschul et al. 1990). Only reliably identified and specific protein hits were selected for final presentation. Individual protein sequences were evaluated in SignalIP 5.0 for the prediction of signal peptides (Almagro Armenteros et al. 2019). Furthermore, the protein sequences of *B. terestris* were searched in NCBI, and the first/best result related to *A. mellifera* was selected. The corresponding

sequences were individually compared. Gene names were selected for both *B. terrestris* and *A. mellifera* accessions. In addition, conserved domains (CCDs) (Lu et al. 2020) were identified for *B. terrestris* accessions. In addition, honey bee–related protein identifications in *A. mellifera* honey from our previous studies (Erban et al. 2019, 2021) were individually evaluated to assess consistency with bumblebee-derived proteins found to be similar in *B. terrestris* honey. Further, among the similar proteins were identified those denoted in our previous study (Erban et al. 2019) as 71 major or trace protein hits.

2.5. pH, water and protein content, and sugar analysis

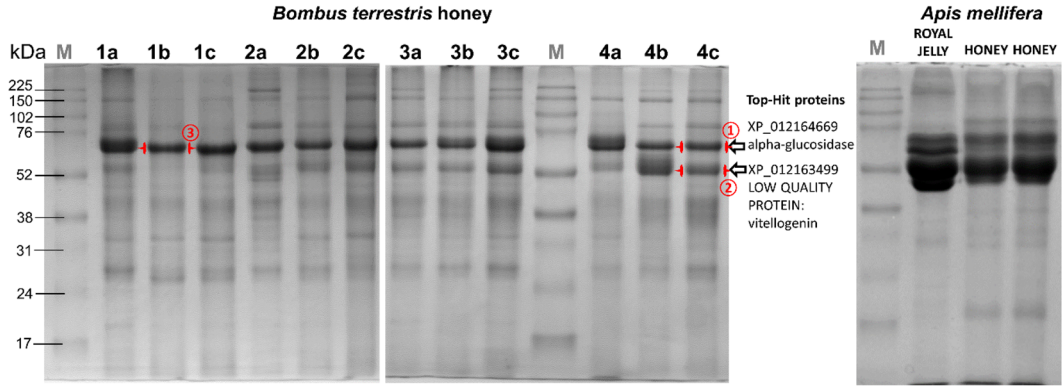
To determine the pH of honey, 1 g was diluted in 10 mL of 0.2-µm-filtered Nanopure water. The analysis was performed immediately after dilution using an Orion Star A111 pH meter (Thermo). The water content was determined using the lyophilization technique. Honey samples were placed into 50-mL sterile centrifugal tubes and lyophilized overnight in PowerDry LL3000 (Thermo). The water content was calculated as the difference between the tube and the sample before lyophilization and after lyophilization. The protein content per g of honey sample was calculated from the analyses in aliquots, which were analyzed using the Bradford reagent (Sect. 2.2). Sugar analysis was performed using the HPLC-ELSD technique as described previously (Erban et al. 2019) with a slight modification of the isocratic elution that was performed with 85:15 ACN/H_2O.

3. RESULTS AND DISCUSSION

In this study, we provide the first proteomic analysis of honey from the bumblebee *B. terrestris*. The difference in the proteomes between *B. terrestris* and *A. mellifera* honey is visually illustrated with the 1D-E SDS–PAGE profile (Figure 1). Our comprehensive shot-gun label-free proteomic analysis reliably (i.e., after applying the cutoff threshold and individual verification of results; see Table S1) identified more than one hundred bumblebee-derived proteins in the honey samples (Table I and Figure 2). This number is greater than that of the *A. mellifera* honey proteome analyzed using the same shotgun proteomics method in our previous studies (Erban et al. 2019, 2021). Again, we stress that the same protein content was used to analyze each sample using nano-LC–MS/MS (Erban et al. 2019, 2021). Furthermore, according to our data, plant-derived proteins (Figure 2) are more abundant in the bumblebee honey proteome than in the honey bee proteome (Erban et al. 2021). The top-hit plant-derived proteins ranked approximately 10^{th} in abundance based on intensity and 6^{th} in abundance based on MS/MS counts. The array of the top-hit plant-derived proteins is listed in Figure 2, and the complete list is provided in Table S2. Overall, the array of predominantly identified plant-derived proteins agrees with the honey collection for our analyses at the time of rapeseed flowering.

An important parameter of honey is total protein content (Rossano et al. 2012; Chua et al. 2013; Erban et al. 2019). Our analysis shows that this factor is similar in bumblebees given that the average accounted for 0.4 mg proteins per g of honey with approximately 11% water content as determined by the lyophilization technique (Table II). However, the protein content is thought to differ based on botanical origin, which is similar to that noted for honey bee honey (Erban et al. 2019). Honey moisture will likely be affected by botanical origin and weather/season. It is necessary to consider that bumblebees have not been observed collecting water (Crane 1972); hence, the supposition is that nectar is the source of water for bumblebees.

We suggest two general explanations for the different honey proteome structure of the bumblebee compared to honey bee. Bumblebee honey has higher total number of bee-derived proteins and increased relative abundance of plant-derived proteins. The most important difference is the lack of high-abundance proteins that have been identified mutually in RJ and honey of honey bees (Erban et al. 2019). These



Three Top-Hit proteins identified using nanoLC-MS/MS according to MS/MS counts

		Intensity	MS/MS count	Fasta headers
①	Band 1	59827000000	162	gi|808122794|ref|XP_012164669.1| alpha-glucosidase [*Bombus terrestris*]
		242920000	73	gi|808120072|ref|XP_012163499.1| LOW QUALITY PROTEIN: vitellogenin [*Bombus terrestris*]
		2606000000	46	gi|340729195|ref|XP_003402892.1| LOW QUALITY PROTEIN: glucosylceramidase [*Bombus terrestris*]
②	Band 2	514200000	93	gi|808120072|ref|XP_012163499.1| LOW QUALITY PROTEIN: vitellogenin [*Bombus terrestris*]
		443160000	42	gi|808122794|ref|XP_012164669.1| alpha-glucosidase [*Bombus terrestris*]
		142390000	19	gi|340726778|ref|XP_003401730.1| regucalcin [*Bombus terrestris*]
③	Band 3	75068000000	183	gi|808122794|ref|XP_012164669.1| alpha-glucosidase [*Bombus terrestris*]
		300590000	77	gi|808120072|ref|XP_012163499.1| LOW QUALITY PROTEIN: vitellogenin [*Bombus terrestris*]
		443360000	54	gi|1249743647|ref|XP_013706042.2| uncharacterized protein LOC106410089 [*Brassica napus*]

Figure 1. Differences in the proteomes between *B. terrestris* and *A. mellifera* honey based on the Coomassie-stained 1D-E SDS–PAGE profile. In addition, the example of 1D-E profile of royal jelly produced exclusively by honey bees is shown for comparison. 1D-E profiles of *B. terrestris* honey collected from 12 colonies overall (four Tripol hives 1–4) are shown. Nano-LC–MS/MS of three bands (①, ②, ③) excised from the gel confirmed the results of label-free shotgun analysis (Table I, Figure 2) that the most abundant protein in *B. terrestris* honey is alpha-glucosidase (GenBank: XP_012164669). The nano-LC–MS/MS analysis results of the three bands showed that many proteins can be identified in the two strongest bands (Tables S3–S5). Note that the proteins of 12 bumblebee honey samples were separated in two gels. Thus, the two images are separated by white space between 2c and 3a samples.

proteins mainly include MRJPs, glucose oxidase (GOx) and alpha-amylase. The lack of these proteins is consistent with the absence of RJ production, reduced HGs in bumblebees (Albert et al. 2014), and differences in the gene arsenal between the species (Sadd et al. 2015). Furthermore, there are different storage and flow of nutrients in the perennial and annual colonies of honey bees and bumblebees (Judd 2011). Overall, it appears that secretion of proteins to honey is more precisely regulated in colonies of honey bees compared with bumblebees. Indeed, honey bees have evolved a food-receiving/storing caste that has not been reported in bumblebees (Seeley et al. 1996; Noll 2002). Our results indicate that compared to bumblebees, honey bees invest more energy in honey in the form of a higher proportion of self-produced proteins within the proteome structure. We suggest that providing honey/food stores with more self-produced proteins may represent a further adaptation for the "highly eusocial" perennial colonies.

3.1. Low array of similar proteins in A. *mellifera* and B. *terrestris* honey

Sequence matches between *A. mellifera*- and *B. terrestris*-derived proteins in honey samples that were similar between the two species were limited (Table I). Specifically, approximately 40 similar (likely homologous) proteins were reliably identified in the honeys. However, the number of unique proteins in *B. terrestris* honey (see

Table I 107 bumblebee-derived protein hits that were reliably identified in the 12 honey samples analyzed using nano-LC–MS/MS

[a]MS/MS count	[b]SignalP-5.0	[c]Fasta headers [*Bombus terrestris*]	[d]*Apis mellifera* honey	[e]Note *A. m.* honey
2816	X	**XP_012164669.1**| alpha-glucosidase	Yes	71 major
690		**XP_003398432.1**| pancreatic lipase-related protein 2	Yes	similar
1087		**XP_020718843.1**| uncharacterized protein LOC100644055	Yes	71 major
1755	X	**XP_012163499.1**| LOW QUALITY PROTEIN: vitellogenin	Yes	rare/trace
399	X	**XP_003402892.1**| LOW QUALITY PROTEIN: glucosylceramidase	Yes	71 major
409		**XP_003398436.1**| pancreatic lipase-related protein 2		
326	X	**XP_020719667.1**| uncharacterized protein LOC100651683		
298	X	**XP_003398435.1**| pancreatic lipase-related protein 2		
341	X	**XP_003401730.1**| regucalcin	Yes	71 major
220	X	**XP_012168805.1**| pancreatic lipase-related protein 2	Yes	71 major
541	X	**XP_020719676.1**| uncharacterized protein LOC100651249		
238	X	**XP_012169771.1**| uncharacterized protein LOC100644337		
253	X	**XP_012176421.1**| venom acid phosphatase Acph-1	Yes	71 major
218		**XP_012171910.2**| mucin-5AC		
280	X	**NP_001267823.1**| venom serine protease precursor	Yes	71 major
277	X	**XP_020721948.1**| transmembrane protease serine 9	Yes	rare/trace
210		**XP_003397297.1**| esterase FE4		
111	X	**XP_003401445.1**| carbonic anhydrase 2		
131		**XP_003398135.1**| glyceraldehyde-3-phosphate dehydrogenase 2	Yes	rare/trace
163	X	**XP_012174762.1**| glucosylceramidase isoform X2	Yes	71 major
156	X	**XP_003393535.1**| chymotrypsin-1	Yes	rare/trace
63	X	**XP_012169491.2**| LOW QUALITY PROTEIN: RNA polymerase-associated protein LEO1		
60	X	**XP_003401255.1**| cysteine-rich venom protein 6	Yes	71 major
138		**XP_012172464.1**| peroxiredoxin 1	Yes	rare/trace
144	X	**XP_012175191.1**| chitinase-like protein Idgf4 isoform X2	Yes	71 major
118	X	**XP_003396094.1**| chymotrypsin-2-like		
98		**XP_020721112.1**| allergen Cr-PI-like isoform X2	Yes	71 major
186		**XP_012170810.1**| enolase		
79	X	**XP_003396228.1**| icarapin-like	Yes	71 major
124	X	**XP_003396093.1**| chymotrypsin-2-like		
125		**XP_003396990.1**| actin-5C		
74	X	**XP_020719382.1**| protein G12 isoform X2		
150		**XP_012173844.2**| uncharacterized protein LOC100643020		

Table I (continued)

[a]MS/MS count	[b]SignalP-5.0	[c]Fasta headers [*Bombus terrestris*]	[d]*Apis mellifera* honey	[e]Note *A. m.* honey
116	X	**XP_003394838.1**| leucine-rich repeat-containing protein 70		
28		**XP_003402547.3**| glucosylceramidase	Yes	71 major
77	X	**XP_003402915.1**| trypsin-2		
120		**XP_003395914.2**| maltase 1		
62	X	**XP_020722252.1**| sialidase		
102	X	**XP_003401050.2**| serine protease 53-like		
125	X	**XP_003400098.1**| venom serine carboxypeptidase		
41	X	**XP_003395447.1**| epididymal secretory protein E1-like	Yes	rare/trace
63	X	**XP_003394143.1**| laccase-1	Yes	71 major
83		**XP_003403377.2**| glucose dehydrogenase [FAD, quinone]		
115		**XP_003401502.1**| arginine kinase		
61	X	**XP_003402225.2**| maltase A1	Yes	rare/trace
61	X	**XP_003401778.1**| hexamerin	Yes	71 major
51	X	**XP_003402742.1**| chymotrypsin-1		
56		**XP_012175036.1**| xanthine dehydrogenase/oxidase isoform X2	Yes	71 major
54	X	**XP_012165909.1**| uncharacterized protein LOC105665936		
72		**XP_003395180.1**| tubulin beta-1 chain	Yes	rare/trace
98		**XP_003397462.1**| heat shock 70 kDa protein cognate 4		
74	X	**XP_003402441.1**| venom serine protease 34	Yes	71 major
57		**XP_003399700.1**| tubulin alpha-1 chain	Yes	rare/trace
75		**XP_003396897.1**| heat shock protein 83	Yes	rare/trace
37	X	**XP_012172139.1**| uncharacterized protein LOC100650343 isoform X2		
69	X	**XP_012174011.1**| zinc carboxypeptidase		
31		**XP_020723033.1**| elongation factor 1-alpha	Yes	71 major
40	X	**XP_003401782.1**| hexamerin	Yes	71 major
46		**XP_003397695.1**| peroxiredoxin-6	Yes	rare/trace
67		**XP_003401321.1**| cytosol aminopeptidase isoform X2		
47		**XP_012166688.1**| superoxide dismutase [Cu–Zn]		
31	X	**XP_003394058.1**| probable salivary secreted peptide		
42		**XP_003399358.1**| adenosylhomocysteinase		
49		**XP_012165896.1**| uncharacterized protein LOC105665627		
36	X	**XP_012172140.1**| transferrin	Yes	71 major
41		**XP_003393531.1**| L-galactose dehydrogenase isoform X1		
34		**XP_003397389.1**| aldehyde dehydrogenase, mitochondrial		
47	X	**XP_003394487.1**| angiotensin-converting enzyme		
40		**XP_012174422.1**| putative ATP-dependent RNA helicase me31b		
36		**XP_012163601.1**| glutathione S-transferase		

Table I (continued)

[a]MS/MS count	[b]SignalP-5.0	[c]Fasta headers [*Bombus terrestris*]	[d]*Apis mellifera* honey	[e]Note *A. m.* honey
21		**XP_003395091.1**| alcohol dehydrogenase class-3		
30	X	**XP_003397865.1**| uncharacterized protein LOC100645203		
15	X	**XP_003393467.1**| uncharacterized protein LOC100648425		
36		**XP_003399519.1**| lambda-crystallin homolog		
32	X	**XP_020721863.1**| uncharacterized protein LOC110119815		
37		**XP_020723251.1**| aminopeptidase Ey		
34		**XP_003399629.1**| LOW QUALITY PROTEIN: 60 kDa heat shock protein, mitochondrial		
21		**XP_003394144.1**| laccase-1	Yes	71 major
45	X	**XP_012172440.2**| LOW QUALITY PROTEIN: alkaline phosphatase 4		
33	X	**XP_012170795.1**| peptidoglycan-recognition protein SC2		
30	X	**XP_012171976.1**| multiple inositol polyphosphate phosphatase 1		
19	X	**XP_003400637.1**| probable chitinase 2		
30		**XP_003400614.1**| ATP synthase subunit beta, mitochondrial	Yes	rare/trace
24		**XP_012172367.1**| aldose reductase		
33		**XP_003396735.1**| ATP synthase subunit alpha, mitochondrial	Yes	71 major
20		**XP_012168040.1**| protein LSM14 homolog A isoform X2		
21		**XP_012174451.1**| fructose-bisphosphate aldolase isoform X2	Yes	rare/trace
15		**XP_020718568.1**| nucleoplasmin-like protein isoform X2		
28	X	**XP_003395393.1**| chitotriosidase-1		
14	X	**XP_012167824.1**| ferritin subunit	Yes	71 major
13	X	**XP_020720525.1**| uncharacterized protein LOC100650672 isoform X2		
12		**XP_003395087.1**| inorganic pyrophosphatase		
20	X	**XP_012167789.1**| poly(U)-specific endoribonuclease homolog isoform X2		
19		**XP_012168294.1**| 14–3-3 protein zeta isoform X1		
21	X	**XP_012175087.1**| N(4)-(Beta-N-acetylglucosaminyl)-L-asparaginase		
17		**XP_012172113.1**| protein D2 isoform X2		
15	X	**XP_003400963.1**| carboxypeptidase B		
18	X	**XP_012174753.1**| endochitinase		
16		**XP_003396233.1**| glucose-6-phosphate isomerase		
16	X	**XP_012167818.1**| ferritin-3, chloroplastic	Yes	71 major
15		**XP_003398335.1**| 15-hydroxyprostaglandin dehydrogenase [NAD(+)]		
11	X	**XP_003401048.1**| trypsin-7		
12	X	**XP_003398424.1**| serine protease inhibitor 88Ea	Yes	71 major
15		**XP_012166540.1**| superoxide dismutase [Cu–Zn], chloroplastic		

Table I (continued)

[a]MS/MS count	[b]SignalP-5.0	[c]Fasta headers [*Bombus terrestris*]	[d]*Apis mellifera* honey	[e]Note *A. m.* honey
16	X	**NP_001318040.1**| vitellogenin-like precursor		
10		**XP_012169580.1**| transitional endoplasmic reticulum ATPase TER94		
11	X	**XP_012174456.1**| digestive cysteine proteinase 1		

[f]FASTA header (first/best *Apis mellifera* (taxid:7460))	[g]Query cover (%)	[h]Per. Ident. (%)	[i]GENE (*Apis mellifera*)
NP_001011608.1| alpha-glucosidase precursor	99	59.86	*Hbg3*
XP_026298886.1| pancreatic triacylglycerol lipase-like	99	58.81	*LOC113219028*
XP_006567694.1| glucose dehydrogenase [FAD, quinone] isoform X1	94	77.8	*Gld2*
NP_001011578.1| vitellogenin precursor	99	51.94	*Vg*
XP_393208.1| putative glucosylceramidase 4	99	62.29	*LOC409709*
XP_026298886.1| pancreatic triacylglycerol lipase-like	91	54.64	*LOC113219028*
XP_006558991.2| yellow-e3 isoform X1	94	77.25	*Y-e3*
XP_026298886.1| pancreatic triacylglycerol lipase-like	99	56.35	*LOC113219028*
XP_026298038.1| uncharacterized protein LOC413627	99	75.96	*LOC413627*
XP_026299638.1| lipase member H-A	93	51.43	*LOC727193*
XP_001123230.2| uncharacterized protein LOC727522	95	59.76	*LOC727522*
XP_016770286.1| uncharacterized protein LOC408320	99	53.85	*LOC408320*
XP_001122458.2| venom acid phosphatase Acph-1	98	72.19	*LOC726737*
XP_026300678.1| mucin-2 isoform X2	57	67.69	*LOC410509*
XP_006560620.1| venom serine protease Bi-VSP	98	61.8	*SP3*
XP_016771183.1| transmembrane protease serine 11B-like protein	100	74.07	*SP17*
XP_393293.2| esterase E4	97	54.53	*LOC409801*
XP_003249463.1| carbonic anhydrase 2	100	55.91	*LOC727237*
XP_393605.1| glyceraldehyde-3-phosphate dehydrogenase 2	99	84.34	*Gapdh*
XP_016768147.1| glucosylceramidase-like isoform X1	99	65.5	*LOC409708*
XP_394370.2| chymotrypsin-1	99	70.23	*LOC410894*
No significant similarity found			
XP_006563421.1| chymotrypsin inhibitor-like	90	51.35	*LOC113218518*
XP_003249289.2| peroxiredoxin 1	98	91.19	*Tpx-1*
XP_016769016.1| chitinase-like protein Idgf4 isoform X2	100	89.63	*LOC413324*
XP_393127.4| chymotrypsin-2	90	52.12	*SP40*
ABU92559.1| hexamerin 110	81	81.23	*hex110*
XP_026300411.1| enolase	99	87.04	*LOC552678*
XP_006563266.1| icarapin-like isoform X1	100	66.81	*LOC503505*
XP_393127.4| chymotrypsin-2	100	67.19	*SP40*
NP_001172074.1| actin related protein 1	100	99.73	*Arp1*
XP_006570633.1| uncharacterized protein LOC102654405	99	45.41	*LOC102654405*
XP_016768511.2| putative aminopeptidase-2	98	64.79	*LOC409619*

Table I (continued)

[f]FASTA header (first/best *Apis mellifera* (taxid:7460))	[g]Query cover (%)	[h]Per. Ident. (%)	[i]GENE (*Apis mellifera*)
XP_397526.2| uncharacterized protein LOC408807	100	72.1	*LOC408807*
XP_393208.1| putative glucosylceramidase 4	100	61.76	*LOC409709*
XP_001120112.3| serine protease 53	100	63.49	*SPH57*
NP_001035326.1| alpha glucosidase 2 precursor	91	60.66	*AGLU2*
XP_026300677.1| mucin-2 isoform X1	42	49.74	*LOC410509*
XP_026301256.1| trypsin-7-like	95	77.86	*LOC725250*
XP_016771167.1| venom serine carboxypeptidase isoform X1	100	81.62	*LOC410451*
XP_001120220.1| NPC intracellular cholesterol transporter 2	100	74.5	*LOC724386*
XP_625189.3| laccase-5	98	67.33	*LOC552811*
XP_006565041.1| glucose dehydrogenase [FAD, quinone] isoform X2	99	66.24	*GMCOX1*
XP_006559245.1| arginine kinase isoform X2	100	95.19	*Argk*
NP_001035349.1| alpha-glucosidase precursor	99	69	*Hbg2*
NP_001011600.1| hexamerin 70b precursor	98	74.16	*HEX70b*
XP_026297199.1| chymotrypsin-1	87	69.16	*SP35*
XP_016768886.2| xanthine dehydrogenase	100	76.35	*LOC724933*
XP_001123230.2| uncharacterized protein LOC727522	98	47.17	*LOC727522*
XP_392313.1| tubulin beta-1 chain	100	100	*LOC408782*
NP_001153522.1| heat shock protein cognate 4	100	98.77	*Hsc70-4*
XP_026296056.1| venom serine protease 34 isoform X2	98	63.41	*SP28*
XP_006567025.1| tubulin alpha-1 chain	100	100	*LOC408388*
NP_001153536.1| heat shock protein 90	100	98.34	*Hsp90*
XP_006563622.1| uncharacterized protein LOC100576330	46	29.52	*LOC100576330*
XP_623922.2| zinc carboxypeptidase	100	76.59	*LOC551524*
XP_006569953.1| elongation factor 1-alpha isoform X1	100	99.13	*EF1a-F2*
ABR45905.1| hexamerin	99	77.49	*Hex70c*
NP_001164444.1| peroxiredoxin-like protein	100	90	*Tpx-4*
XP_016772258.2| cytosol aminopeptidase isoform X2	100	90.43	*LOC552109*
NP_001171498.1| superoxide dismutase 1	98	84.56	*Sod1*
XP_006564764.1| probable salivary secreted peptide	96	67.42	*LOC100579019*
XP_391917.2| adenosylhomocysteinase	100	90.07	*LOC408368*
XP_006570633.1| uncharacterized protein LOC102654405	87	46.33	*LOC102654405*
NP_001011572.1| transferrin 1 precursor	100	77.67	*Tsf1*
XP_006561160.1| L-galactose dehydrogenase isoform X1	93	81.5	*LOC551849*
XP_623084.1| aldehyde dehydrogenase, mitochondrial	100	88.04	*Aldh*
XP_006569964.1| DNA-directed RNA polymerase III subunit RPC1 isoform X2	98	79.15	*LOC725552*
XP_026301917.1| putative ATP-dependent RNA helicase me31b	100	99.55	*LOC552206*
XP_026295805.1| uncharacterized protein LOC552283	99	67.34	*LOC552283*
XP_393266.1| alcohol dehydrogenase class-3	100	87.8	*Fdh*
NP_001035311.1| odorant binding protein 3 precursor	97	36.09	*Obp3*

Table I (continued)

[f]FASTA header (first/best *Apis mellifera* (taxid:7460))	[g]Query cover (%)	[h]Per. Ident. (%)	[i]GENE (*Apis mellifera*)
XP_026300423.1| balbiani ring protein 3	49	62.5	*LOC100576248*
NP_001129207.1| lambda crystallin-like protein	95	83.11	*Cryl1*
XP_026300423.1| balbiani ring protein 3 (lower similarity: protein obstructor E XP_397120.4)	97	43.63	*LOC100576248*
XP_006565537.1| aminopeptidase Ey isoform X1	100	86.66	*LOC412808*
not found in *A. mellifera*, but other bees (*A. dosata*, *A. cerana*, *A. florea*)			
XP_625189.3| laccase-5	98	65.49	*LOC552811*
XP_026294961.1| uncharacterized protein LOC551687	93	83.88	*LOC551687*
NP_001157188.1| peptidoglycan-recognition protein S2 precursor	100	77.84	*Pgrp-s2*
XP_006565013.1| multiple inositol polyphosphate phosphatase 1	96	53.2	*LOC409751*
XP_026300868.1| probable chitinase 2	98	64	*LOC551344*
XP_006564892.1| ATP synthase subunit beta, mitochondrial	100	96.21	*Atp5b*
XP_624353.1| aldose reductase	100	83.96	*LOC551968*
XP_392639.1| ATP synthase subunit alpha, mitochondrial	100	96.89	*Atp5a1*
XP_006560173.1| protein LSM14 homolog A isoform X2	100	97.28	*tral*
XP_006563323.1| fructose-bisphosphate aldolase	100	93.97	*LOC550785*
XP_006570877.1| nucleoplasmin-like protein isoform X2	100	99.42	*LOC102655896*
XP_006562163.1| acidic mammalian chitinase isoform X1	100	68.84	*LOC413705*
XP_026300991.1| ferritin subunit	100	62.5	*Fer1HCH*
XP_001120347.3| uncharacterized protein LOC725273 isoform X1	100	97.86	*CTL1*
XP_003249382.1| inorganic pyrophosphatase	100	87.65	*LOC100578006*
XP_026299154.1| LOW QUALITY PROTEIN: chromatin modification-related protein eaf-1 [Apis mellifera]	74	64.21	*LOC102655712*
XP_006566156.1| 14–3-3 protein zeta isoform X1	100	99.6	*LOC102655329*
XP_026298036.1| protein Red	100	81.41	*LOC411392*
XP_623194.2| protein D2 isoform X2	100	82.97	*LOC408516*
XP_623727.2| carboxypeptidase B	100	78.12	*LOC551327*
XP_026296938.1| uncharacterized protein LOC408874 isoform X9	96	84.48	*LOC408874*
XP_623552.1| glucose-6-phosphate isomerase	100	92.64	*LOC551154*
XP_624076.1| ferritin heavy polypeptide-like 17	100	53.1	*Fer2LCH*
XP_026299150.1| uncharacterized protein LOC724838	99	38.7	*LOC724838*
XP_016772200.1| trypsin-5 isoform X1	80	72.6	*LOC102656186*
XP_006562425.1| serine protease inhibitor 88Ea isoform X1	99	72.54	*LOC413749*
XP_026298263.1| superoxide dismutase [Cu–Zn], chloroplastic-like	85	71.88	*LOC113218958*
NP_001318046.1| larval-specific very high density lipoprotein precursor	99	61.57	*Vhdl*

Table I (continued)

[f]FASTA header (first/best *Apis mellifera* (taxid:7460))	[g]Query cover (%)	[h]Per. Ident. (%)	[i]GENE (*Apis mellifera*)
XP_006563745.1| transitional endoplasmic reticulum ATPase TER94	100	99.5	*TER94*
XP_006562600.2| digestive cysteine proteinase 1	100	86.34	*LOC409517*

Twelve *B. terrestris* honeys from four Tripol hives were included in the study

[a]Total values of MS/MS counts for each marker are shown as quantitative value, although the proteins in the table are ordered based on LFQ intensity. Note that the wide database that included plant-derived proteins was used in data evaluation. The values generally correspond to the heatmap presented in Fig. 2, where the top-hit bumblebee-derived proteins are presented together with plant-derived proteins

[b]The *B. terrestris* proteins for which analysis using SignalIP-5 (Almagro Armenteros et al. 2019) showed the presence of a signal peptide, which is indicative of secretion, are indicated by X

[c]Protein identifiers, such as the representative GenBank access no. and fasta header for reliable *B. terrestris* identifications are provided. In addition, we provide information on the

[d]Information on the similar (homologous) proteins that were identified in previous studies in *A. mellifera* honey (Erban et al. 2019, 2021) are indicated by Yes

[e]The similar (homologous) proteins are divided into 71 major or trace proteins as they were identified in *A. mellifera* honey in a previous study (Erban et al. 2019). Note that the abundance can be different for similar proteins (homologs) in bumblebee and honey bee honey

[f]The first/best *A. mellifera* protein sequences with the highest identity to *B. terrestris* proteins in honey found using Blastp (Altschul et al. 1990) are shown

[g]Query coverage, which is the percentage of [c]*B. terrestris* sequence aligned to the first [f]*A. mellifera* sequence

[h]Percentage identity, which is the percentage of [c]*B. terrestris* sequence aligned to the first [f]*A. mellifera* sequence

[i]A gene for the best *A. mellifera* relative to the *B. terrestris* protein reliably identified in bumblebee honey is provided.

For further details, see Table S1, Supplementary information

Table I) prevailed over those that we identified to be similar given that 107 proteins were reliably identified (Table I). We must consider that while some protein hits are major (high-intensity) in *A. mellifera* or *B. terrestris* honey, the homologs in the honey from the second organism were only present at trace levels or were missing in the analysis. Thus, important examples include increased relative abundances of vitellogenin, lipases, peroxiredoxin and regucalcin in bumblebee honey. Some of the different features, such as the lack of MRJP, could be revealed by a simple comparison of their genomes (Sadd et al. 2015), but the focus on differences in honey proteins was not noted until the present study. Indeed, the lack of alpha-amylase and GOx in honey is also key newly observed features. Thus, here, we provide novel insights into eusociality differences by comparing honey proteomes as representatives of gland secretions.

The similar sequences in honey proteomes of the two species (Table I) are likely true homologs (Krishna and Grishin 2004) that remained in the honeys of the two species after divergence from a common ancestor (Bossert et al. 2019; Porto and Almeida 2021). Future functional studies are necessary to uncover how these proteome similarities relate to the level of eusociality. For instance, the similarities as well as differences in protein secretions into honey could explain the need for the preservation and processing of honey (Erban et al. 2019; Lewkowski et al. 2019) between the annual and perennial colonies. Further understanding may be obtained regarding different brood rearing, immunity and/or signal transduction mechanisms.

3.2. Bumblebee-derived proteins among ~ 30 top hits

Due to the high number of bumblebee-derived proteins identified, for simplification, we focus

Figure 2. The top-hit plant-derived proteins identified among the bumblebee-derived proteins in *B. terrestris* honey. The first 70 protein hits were visualized and selected based on MS/MS counts. Legend: P – plant-derived proteins.

in this section on those that were among the ~30 top-hit results (based on LFQ intensity in Table I). These relatively high-abundance proteins constitute an important difference between the honey of bumblebees and honey bees.

According to label-free shot gun proteomic analysis (Table I, Figure 2) and analysis of bands from 1D-E (Figure 1), the dominant protein in bumblebee honey is alpha-glucosidase (XP_012164669). The alpha-glucosidase

Table II Characteristics of the bumblebee honey samples on protein, water, pH and sugars

Honey sample		**[a]Protein**		**[b]Water**	**[c]pH**	**[d]Sugars**				
		[mg/g]	%	%		fru [mg/g]	glu [mg/g]	% fru	% glu	fru/glu
Tripol 1	1a	0.26	0.026	10.4	4.4	353	246	35.3	24.6	1.44
	1b	0.21	0.021	12.0	4.7	483	355	48.3	35.5	1.36
	1c	0.19	0.019	10.6	4.1	365	231	36.5	23.1	1.58
Tripol 2	2a	0.33	0.033	10.6	4.9	356	258	35.6	25.8	1.38
	2b	0.27	0.027	10.5	4.6	387	309	38.7	30.9	1.25
	2c	0.19	0.019	12.1	5.0	389	216	38.9	21.6	1.80
Tripol 3	3a	0.28	0.028	10.8	4.9	345	303	34.5	30.3	1.14
	3b	0.37	0.037	12.3	4.7	420	372	42.0	37.2	1.13
	3c	0.27	0.027	10.9	4.3	567	329	56.7	32.9	1.72
Tripol 4	4a	0.26	0.026	10.9	4.7	608	364	60.8	36.4	1.67
	4b	0.55	0.055	11.0	4.9	590	356	59.0	35.6	1.66
	4c	0.43	0.043	9.5	4.1	630	263	63.0	26.3	2.39
Average (± SD)		0.3 ± 0.11	0.03 ± 0.01	11 ± 0.8	4.6 ± 0.3	458 ± 111	300 ± 56	45.8 ± 11.1	30.0 ± 5.6	1.54 ± 0.35

[a]The proteins were determined using the Bradford reagent, and bovine serum albumin served as a standard after sample cleaning by PD MidiTrap G-25 columns

[b]Water was determined using the lyophilization technique

[c]pH was determined after dissolving honey 1/10

[d]Sugars were determined using the HPLC-ELSD technique

homolog in honey bees (NP_001011608; *Hbg3* gene) is secreted from hypopharyngeal glands (Kubota et al. 2004), and it is among the most abundant and key proteins in honey bee honey (Di Girolamo et al. 2012; Rossano et al. 2012; Erban et al. 2019; Zhang et al. 2019). Our analysis of sugar composition (Table II; Figure S1) showed that fructose and glucose are the main sugars in bumble bee honey, and the resulting fru/glu ratio (1.5) also appears similar (compare with Table II in Erban et al. (2019)). Due to the lack of major nectar disaccharide sucrose (Wolff 2006), it is likely that the alpha-glucosidase added by bumblebees to honey is invertase that functions in the same manner as honey bee Hbg3 (Kubota et al. 2004).

The second most abundant protein was pancreatic lipase-related protein 2 (XP_003398432), but three additional similar protein hits with the same name were identified among the top protein hits (XP_003398436; XP_003398435; XP_012168805). The latter sequence XP_012168805 had the highest similarity to *A. mellifera* lipase member H-A (XP_026299638; query cover 93%; identity 51.43%). Thus, these lipases are relatively similar in honey bee and bumblebee honey, but our results indicate that more isoforms are present as top hits in *B. terrestris* honey. The question is whether lipases can produce sugar esters (Siebenhaller et al. 2018).

Another high-abundance protein was uncharacterized protein LOC100644055 (Gdh; XP_020718843), which is similar to honey *A. mellifera* glucose dehydrogenase [FAD, quinone] (XP_006567694/XP_016767722; *Gld2* gene; Query cover 94%, identity 77.8%) (Erban et al. 2019, 2021). Importantly, our proteomic data in this paper do not indicate that bumblebees secrete GOx into honey, which is one of the key enzymes secreted from honey bee HGs into honey (Bucekova et al. 2014). The question is to what extent the putative bumblebee "Gdh" (XP_020718843) substitutes or compensates for the function of GOx in the production of D-glucono-1,5-lactone/gluconic acid (Ferri et al. 2011; Milton et al. 2013; Erban et al. 2019). Gluconic acid is a major acid in honey (Mato et al. 2003; Ramachandran et al. 2006) and contributes to acidity that prevents the growth of bacteria and spoilage. The pH in our bumblebee honey samples was also acidic. The average pH of 4.6 (Table II) was approximately in the middle of the common pH range (between 3.5 and 5.5) for honey bee honey (Sereia et al. 2017). Thus, it is possible that bumblebees adjust the pH of honey, but whether gluconic acid contributes is unknown. The absence of GOx is also important because it is one of the key enzymes that maintains H_2O_2 in honey bee honey (Bucekova et al. 2014). Based on that connection, the relatively high abundance of peroxiredoxin 1 (XP_012172464) in *B. terrestris* honey appears to be important because as an antioxidant enzyme, it should be associated with reducing H_2O_2 levels (Neumann et al. 2009). Due to the high abundance, peroxiredoxin may play an important role in cell signaling (Neumann et al. 2009). A different situation has been observed in *A. mellifera* honey, in which peroxiredoxin 1 (XP_003249289) was only present in trace amounts (Erban et al. 2019, 2021). Secretion regulation of proteins that function to maintain H_2O_2 and their antagonists, such as peroxiredoxin, is an open question. It is also possible that these regulations occur due to environmental exposure, and one possible trigger may be microbes in the food sources of bees (Brudzynski 2021). Overall, bumblebees may utilize a different mechanism to preserve honey than bumblebees, i.e., H_2O_2 level maintenance.

We also identified vitellogenin (XP_01216 3499), which is somewhat similar to *A. mellifera* vitellogenin (NP_001011578; query cover 99%, identity 51.94%) among the top-hit proteins of *B. terrestris* honey. However, vitellogenin was identified only among the trace protein hits of *A. mellifera* honey (Erban et al. 2019, 2021). In the *A. mellifera* colony, vitellogenin was found to be multifunctional. Various functions of honey bee vitellogenin have been recognized (Amdam et al. 2012). Thus, this protein might function in immunity, oxidative stress and lifespan regulation. Although vitellogenin is known to interact with juvenile hormone in honey bees, a similar function was not indicated in bumblebees (Amsalem et al. 2014); instead,

social interactions affect vitellogenin levels more strongly than a worker's reproductive physiological state (Amsalem et al. 2014). Thus, we suggest that the difference in vitellogenin abundance in *B. terrestris* honey and the lack in *A. mellifera* honey is an important attribute corresponding to the intermediate stage of eusociality evolution in bumblebees.

The 7th top-hit protein (yellow-e3-like) in Table I is discussed in a separate section (Sect. 3.3).

Our results show that regucalcin (XP_00340 1730) is the fundamental *B. terrestris* honey protein component, which is similar to that noted for *A. mellifera* (XP_026298038; unch. protein LOC413627) honey (Erban et al. 2019, 2021). Regucalcin is interesting because it is a senescence marker in insects and mammals (Nakajima and Natori 2000). Regucalcin may function in honey to modulate biological processes by affecting signal transduction, and it affects cell growth by regulating apoptosis/proliferation (Izumi and Yamaguchi 2004; Yamaguchi 2013; Feng et al. 2015; Yamaguchi and Murata 2015).

Additional important proteins in *A. mellifera* honey are proteases (Di Girolamo et al. 2012; Rossano et al. 2012; Erban et al. 2019, 2021). Our proteome results indicate differences and similarities in protease proteins between *B. terrestris* and *A. mellifera*. Venom acid phosphatase Acph-1 (XP_012176421) and venom serine protease precursor (NP_001267823) represent significant and reliably identified proteases in bumblebee honey. In *A. mellifera* honey, homologs of these two proteases are reliably identified venom-like proteins (XP_001122458 and XP_006560620, respectively) (Erban et al. 2019, 2021). However, a protein (XP_016771183) homologous to *B. terrestris* transmembrane protease serine 9 (XP_020721948) was detected in only trace amounts in *A. mellifera* honey (Erban et al. 2019). A relatively substantial difference in chymotrypsin proteases was observed. Specifically, a high level of chymotrypsin-1 (XP_003393535) was identified in *B. terrestris* honey and was only rarely detected in *A. mellifera* (XP_394370) honey (Erban et al. 2019); additionally, chymotrypsin-2-like (XP_003396094; XP_003396093) homologous proteins (XP_393127) were not identified in *A. mellifera* honey (Erban et al. 2019, 2021). However, our results indicate the presence of proteins/peptides having potential to inhibit protease activity in *B. terrestris* honey, such as cysteine-rich venom protein 6 (XP_003401255), which is similar to *A. mellifera* chymotrypsin inhibitor-like (XP_006563421) (Erban et al. 2019, 2021).

Among the relatively high-intensity proteins identified were additional proteins whose homologs were not identified in *A. mellifera* honey, but these proteins are also of interest. Based on the domain architecture of multiple Ins_allergen domains in the uncharacterized protein LOC100651249 (XP_020719676), this protein might be linked to the chemical interaction of bumblebees with plants. For instance, similar proteins may be found in brassicaceous-feeding insects (Fischer et al. 2008). Notably, the bumblebee honey examined in this study was produced at the time of rapeseed *B. napus* flowering, which supports our interesting supposition that it might have evolved as an adaptation to detoxify certain plant compounds.

Other top-hit proteins are also of interest. The uncharacterized protein LOC100644337 (XP_012169771) contains a JHBP s.f. domain. The domain architecture of mucin-5AC (XP_012171910) has multiple chitin-binding peritrophin-A domains, serine-rich domains and a herpes_BLLF1 domain. Mucin-5AC alters host-microbiome interactions, bacterial adhesion and colonization (Quintana-Hayashi et al. 2015; Ramsey et al. 2017). Esterase FE4 (XP_003397297) contributes to the transformation of various compounds and the detoxification and elimination of environmental stressors (Ma et al. 2018). Carbonic anhydrase 2 (XP_003401445) is an enzyme that reversibly catalyzes carbon dioxide transformation. The enzyme can affect various physiological properties of honey, including pH regulation (Soydan et al. 2020). LOW QUALITY PROTEIN: RNA polymerase-associated protein LEO1 (XP_012169491) is classified as a prolipoprotein diacylglyceryl transferase based on

its domain architecture (Lu et al. 2020). We did not detect any similar sequence in honey bees; therefore, this protein may be specific to the *B. terrestris* genome.

3.3. Presence of yellow-e3-like in the absence of MRJPs

The lack of MRJPs has individual consequences. For instance, the lack of MRJP1 and its "true" homolog has consequences for the antimicrobial peptide arsenal given that jelleins are derived from MRJP1 and contribute to antimicrobial functions in honey (Brudzynski and Sjaarda 2015; Brudzynski et al. 2015). Furthermore, bumblebees lack the functional potential of MRJP3 that participates in RNA uptake in honey bees (Maori et al. 2019). Therefore, an interesting question is whether some MRJP homologs(s) are present in bumblebee honey.

Importantly, a detailed investigation of our data showed that MRJP sequence homolog (XP_020719667.1| uncharacterized protein LOC100651683), which is a yellow-e3-like protein with two MRJP domains (CCD analysis (Lu et al. 2020)), is present among the high-abundance (top ten protein hits in Table I) bumblebee honey proteins. This protein has been denoted BtRJPL (XP_020719667/ADW82102). A nonnutritive function has been suggested for BtRJPL (Kupke et al. 2012), and its expression in the bumblebee brain has been reported (Kupke et al. 2012; Albert et al. 2014). In addition, this yellow-e3-like protein has been reported in *B. terrestris* venom (GenBank removed record gi|340,716,434|ref|XP_003396703.1–100% identity to XP_020719667). However, in the reporting study (Van Vaerenbergh et al. 2015), its expression was presented relative to MRJP9 in *A. mellifera*. According to our analysis, the most similar protein related to BtRJPL in *A. mellifera* is yellow-e3 (XP_006558991; Query cover 94% and Identity 77.25%). Our comparison with the *A. mellifera* honey proteome (Erban et al. 2019, 2021) showed that yellow-e3 is not a honey protein in *A. mellifera*. Therefore, these proteins with similar sequences likely have different functions in the context of eusociality level adaptations.

In general, the primary protein structure (sequence) of all MRJPs is highly similar to that of the yellow gene family (Buttstedt et al. 2014). Conceivably, BtRJPL would be replaced by MRJPs in evolution, or these proteins would have no direct evolutionary connection. Incidentally, our hypothesis may be related to the substantial change in the morphology of HGs of honey bees due to specific food production linked to RJ production (Kupke et al. 2012; Albert et al. 2014).

3.4. Lack of alpha-amylase in bumblebee honey

One important component of honey bee honey is alpha-amylase. Alpha-amylase activity is expressed as a diastase number (DN) (Bodganov et al. 1997; Pasias et al. 2017), which has become an internationally valid measure of freshness and quality, enabling the marketing of honey (Bogdanov et al. 1999; FAO and WHO 2001; Council of the European Union 2002). However, we did not identify homologous sequences to alpha-amylase in *B. terrestris* honey despite the fact that the expected sequence was in our search database. Thus, alpha-amylase does not appear to be functionally required in honey for bumblebee colonies. This shortage can again be explained by the lack of RJ production (Albert et al. 2014). Indeed, similar to MRJPs and GOx, alpha-amylase is among the main *A. mellifera* honey components that are also found in RJ (Erban et al. 2019).

3.5. Note on other proteins processing carbohydrates

As mentioned above (Sect. 3.2), in *B. terrestris* honey, we identified alpha-glucosidase (XP_012164669), which is homologous to *A. mellifera* Hbg3. The homologous proteins/enzymes of Hbg3 alpha-glucosidase are major protein components in honeys from

both species. Further, we reliably identified maltase 1 (XP_003395914) and maltase A1 (XP_003402225), which are similar to *A. mellifera* Hbg1 and Hbg2 (Table I), respectively. Although the two alpha-glucosidases are of interest in *A. mellifera* (Kimura et al. 1990; Takewaki et al. 1993; Kubota et al. 2004), they were not identified to be components of honey proteome (Erban et al. 2019, 2021). It is in agreement with that they are not secreted from honey bee HGs (Kubota et al. 2004), which are reduced in bumblebees (Albert et al. 2014). On the other hand, Hbg1 and Hbg2 are present in ventriculus and hemolymph of honey bees (Kubota et al. 2004). Taken together, we suggest that identification of maltase 1 and maltase A1 in *B. terrestris* honey can be due to different ways of secretion to honey.

Overall, important differences exist in the detection of alpha-amylase and alpha-glucosidases in *A. mellifera* and *B. terrestris* honey. Although alpha-amylase is absent in *B. terrestris* honey, homologous sequences to all three alpha-glucosidases (Hbg1, 2 and 3) that are of interest in honey bees were reliably detected.

3.6. The differences in antimicrobial peptides and protease inhibitors

One of the important components of *A. mellifera* honey is antimicrobial peptides (AMPs). Based on our previous conclusion from a study of the *A. mellifera* honey proteome, serine protease inhibitors might also be involved in antimicrobial activity (Erban et al. 2019). Interestingly, a genome analysis showed that *B. terrestris* only contains a single copy of the defensin gene compared to two copies in *A. mellifera*. On the other hand, serine protease inhibitors are expanded in the *B. terrestris* genome compared to the *A. mellifera* genome (Sadd et al. 2015). We were not able to identify homologs of defensin-1, which is an important and well-known AMP in *A. mellifera* honey (Kwakman et al. 2010; Di Girolamo et al. 2012; Sojka et al. 2016; Bucekova et al. 2017; Erban et al. 2019, 2021). In addition, we did not identify a hymenoptaecin homolog that was recently identified to be commonly present in *A. mellifera* honey (Erban et al. 2019, 2021). These results indicate lack of AMPs in *B. terrestris* honey. On the other hand, a striking resemblance to the *A. mellifera* honey proteome showed identification of homologs of *B. terrestris* serine protease inhibitors, such as serine protease inhibitor 88Ea (XP_003398424) and cysteine-rich venom protein 6 (XP_003401255).

Further, an immune-related function was suggested for the *B. terrestris* peptidoglycan-recognition protein (PGRP) SC2 (XP_012170795) (Barribeau et al. 2015), which we identified in *B. terrestris* honey. This protein might be of interest because an additional PGRP was identified in *B. impatiens* (XP_003487752), which is missing in both *B. terrestris* and *A. mellifera* (Barribeau et al. 2015).

Finally, we identified a "probable salivary secreted peptide" (XP_003394058) that has a homolog in *A. mellifera* but was not detected in its honey. This peptide belongs to the MBF2 superfamily. In *Bombyx mori,* this peptide acts as a transcriptional activator through interaction with TFIIA (Li et al. 1997; Liu et al. 1998, 2000), which functions in defense against reactive oxygen species (Kraemer et al. 2006).

3.7. Plant proteins in bumblebee honey

Based on our analyses, plant-derived proteins appear more abundant (Figure 2) in the bumblebee honey proteome than in the honey bee (Erban et al. 2021) honey proteome; however, the total protein content appears in a similar quantities, i.e., tenths mg of protein per g (Table II). Providing honey with more secreted proteins is associated with a higher energy cost of protein synthesis. Such an energy investment must be highly beneficial. Based on the structure of the honey proteome, honey bees invest more in protein secretion than bumblebees. Notably, RJ-related proteins, mainly MRJPs, are also dominant in honey (Won et al. 2008; Di Girolamo et al. 2012; Chua et al. 2015; Erban et al. 2019, 2021). The lower proportion of bumblebee-derived proteins in honey might be due to a simpler flow of nutrients in the annual colonies (Noll 2002; Judd 2011).

The first/most abundant non-bumblebee protein hit in our analysis was identified as 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MetE); based on the total MS/MS counts, this protein hit was 6th among all proteins (Figure 2, Table S2). In *A. mellifera* honeys, MetE was also observed to be the most abundant plant protein group, whereas it was 24th based on the MS/MS counts in a dataset (Erban et al. 2021). MetE and number of other plant-derived proteins that we identified in the honey of *B. terrestris* according to taxonomy identity assigned to rapeseed origin. This result is consistent with the fact that rapeseed flowered at the time of sampling and that it was near our experimental colonies. Of note, some rather rarely identified proteins were also specific to apple trees, which also flowered at the sampling time.

These results regarding plant-derived proteins are useful for the future development of methods to analyze honey proteins that are not provided by bumblebees. The applicability of this proteomic approach is similar to that used for the honey of honey bees, which is elucidation of honey properties and analyses of differences in honey composition and origin (Erban et al. 2021). Finally, some proteins provided by bumblebee workers are potentially affected by the plant source; e.g., the aforementioned results suggested an interaction between a protein with multiple Ins_allergen domains and brassicaceous-defensive compounds (Fischer et al. 2008; Lu et al. 2020).

3.8. Markers for future interdisciplinary research

We mainly targeted similarities and differences in honey bee and bumblebee honey proteins. Although important differences have been previously inferred from comparison of genomes (Sadd et al. 2015), the proteome comparison of honey provided functional markers specific to proteins that the two species of bees provide to honey. Importantly, some of the markers that indicate interspecies difference at qualitative proteome level (present/absent in a sample) in honey were found in genomes (Sadd et al. 2015) of both *A. mellifera* and *B. terrestris*. Thus, our proteome study provided differentiation markers that could not be identified from comparison of genomes. A nice example is AMPs defensin and hymenoptaecin, which were identified only in *A. mellifera* honey proteome (Erban et al. 2019, 2021). This difference clearly relates to immunity adaptation. The most important qualitative markers are due to the absence of RJ production in bumblebees. Indeed, results of this study show that the difference in RJ production is projected to honey proteomes. This difference has multiple direct consequences such as nutritional adaptation (e.g., MRJPs), immunity (e.g. MRJP1 is the source of AMPs Brudzynski and Sjaarda 2015; Brudzynski et al. 2015)), and physical properties (e.g., GOx). An open question is if bumblebees substitute the missing proteins by other proteins in their honey or whether the protein functions are not required at the lower eusociality level. This hypothesis may be valid also vice versa, because some adaptations could be lost due to higher eusociality level of honey bees. Whether the interspecies differences are qualitative or quantitative, both can have roots at the level of evolutionary adaptations. Overall, the comparison and acquired markers are useful for future research in bee biology and evolution. Furthermore, the identification of bee-derived and other proteins is useful for the determination of protein flow in honeys of different bee species depending on environmental and other biological factors.

The new complex knowledge acquired in this study may also be applied to pharmacological research. Honey bee honey has become a subject of increasing interest due to its broad medial applications (Molan 2001; Hidaka et al. 2008; Othman 2012; Ahmed and Othman 2013; Majtan 2014; Ranzato and Martinotti 2014; Martinotti et al. 2017; Samarghandian et al. 2017) in addition to its use as a common first aid measure or for the prevention of cold and upper respiratory infection in general (Samarghandian et al. 2017; The Lancet 2018; Abuelgasim et al. 2021). Honey is also a very important matrix for mining markers for potential use to treat various diseases (Majtan 2014; Samarghandian et al. 2017; Erban

et al. 2019). However, there is a lack of knowledge in this research area regarding the honey of bumblebees.

4. CONCLUSIONS

In conclusion, we provide the first in-depth insights into honey from the bumblebee *B. terrestris*. We also provide pilot data on total protein, moisture, pH and sugar (fru/glu ratio) characteristics. The results reveal similarities and differences in proteins secreted into honey by bumblebee and honey bee colonies. Similarities and differences in bee-derived proteins are useful in future studies related to the determination of eusociality differences. Furthermore, the approach and data are useful to study seasonal and regional variances within bumblebee species and variances due to different bumble species. The major difference reported here at the proteome level is the lack of major RJ proteins, mainly MRJPs, in bumblebee honey. A dispute exists whether the yellow-3-like protein might represent the prediversification stage. However, a number of other proteins that show differences in these honeys were identified. Importantly, with the exception of results for ~ 40 proteins, we were not able to identify *A. mellifera* honey-related homologs in the majority of the overall array of 107 reliably identified *B. terrestris* honey-related proteins. The key missing proteins include alpha-amylase and GOx, which are also important components of honey bee RJ and honey. An important open question is how bumblebees regulate the physical properties of honeys in the absence of various proteins, such as GOx. In this connection, the presence of carbonic anhydrase 2 in bumblebee honey may be important. However, further studies are needed to confirm the functions of these and other proteins. The results of this study and future studies of the bumblebee honey proteome should help to better understand how secretion of these proteins is regulated in bumblebees, which have substantially reduced HGs.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/s13592-022-00928-3.

ACKNOWLEDGEMENTS

The authors would like to thank Martin Markovic for his valuable help. We thank the editor and two anonymous reviewers whose comments/suggestions helped improve and clarify this manuscript.

AUTHOR CONTRIBUTION

T.E. developed the study, evaluated the data in detail, and wrote the main manuscript. E.S. prepared samples and performed the experiments. P.T. and K.H. performed the nano-LC–MS/MS analysis. E.S. performed electrophoresis, sugar analysis using HPLC-ELSD, and moisture and total protein analyses. All authors discussed the results and commented on the manuscript.

FUNDING

This study was supported by Grant No. QK1820088 and RO0418 of the Ministry of Agriculture of the Czech Republic.

DATA AVAILABILITY

The raw nano-LC–MS/MS runs reported in this paper can be accessed via MassIVE (ID: MSV000088979; https://doi.org/10.25345/C54J09Z9W) or ProteomeXchange (ID: PXD032026). Furthermore, we provide the entire "combined/txt" folder from MaxQuant data processing and the protein databases used for the search for download.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interest.

REFERENCES

Abuelgasim H, Albury C, Lee J (2021) Effectiveness of honey for symptomatic relief in upper respiratory tract infections: a systematic review and

meta-analysis. BMJ Evid Based Med 26:57–64. https://doi.org/10.1136/bmjebm-2020-111336

Ahmed S, Othman NH (2013) Honey as a potential natural anticancer agent: a review of its mechanisms. Evid Based Complement Alternat Med 2013:829070. https://doi.org/10.1155/2013/829070

Albert S, Spaethe J, Grubel K, Rossler W (2014) Royal jelly-like protein localization reveals differences in hypopharyngeal glands buildup and conserved expression pattern in brains of bumblebees and honeybees. Biol Open 3:281–288. https://doi.org/10.1242/bio.20147211

Almagro Armenteros JJ, Tsirigos KD, Sonderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H (2019) SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 37:420–423. https://doi.org/10.1038/s41587-019-0036-z

Altaye SZ, Meng L, Li J (2019) Molecular insights into the enhanced performance of royal jelly secretion by a stock of honeybee (*Apis mellifera ligustica*) selected for increasing royal jelly production. Apidologie 50:436–453. https://doi.org/10.1007/s13592-019-00656-1

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Amdam GV, Fennern E, Havukainen H (2012) Vitellogenin in honey bee behavior and lifespan. In: Galizia CG, Eisenhardt D, Giurfa M (eds) Honeybee neurobiology and behavior: a tribute to Randolf Menzel, Springer, Dordrecht, pp 17–29. https://doi.org/10.1007/978-94-007-2099-2_2

Amsalem E, Malka O, Grozinger C, Hefetz A (2014) Exploring the role of juvenile hormone and vitellogenin in reproduction and social behavior in bumble bees. BMC Evol Biol 14:45. https://doi.org/10.1186/1471-2148-14-45

Babacan S, Rand AG (2005) Purification of amylase from honey. J Food Sci 70:C413–C418. https://doi.org/10.1111/j.1365-2621.2005.tb11439.x

Babacan S, Rand AG (2007) Characterization of honey amylase. J Food Sci 72:C050–C055. https://doi.org/10.1111/j.1750-3841.2006.00215.x

Barribeau SM, Sadd BM, du Plessis L, Brown MJF, Buechel SD, Cappelle K, Carolan JC, Christiaens O, Colgan TJ, Erler S, Evans J, Helbing S, Karaus E, Lattorff HMG, Marxer M, Meeus I, Napflin K, Niu J, Schmid-Hempel R, Smagghe G, Waterhouse RM, Yu N, Zdobnov EM, Schmid-Hempel P (2015) A depauperate immune repertoire precedes evolution of sociality in bees. Genome Biol 16:83. https://doi.org/10.1186/s13059-015-0628-y

Bogdanov S, Lullmann C, Martin P, von der Ohe W, Russmann H, Vorwohl G, Persano Odo L, Sabatini A-G, Marcazzan GL, Piro R, Flamini C, Morlot M, Lheritier J, Borneck R, Marioleas P, Tsigouri A, Kerkvliet J, Ortiz A, Ivanov T, D'Arcy B, Mossel B, Vit P (1999) Honey quality and international regulatory standards: review by the International Honey Commission. Bee World 80:61–69. https://doi.org/10.1080/0005772X.1999.11099428

Bodganov S, Martin P, Lullmann C (1997) Harmonised methods of the European Honey Commission. Apidologie APIDGB5(extra issue):1–59

Bong J, Middleditch M, Loomes KM, Stephens JM (2021) Proteomic analysis of honey. Identification of unique peptide markers for authentication of NZ manuka (*Leptospermum scoparium*) honey. Food Chem 350:128442. https://doi.org/10.1016/j.foodchem.2020.128442

Bossert S, Murray EA, Almeida EAB, Brady SG, Blaimer BB, Danforth BN (2019). Combining transcriptomes and ultraconserved elements to illuminate the phylogeny of Apidae. Mol Phylogenet Evol 130:121–131. https://doi.org/10.1016/j.ympev.2018.10.012

Brudzynski K (2020) A current perspective on hydrogen peroxide production in honey. Food Chem 332:127229. https://doi.org/10.1016/j.foodchem.2020.127229

Brudzynski K (2021) Honey as an ecological reservoir of antibacterial compounds produced by antagonistic microbial interactions in plant nectars, honey and honey bee. Antibiotics (Basel) 10:551. https://doi.org/10.3390/antibiotics10050551

Brudzynski K, Sjaarda C (2015) Honey glycoproteins containing antimicrobial peptides, jelleins of the major royal jelly protein 1, are responsible for the cell wall lytic and bactericidal activities of honey. PLoS One 10:e0120238. https://doi.org/10.1371/journal.pone.0120238

Brudzynski K, Sjaarda C, Lannigan R (2015) MRJP1-containing glycoproteins isolated from honey, a novel antibacterial drug candidate with broad spectrum activity against multi-drug resistant clinical isolates. Front Microbiol 6:711. https://doi.org/10.3389/fmicb.2015.00711

Bucekova M, Sojka M, Valachova I, Martinotti S, Ranzato E, Szep Z, Majtan V, Klaudiny J, Majtan J (2017) Bee-derived antibacterial peptide, defensin-1, promotes wound re-epithelialisation *in vitro* and *in vivo*. Sci Rep 7:7340. https://doi.org/10.1038/s41598-017-07494-0

Bucekova M, Valachova I, Kohutova L, Prochazka E, Klaudiny J, Majtan J (2014) Honeybee glucose oxidase—its expression in honeybee workers and comparative analyses of its content and H_2O_2-mediated antibacterial activity in natural honeys. Naturwissenschaften 101:661–670. https://doi.org/10.1007/s00114-014-1205-z

Buttstedt A, Moritz RFA, Erler S (2014) Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the *yellow* gene family. Biol Rev Camb Philos Soc 89:255–269. https://doi.org/10.1111/brv.12052

Chua LS, Lee JY, Chan GF (2013) Honey protein extraction and determination by mass spectrometry. Anal Bioanal Chem 405:3063–3074. https://doi.org/10.1007/s00216-012-6630-2

Chua LS, Lee JY, Chan GF (2015) Characterization of the proteins in honey. Anal Lett 48:697–709. https://doi.org/10.1080/00032719.2014.952374

Council of the European Union (2002) Council Directive 2001/110/EC of 20 December 2001 relating to honey. Off J Eur Union OJ L 10:47–52. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32001L0110. Accessed 3 Mar 2022

Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol Cell Proteomics 13:2513−2526. https://doi.org/10.1074/mcp.M113.031591

Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26:1367–1372. https://doi.org/10.1038/nbt.1511

Crane E (1972) Bumble bee honeys and others. Bee World 53:38–39. https://doi.org/10.1080/0005772X.1972.11097399

de Ruijter A (1997). Commercial bumblebee rearing and its implications. Acta Hortic 437:261–269. https://doi.org/10.17660/ActaHortic.1997.437.30

Dehon M, Engel MS, Gerard M, Aytekin AM, Ghisbain G, Williams PH, Rasmont P, Michez D (2019) Morphometric analysis of fossil bumble bees (Hymenoptera, Apidae, Bombini) reveals their taxonomic affinities. Zookeys 891:71–118. https://doi.org/10.3897/zookeys.891.36027

Di Girolamo F, D'Amato A, Righetti PG (2012) Assessment of the floral origin of honey via proteomic tools. J Proteomics 75:3688–3693. https://doi.org/10.1016/j.jprot.2012.04.029

Erban T, Shcherbachenko E, Talacko P, Harant K (2019) The unique protein composition of honey revealed by comprehensive proteomic analysis: allergens, venom-like proteins, antibacterial properties, royal jelly proteins, serine proteases, and their inhibitors. J Nat Prod 82:1217–1226. https://doi.org/10.1021/acs.jnatprod.8b00968

Erban T, Shcherbachenko E, Talacko P, Harant K (2021) A single honey proteome dataset for identifying adulteration by foreign amylases and mining various protein markers natural to honey. J Proteomics 239:104157. https://doi.org/10.1016/j.jprot.2021.104157

Erler S, Denner A, Bobis O, Forsgren E, Moritz RFA (2014) Diversity of honey stores and their impact on pathogenic bacteria of the honeybee, *Apis mellifera*. Ecol Evol 4:3960–3967. https://doi.org/10.1002/ece3.1252

FAO, WHO (2001) Standard for honey (CODEX STAN 12–1981). Adopted in 1981. Revisions 1987 and 2001. In: FAO, WHO (eds) *Codex alimentarius*: international food standards, Food and Agriculture Organization of the United Nations (FAO), Rome and World Health Organization (WHO), Geneva. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B12-1981%252FCXS_012e.pdf

Feng M, Fang Y, Han B, Xu X, Fan P, Hao Y, Qi Y, Hu H, Huo X, Meng L, Wu B, Li J (2015) In-depth N-glycosylation reveals species-specific modifications and functions of the royal jelly protein from western (*Apis mellifera*) and eastern honeybees (*Apis cerana*). J Proteome Res 14:5327–5340. https://doi.org/10.1021/acs.jproteome.5b00829

Ferri S, Kojima K, Sode K (2011) Review of glucose oxidases and glucose dehydrogenases: a bird's eye view of glucose sensing enzymes. J Diabetes Sci Technol 5:1068–1076. https://doi.org/10.1177/193229681100500507

Fischer HM, Wheat CW, Heckel DG, Vogel H (2008) Evolutionary origins of a novel host plant detoxification gene in butterflies. Mol Biol Evol 25:809–820. https://doi.org/10.1093/molbev/msn014

Goulson D (2003) Conserving wild bees for crop pollination. J Food Agric Environ 1:142–14

Hidaka S, Okamoto Y, Ishiyama K, Hashimoto K (2008) Inhibition of the formation of oral calcium phosphate precipitates: the possible effects of certain honeybee products. J Periodontal Res 43:450–458. https://doi.org/10.1111/j.1600-0765.2008.01088.x

Hung K-LJ, Kingston JM, Albrecht M, Holway DA, Kohn JR (2018) The worldwide importance of honey bees as pollinators in natural habitats. Proc Biol Sci 285:20172140. https://doi.org/10.1098/rspb.2017.2140

Izumi T, Yamaguchi M (2004) Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-alpha or thapsigargin. J Cell Biochem 92:296–306. https://doi.org/10.1002/jcb.20056

Judd TM (2011) The role of food storage and communication in the evolution of perennial social hymenopteran colonies. In: Stewart EM (ed) Social insects: structure, function, and behavior, Nova Science Publishers, Hauppauge, NY, pp 1–23

Kimura A, Takewaki S-i, Matsui H, Kubota M, Chiba S (1990) Allosteric properties, substrate specificity, and subsite affinities of honeybee alpha-glucosidase I. J Biochem 107:762–768. https://doi.org/10.1093/oxfordjournals.jbchem.a123122

Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T (2007) Importance of pollinators in changing landscapes

for world crops. Proc Biol Sci 274:303–313. https://doi.org/10.1098/rspb.2006.3721

Knapp JL, Becher MA, Rankin CC, Twiston-Davies G, Osborne JL (2019) *Bombus terrestris* in a mass-flowering pollinator-dependent crop: a mutualistic relationship? Ecol Evol 9:609–618. https://doi.org/10.1002/ece3.4784

Kolayli S, Keskin M (2020) Chapter 7 - Natural bee products and their apitherapeutic applications. Stud Nat Prod Chem 66:175–196. https://doi.org/10.1016/B978-0-12-817907-9.00007-6

Konzmann S, Lunau K (2014) Divergent rules for pollen and nectar foraging bumblebees – a laboratory study with artificial flowers offering diluted nectar substitute and pollen surrogate. PLoS One 9:e91900. https://doi.org/10.1371/journal.pone.0091900

Kraemer SM, Goldstrohm DA, Berger A, Hankey S, Rovinsky SA, Moye-Rowley WS, Stargell LA (2006) TFIIA plays a role in the response to oxidative stress. Eukaryot Cell 5:1081–1090. https://doi.org/10.1128/EC.00071-06

Krishna SS, Grishin NV (2004) Structurally analogous proteins do exist! Structure 12:1125–1127. https://doi.org/10.1016/j.str.2004.06.004

Kubota M, Tsuji M, Nishimoto M, Wongchawalit J, Okuyama M, Mori H, Matsui H, Surarit R, Svasti J, Kimura A, Chiba S (2004) Localization of alpha-glucosidases I, II, and III in organs of European honeybees, *Apis mellifera* L., and the origin of alpha-glucosidase in honey. Biosci Biotechnol Biochem 68:2346–2352. https://doi.org/10.1271/bbb.68.2346

Kupke J, Spaethe J, Mueller MJ, Rossler W, Albert S (2012) Molecular and biochemical characterization of the major royal jelly protein in bumblebees suggest a non-nutritive function. Insect Biochem Mol Biol 42:647–654. https://doi.org/10.1016/j.ibmb.2012.05.003

Kwakman PHS, te Velde AA, de Boer L, Speijer D, Vandenbroucke-Grauls CMJE, Zaat SAJ (2010) How honey kills bacteria. FASEB J 24:2576–2582. https://doi.org/10.1096/fj.09-150789

Lewkowski O, Muresan CI, Dobritzsch D, Fuszard M, Erler S (2019) The effect of diet on the composition and stability of proteins secreted by honey bees in honey. Insects 10:282. https://doi.org/10.3390/insects10090282

Li F-Q, Takemaru K, Goto M, Ueda H, Handa H, Hirose S (1997) Transcriptional activation through interaction of MBF2 with TFIIA. Genes Cells 2:143–153. https://doi.org/10.1046/j.1365-2443.1997.1090306.x

Liu Q-X, Ueda H, Hirose S (1998) Comparison of sequences of a transcriptional coactivator MBF2 from three Lepidopteran species *Bombyx mori*, *Bombyx mandarina* and *Samia cynthia*. Gene 220:55–59. https://doi.org/10.1016/s0378-1119(98)00428-4

Liu Q-X, Ueda H, Hirose S (2000) MBF2 is a tissue- and stage-specific coactivator that Is regulated at the step of nuclear transport in the silkworm *Bombyx mori*. Dev Biol 225:437–446. https://doi.org/10.1006/dbio.2000.9836

Lu S, Wang J, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Marchler GH, Song JS, Thanki N, Yamashita RA, Yang M, Zhang D, Zheng C, Lanczycki CJ, Marchler-Bauer A (2020) CDD/SPARCLE: the conserved domain database in 2020. Nucleic Acids Res 48:D265–D268. https://doi.org/10.1093/nar/gkz991

Ma M, Jia H, Cui X, Zhai N, Wang H, Guo X, Xu B (2018) Isolation of carboxylesterase (esterase FE4) from *Apis cerana cerana* and its role in oxidative resistance during adverse environmental stress. Biochimie 144:85–97. https://doi.org/10.1016/j.biochi.2017.10.022

Majtan J (2014) Honey: an immunomodulator in wound healing. Wound Repair Regen 22:187–192. https://doi.org/10.1111/wrr.12117

Maori E, Navarro IC, Boncristiani H, Seilly DJ, Rudolph KLM, Sapetschnig A, Lin C-C, Ladbury JE, Evans JD, Heeney JL, Miska EA (2019) A secreted RNA binding protein forms RNA-stabilizing granules in the honeybee royal jelly. Mol Cell 74:598–608. e596. https://doi.org/10.1016/j.molcel.2019.03.010

Martinotti S, Calabrese G, Ranzato E (2017) Honeydew honey: biological effects on skin cells. Mol Cell Biochem 435:185–192. https://doi.org/10.1007/s11010-017-3067-0

Mato I, Huidobro JF, Simal-Lozano J, Sancho MT (2003) Significance of nonaromatic organic acids in honey. J Food Prot 66:2371–2376. https://doi.org/10.4315/0362-028x-66.12.2371

Milton RD, Giroud F, Thumser AE, Minteer SD, Slade RCT (2013) Hydrogen peroxide produced by glucose oxidase affects the performance of laccase cathodes in glucose/oxygen fuel cells: FAD-dependent glucose dehydrogenase as a replacement. Phys Chem Chem Phys 15:19371–19379. https://doi.org/10.1039/C3CP53351D

Molan PC (2001) Potential of honey in the treatment of wounds and burns. Am J Clin Dermatol 2:13–19. https://doi.org/10.2165/00128071-200102010-00003

Nakajima Y, Natori S (2000) Identification and characterization of an anterior fat body protein in an insect. J Biochem 127:901–908. https://doi.org/10.1093/oxfordjournals.jbchem.a022685

Neumann CA, Cao J, Manevich Y (2009) Peroxiredoxin 1 and its role in cell signaling. Cell Cycle 8:4072–4078. https://doi.org/10.4161/cc.8.24.10242

Noll FB (2002) Behavioral phylogeny of corbiculate Apidae (Hymenoptera; Apinae), with special reference to social behavior. Cladistics 18:137–153. https://doi.org/10.1111/j.1096-0031.2002.tb00146.x

Othman NH (2012) Does honey have the characteristics of natural cancer vaccine? J Tradit Complement Med 2:276–283. https://doi.org/10.1016/S2225-4110(16)30113-4

Pasias IN, Kiriakou IK, Proestos C (2017) HMF and diastase activity in honeys: a fully validated approach and a chemometric analysis for identification of honey freshness and adulteration. Food Chem 229:425–431. https://doi.org/10.1016/j.foodchem.2017.02.084

Porto DS, Almeida EAB (2021) Corbiculate bees (Hymenoptera: Apidae): exploring the limits of morphological data to solve a hard phylogenetic problem. Insect Syst Diver 5:2. https://doi.org/10.1093/isd/ixab008

Quintana-Hayashi MP, Mahu M, De Pauw N, Boyen F, Pasmans F, Martel A, Premaratne P, Fernandez HR, Teymournejad O, Vande Maele L, Haesebrouck F, Linden SK (2015) The levels of *Brachyspira hyodysenteriae* binding to porcine colonic mucins differ between individuals, and binding is increased to mucins from infected pigs with *de novo* MUC5AC synthesis. Infect Immun 83:1610–1619. https://doi.org/10.1128/iai.03073-14

Ramachandran S, Fontanille P, Pandey A, Larroche C (2006) Gluconic acid: properties, applications and microbial production. Food Technol Biotechnol 44:185–195

Ramsey JS, Chavez JD, Johnson R, Hosseinzadeh S, Mahoney JE, Mohr JP, Robison F, Zhong X, Hall DG, MacCoss M, Bruce J, Cilia M (2017) Protein interaction networks at the host–microbe interface in *Diaphorina citri*, the insect vector of the citrus greening pathogen. R Soc Open Sci 4:160545. https://doi.org/10.1098/rsos.160545

Ranzato E, Martinotti S (2014) Cellular and molecular mechanisms of honey wound healing. Nova Science Publishers, Hauppauge, NY

Rossano R, Larocca M, Polito T, Perna AM, Padula MC, Martelli G, Riccio P (2012) What are the proteolytic enzymes of honey and what they do tell us? A fingerprint analysis by 2-D zymography of unifloral honeys. PLoS One 7:e49164. https://doi.org/10.1371/journal.pone.0049164

Sadd BM, Barribeau SM, Bloch G, de Graaf DC, Dearden P, Elsik CG, Gadau J, Grimmelikhuijzen CJ, Hasselmann M, Lozier JD, Robertson HM, Smagghe G, Stolle E, Van Vaerenbergh M, Waterhouse RM, Bornberg-Bauer E, Klasberg S, Bennett AK, Camara F, Guigo R, Hoff K, Mariotti M, Munoz-Torres M, Murphy T, Santesmasses D, Amdam GV, Beckers M, Beye M, Biewer M, Bitondi MM, Blaxter ML, Bourke AF, Brown MJ, Buechel SD, Cameron R, Cappelle K, Carolan JC, Christiaens O, Ciborowski KL, Clarke DF, Colgan TJ, Collins DH, Cridge AG, Dalmay T, Dreier S, du Plessis L, Duncan E, Erler S, Evans J, Falcon T, Flores K, Freitas FC, Fuchikawa T, Gempe T, Hartfelder K, Hauser F, Helbing S, Humann FC, Irvine F, Jermiin LS, Johnson CE, Johnson RM, Jones AK, Kadowaki T, Kidner JH, Koch V, Kohler A, Kraus FB, Lattorff HM, Leask M,Lockett GA, Mallon EB, Antonio DS, Marxer M, Meeus I, Moritz RF, Nair A, Napflin K, Nissen I, Niu J, Nunes FM, Oakeshott JG, Osborne A, Otte M, Pinheiro DG, Rossie N, Rueppell O, Santos CG, Schmid-Hempel R, Schmitt BD, Schulte C, Simoes ZL, Soares MP, Swevers L, Winnebeck EC, Wolschin F, Yu N, Zdobnov EM, Aqrawi PK, Blankenburg KP, Coyle M, Francisco L, Hernandez AG, Holder M, Hudson ME, Jackson L, Jayaseelan J, Joshi V, Kovar C, Lee SL, Mata R, Mathew T, Newsham IF, Ngo R, Okwuonu G, Pham C, Pu LL, Saada N, Santibanez J, Simmons D, Thornton R, Venkat A, Walden KK, Wu YQ, Debyser G, Devreese B, Asher C, Blommaert J, Chipman AD, Chittka L, Fouks B, Liu J, O'Neill MP, Sumner S, Puiu D, Qu J, Salzberg SL, Scherer SE, Muzny DM, Richards S, Robinson GE, Gibbs RA, Schmid-Hempel P, Worley KC (2015) The genomes of two key bumblebee species with primitive eusocial organization. Genome Biol 16:76. https://doi.org/10.1186/s13059-015-0623-3

Samarghandian S, Farkhondeh T, Samini F (2017) Honey and health: a review of recent clinical research. Pharmacognosy Res 9:121–127. https://doi.org/10.4103/0974-8490.204647

Schepartz AI, Subers MH (1964) The glucose oxidase of honey I. Purification and some general properties of the enzyme. Biochim Biophys Acta (BBA) Spec Sect Enzymol Subj 85:228–237. https://doi.org/10.1016/0926-6569(64)90243-3

Schmidt JO (1997) Bee products: chemical composition and application. In: Mizrahi A, Lensky Y (eds) Bee products, Springer, Boston, MA, pp 15–26. https://doi.org/10.1007/978-1-4757-9371-0_2

Schmitzova J, Klaudiny J, Albert S, Schroder W, Schreckengost W, Hanes J, Judova J, Simuth J (1998) A family of major royal jelly proteins of the honeybee *Apis mellifera* L. CMLS Cell Mol Life Sci 54:1020–1030. https://doi.org/10.1007/s000180050229

Seeley TD, Kuhnholz S, Weidenmuller A (1996) The honey bee's tremble dance stimulates additional bees to function as nectar receivers. Behav Ecol Sociobiol 39:419–427. https://doi.org/10.1007/s002650050309

Sereia MJ, Marco PH, Perdoncini MRG, Parpinelli RS, de Lima EG, Anjo FA (2017) Techniques for the evaluation of physicochemical quality and bioactive compounds in honey. In: de Toledo VdAA (ed) Honey analysis, IntechOpen, pp 193–214. https://doi.org/10.5772/66839; https://www.intechopen.com/chapters/53469

Siebenhaller S, Gentes J, Infantes A, Muhle-Goll C, Kirschhofer F, Brenner-Weiss G, Ochsenreither K, Syldatk C (2018) Lipase-catalyzed synthesis of

sugar esters in honey and agave syrup. Front Chem 6:24. https://doi.org/10.3389/fchem.2018.00024
Sladen FWL (1912) The humble-bee: its life-history and how to domesticate it: with description of all British species of *Bombus* and *Psithyrus*; Macmillan and Co., London
Sojka M, Valachova I, Bucekova M, Majtan J (2016) Antibiofilm efficacy of honey and bee-derived defensin-1 on multispecies wound biofilm. J Med Microbiol 65:337–344. https://doi.org/10.1099/jmm.0.000227
Soydan E, Olcay AC, Bilir G, Tas O, Senturk M, Ekinci D, Supuran CT (2020) Investigation of pesticides on honey bee carbonic anhydrase inhibition. J Enzyme Inhib Med Chem 35:1923–1927. https://doi.org/10.1080/14756366.2020.1835885
Svanberg I, Berggren A (2018) Bumblebee honey in the Nordic countries. Ethnobiol Lett 9:312–318. https://doi.org/10.14237/ebl.9.2.2018.1383
Takewaki S-I, Kimura A, Kubota M, Chiba S (1993) Substrate specificity and subsite affinities of honeybee alpha-glucosidase II. Biosci Biotechnol Biochem 57:1508–1513. https://doi.org/10.1271/bbb.57.1508
The Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443:931–949. https://doi.org/10.1038/nature05260
The Lancet (2018) Does a spoonful of honey make the medicine go down? Lancet 392:712–712. https://doi.org/10.1016/S0140-6736(18)31986-X
Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, Mann M, Cox J (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13:731–740. https://doi.org/10.1038/nmeth.3901
Van Vaerenbergh M, Debyser G, Smagghe G, Devreese B, de Graaf DC (2015) Unraveling the venom proteome of the bumblebee (*Bombus terrestris*) by integrating a combinatorial peptide ligand library approach with FT-ICR MS. Toxicon 102:81–88. https://doi.org/10.1016/j.toxicon.2013.10.002
Velthuis HHW, van Doorn A (2006) A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie 37:421–451. https://doi.org/10.1051/apido:2006019
Viteri R, Zacconi F, Montenegro G, Giordano A (2021) Bioactive compounds in *Apis mellifera* monofloral honeys. J Food Sci 86:1552–1582. https://doi.org/10.1111/1750-3841.15706
Wolff D (2006) Nectar sugar composition and volumes of 47 species of gentianales from a southern Ecuadorian montane forest. Ann Bot 97:767–777. https://doi.org/10.1093/aob/mcl033
Won S-R, Lee D-C, Ko SH, Kim J-W, Rhee H-I (2008) Honey major protein characterization and its application to adulteration detection. Food Res Int 41:952–956. https://doi.org/10.1016/j.foodres.2008.07.014
Yamaguchi M (2013) Suppressive role of regucalcin in liver cell proliferation: involvement in carcinogenesis. Cell Prolif 46:243–253. https://doi.org/10.1111/cpr.12036
Yamaguchi M, Murata T (2015) Suppressive effects of exogenous regucalcin on the proliferation of human pancreatic cancer MIA PaCa-2 cells *in vitro*. Int J Mol Med 35:1773–1778. https://doi.org/10.3892/ijmm.2015.2164
Zhang Y-Z, Chen Y-F, Wu Y-Q, Si J-J, Zhang C-P, Zheng H-Q, Hu F-L (2019) Discrimination of the entomological origin of honey according to the secretions of the bee (*Apis cerana* or *Apis mellifera*). Food Res Int 116:362–369. https://doi.org/10.1016/j.foodres.2018.08.049