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Original article

Two-dimensional proteomic analysis of honeybee, *Apis mellifera*, winter worker hemolymph

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Abstract – Honeybee (*Apis mellifera* Linnaeus) colonies in temperate zones produce either summer bees, which have a lifespan of 15 to 48 days, or winter bees, which emerge in late summer and live up to 8 months. Winter bees develop unique physiological conditions characterized by changes in protein composition that appear to be major determinants of honeybee lifespan. We analyzed winter honeybee worker hemolymph using a proteomic approach for the first time. Hemolymph collected from the dorsal vessel of winter honeybees using a glass capillary tube was analyzed using two-dimensional gel electrophoresis followed by MALDI TOF/TOF protein identification. Overall, 93 spots were assigned significance ($P<0.05$). Many identified proteins corresponded well with extended lifespan. Vitellogenin subunits (mainly ~180 and ~100 kDa) comprised the major portion of the proteins; however, vitellogenin dominance repressed the signals of the lower-abundance proteins. Future physiological studies related to overwintering bees, including health, immunity, longevity, nutrition, and/or colony losses, can benefit from these results.

***Apis mellifera* / hemolymph / winter honeybee / vitellogenin / proteomics / longevity**

1. INTRODUCTION

Honeybees (*Apis mellifera* Linnaeus) are the most economically valuable pollinators of agricultural crops worldwide. The fact that the honeybee genome was one of the first genomes to be sequenced and annotated (Honeybee Genome Sequencing Consortium 2006) highlights the importance of honeybees to humans. Recently, many researchers have been interested in the as-yet unexplained phenomenon of Colony Collapse Disorder (CCD) (Williams et al. 2010) for which the major cause is thought to be the parasitic mite, *Varroa destructor* (Dainat et al. 2012; van Dooremalen et al. 2012). The study of the biology of wintering bees is important because the main colony losses occur in winter in temperate regions (Dainat et al. 2012; van Dooremalen et al. 2012).

In temperate regions, honeybee colonies produce either short-lived summer worker bees, which have a lifespan between 15 and 48 days, or long-lived winter worker bees, which emerge in late summer and live up to 8 months (Fluri 1990). Because no brood or small numbers of brood are reared during winter, winter workers have low synthesizing activity (Brouwers 1983) in the almost fully developed hypopharyngeal glands (HPGs) (Fluri et al. 1982; Hrassnigg and

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Crailsheim 1998). The unique physiological adaptations that occur in winter bees over a period of 3 to 4 weeks after emergence are associated with worker longevity (Fluri et al. 1982). Most distinctly, winter bees differ from summer worker bees in that they have low juvenile hormone (JH) titer, but high protein titer. Low JH levels increase vitellogenin titers, which negatively regulate JH levels (Fluri et al. 1977, 1982). However, because summer workers are not as homogenous as winter bees, their physiology differs in the role they fulfill in the colony. Dramatic changes occur when nurse bee with high vitellogenin titer becomes forager, a process that decreases vitellogenin titers to an undetectable level (Amdam et al. 2003; Piulachs et al. 2003; Guidugli et al. 2005). Thus, protein composition appears to be a major determinant of honeybee lifespan, and the major hemolymph protein, vitellogenin, seems to play a crucial role in longevity (Amdam and Omholt 2002).

Similar to vertebrate blood, insect hemolymph is responsible for supplying nutrients to tissues and organs. It is composed mainly of water, inorganic salts, carbohydrates, proteins, hormones, lipids, free amino acids, and macrophage-like cells (hemocytes) (Leta et al. 1996; Lavine and Strand 2002; Hrassnigg et al. 2003; Chan et al. 2006; Burmester and Hankeln 2007). The insect hemolymph proteins provide important physiological and immune system information and reflect the health of the organism (Levy et al. 2004; Bogaerts et al. 2009). Moreover, because honeybees are social insects and the protein composition of hemolymph varies between developmental stages, hemolymph proteins can be used to study caste differences as well as development (Chan et al. 2006; Randolt et al. 2008). Several proteomic studies analyzing honeybee hemolymph using one-dimensional gel electrophoresis or two-dimensional gel electrophoresis (2DGE) have been performed (Danty et al. 1998; Chan et al. 2006; Chan and Foster 2008; Randolt et al. 2008; Bogaerts et al. 2009). Bogaerts et al. (2009) were the first group to analyze the hemolymph of summer *A. mellifera carnica* workers using 2DGE and gel-free (2DLC) proteomic approaches (Bogaerts et al. 2009). More recently, a similar 2DGE protein pattern of key hemolymph proteins was found in summer dequeened honeybees (Cardoen et al. 2011).

The objective of the current study was to analyze winter honeybee worker hemolymph using a proteomic approach for the first time. Using a proteomic approach based on 2DGE, together with matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF mass spectrometry analysis, major proteins of long-lived winter *A. mellifera* hemolymph were identified. An insight into the complex proteins of the winter honeybee hemolymph gave us the opportunity to summarize the functions of the proteins that were identified and are thus related to the wintering of the honeybee.

2. MATERIALS AND METHODS

2.1. Biological samples

Winter honeybees, *A. mellifera mellifera,* bred at the Bee Research Institute at Dol in the Czech Republic were used in this study. The beehive was moved in January to a 5×8×4-m flying hall (RH 70–80 %; ~18 °C) for easier manipulation. After a 3-day acclimatization period in the new environment, winter honeybees were collected from the hive and placed in a glass bottle covered with muslin and a cap with 10 mm diameter vent holes. The bees were anesthetized using CO_2. Hemolymph was collected from the dorsal vessel using a 40-μm outer diameter glass capillary tube after puncturing the intersegmental membrane between the fourth and fifth tergites of the adult honeybee abdomen. Approximately 2 μL of hemolymph was collected from each individual. Only transparent hemolymph was used in these studies. Hemolymph that was potentially contaminated by non-hemolymph particles was discarded. The hemolymph was transferred from the glass capillary tube to a 0.5-mL Eppendorf Protein LoBind Tube (Cat. No. Z666491, Sigma-Aldrich) containing 1 μL of protease inhibitor mix (Cat. No. 80-6501-23, GE Healthcare). The samples were stored on ice during collection. To protect the samples from human keratin contamination, sterile plastic single-use gloves were

used throughout the process. Hemolymph extracted from 20 adults was used for analysis.

2.2. Two-dimensional gel electrophoresis

Protein concentration was determined using the Bradford assay (Cat. No. B6916, Sigma-Aldrich), and 250 μg protein was used for the 2DGE. Isoelectric focusing (IEF) was performed on an Ettan IPG Phor 3 instrument (GE Healthcare) controlled by Ettan IPG Phor 3 control software. The separation was performed in 13 cm ceramic strip holders using Immobiline dry strips with a pH range of 3–10 (Cat. No. 17-6001-14, GE Healthcare). A DeStreak Rehydration Solution (Cat. No. 18-1168-31, GE Healthcare) containing 0.5 % IPG buffer, pH3–10 (Cat. No. 17-6000-87, GE Healthcare) was used for active rehydration. The separation program was as follows: step 1—30 V, 10 h; step 2—500 V, 500 Vh; step 3—Grad 1,000 V, 800 Vh; step 4—Grad 6,000 V, 15,000 Vh; and step 5—6,000 V, 16,000 Vh. The duration of the isoelectrofocusing program and the active rehydration was 19 h. Immediately following IEF, the strips were equilibrated for 15 min in equilibration buffer containing dithiothreitol (Cat. No. 43817, Sigma-Aldrich) and for 15 min in buffer containing iodoacetamide (Cat. No. 57670, Sigma-Aldrich). The strips were placed on sodium dodecyl sulfate (SDS)-PAGE gel and fixed with 1 % agarose (Cat. No. A7431, Sigma-Aldrich). The gel was prepared according to the manufacturer's instructions from 37.5:1 acrylamide/bisacrylamide (Cat. No. A3699, Sigma-Aldrich), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Cat. No. T9281, Sigma-Aldrich), SDS (Cat. No. 2326.2, Carl Roth), glycine (Cat. No. 3908.2, Carl Roth), and Tris base (TRIS) (Cat. No. 4855.3, Carl Roth). Nanopure water (0.2 μm filtered) (Barnstead, Thermo) was used throughout the study. A Rainbow Full-Range 12 to 225 kDa molecular weight protein marker (Cat. No. RPN 800E, GE Healthcare) was used for protein mass reference. Ten microliters of the marker was loaded onto the sample application piece (Cat. No. 80-11-29-46, GE Healthcare) positioned near the pH3 (+) end of the strip. The electrophoresis was run at a constant voltage of 100 V for 30 min in an SE 600 Ruby electrophoresis instrument (GE Healthcare) after which the proteins were separated at a constant voltage of 333 V under cooling.

For Coomassie staining, the gel was fixed in a fixing solution (40 % LC-mass spectrometry (MS) grade methanol, 10 % ice-cold acetic acid, 50 % nanopure water) overnight and stained with 0.02 % PhastGel™ Blue R (Cat. No. 17-0518-01, GE Healthcare). Unused fixing solution was used for destaining. The results were visualized using a scanner (CanoScan 8800F, Canon).

2.3. Protein identification

For identification using MS, the proteins were manually excised from the gel. The proteins were destained and further digested with trypsin. Spectra were acquired in the range of 700–4,000 *m*/*z* using a 4800 Plus MALDI TOF/TOF analyzer (AB Sciex) equipped with an Nd:YAG laser (355 nm, firing rate of 200 Hz). Peak lists from the MS spectra were generated using 4000 Series Explorer V 3.5.3 (AB Sciex). The spectra were searched against a database of metazoan protein sequences from GenBank using a local version of Mascot v. 2.1 (Matrix Science). Only hits that were scored as significant ($P<0.05$) and with a score greater than 75 were considered further.

3. RESULTS

In this study, a 2DGE map of hemolymph proteins of winter honeybees, *A. mellifera mellifera*, was created (Figure 1). 2DGE followed by mass spectrometry was used to identify proteins from winter honeybee worker hemolymph (Table I). In total, 121 spots of high abundance were excised after 2DGE and further analyzed by MALDI TOF/TOF mass spectrometry. Overall, 93 proteins with identification scores greater than 75 were assigned significance ($P<0.05$). The proteins identified as significant are denoted in Table I and indicated in Figure 1. The major protein identified in the hemolymph of the winter honeybees was vitellogenin. Its dominance repressed the signals of the lower-abundance proteins (Figure 1).

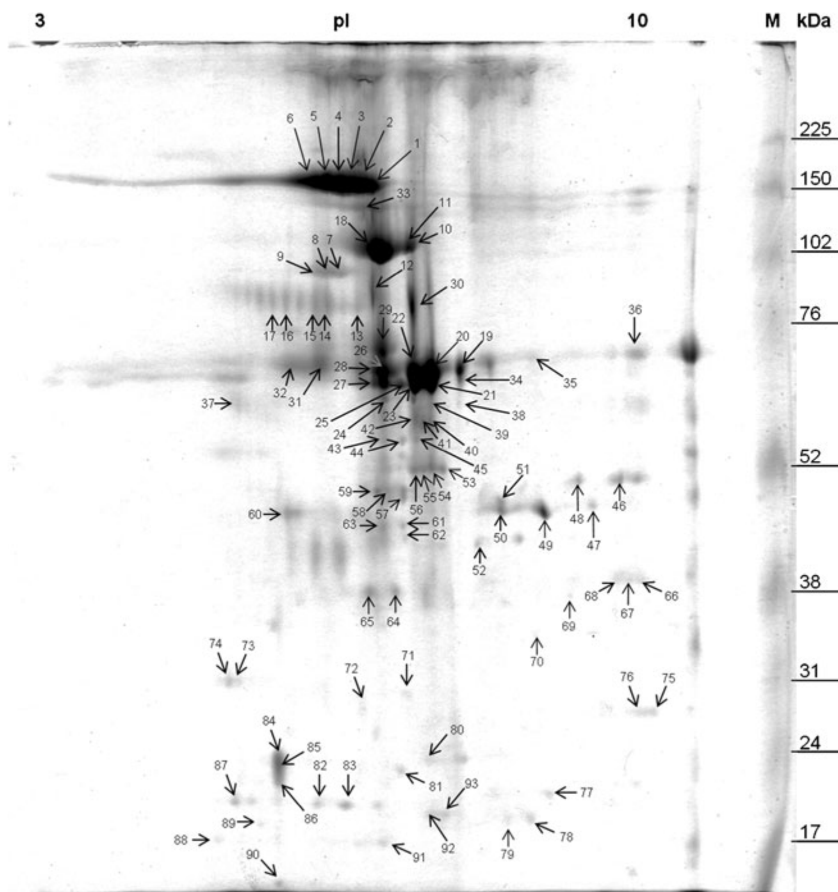

Figure 1. Representative Coomassie-stained 2DGE of winter *Apis mellifera* hemolymph. Spots assigned significance (P<0.05) after MALDI TOF/TOF are marked by a *number* (*1–93*) and denote samples in Table I.

4. DISCUSSION

4.1. Vitellogenin—major protein in the winter honeybee hemolymph

Insect vitellogenins are phospholipoglycoproteins that are synthesized as ~200 kDa precursors and circulate in the hemolymph, usually as dimers (typically 400–600 kDa). The primary insect vitellogenin precursor is composed of large (140–190 kDa) and small (40–60 kDa) subunits that are assembled and secreted into the hemolymph. In some insects, ~100 kDa vitellogenin subunits have been identified (Tufail et al. 2001; Tufail and Takeda 2002, 2008). Honeybee vitellogenin is thought to be a 180-kDa monomer (Engels 1974; Engels and Fahrenhorst 1974; Fluri et al. 1982; Wheeler and Kawooya 1990). The proteomic results of this study showed that vitellogenin subunits of different sizes are detectable in the 2DGE map (Figure 1), mainly as ~180 and ~100 kDa subunits. In addition, we detected a ~40-kDa subunit previously identified in the honeybee abdominal fat body tissue (Havukainen et al. 2011); however, this subunit was the only one identified in the summer honeybee hemolymph 2DGE (Bogaerts et al. 2009). It is possible that the 100- and 40-kDa units are fragmentation products of the unit of higher molecular weight (MW).

Vitellogenins are female-specific precursors of the major egg yolk protein vitellin and are

Table I. Significantly identified ($P<0.05$) MALDI TOF/TOF proteins of winter *Apis mellifera* hemolymph. All 93 spots were taxonomically assigned to *Apis mellifera*.

Spot no.	GI NCBI	Best score	Best mass	Latest accession no.	Description according to latest accession no.		
					Gene	Protein family	Protein description according to accession no.
1	58585104	484	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
2	58585104	412	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
3	58585104	639	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
4	58585104	664	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
5	58585104	543	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
6	58585104	330	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
7	58585104	203	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
8	58585104	264	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
9	58585104	221	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
10	58585104	483	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
11	58585104	573	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
12	58585086	115	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
13	58585104	148	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
14	94400901	209	66,754	NP_001035349.1	Hbg2	*Glycosyl hydrolase 13*	Alpha-glucosidase precursor
15	94400901	298	66,754	NP_001035349.1	Hbg2	*Glycosyl hydrolase 13*	Alpha-glucosidase precursor
16	94400901	108	66,754	NP_001035349.1	Hbg2	*Glycosyl hydrolase 13*	Alpha-glucosidase precursor
17	94400901	410	66,754	NP_001035349.1	Hbg2	*Glycosyl hydrolase 13*	Alpha-glucosidase precursor
18	58585104	463	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
19	58585086	685	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
20	58585086	640	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
21	58585086	465	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
22	58585086	369	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
23	149939403	446	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin
24	58585196	641	80,443	NP_001011627.1	PPO	*Tyrosinase*	Phenoloxidase subunit A3
25	149939403	578	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin

Table I (continued).

Spot no.	GI NCBI	Best score	Best mass	Latest accession no.	Description according to latest accession no.		
					Gene	Protein family	Protein description according to accession no.
26	58585086	503	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
27	149939403	354	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin
28	58585086	195	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
29	110755367	290	76,627	XP_001120678.1	LOC724779	*Toll-like receptor*	Predicted: toll-like receptor 13-like isoform 1
30	58585086	508	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
31	58585104	405	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
32	58585104	469	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
33	58585104	350	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
34	58585086	580	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
35	58585086	142	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
36	110758758	408	376,750	XP_392490.4	Rfabg	*Apolipoprotein*	Predicted: apolipophorins isoform 1
37	66534027 Predicted:	391	40,803	XP_397231.3 hypothetical protein LOC413792	LOC413792	*S-*	*Ribosylhomocysteinase*
38	58585086	238	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
39	58585086	217	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
40	160425209	224	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
41	149939403	200	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin
42	66512983	170	65,835	XP_393293.2	LOC409801	*Carboxylesterase*	Predicted: esterase E4-like
43	149939403	347	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin
44	149939403	440	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin
45	160425209	101	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
46	149939403	268	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin
47	66514614	458	48,996	XP_396769.2	LOC413324	*Glycosyl hydrolase 18*	Predicted: chitinase-like protein Idgf4-like
48	149939403	392	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin
49	66514614	592	48,996	XP_396769.2	LOC413324	*Glycosyl hydrolase 18*	Predicted: chitinase-like protein Idgf4-like

Table I (continued).

Spot no.	GI NCBI	Best score	Best mass	Latest accession no.	Description according to latest accession no.		
					Gene	Protein family	Protein description according to accession no.
50	66514614	464	48,996	XP_396769.2	LOC413324	*Glycosyl hydrolase 18*	Predicted: chitinase-like protein Idgf4-like
51	66514614	437	48,996	XP_396769.2	LOC413324	*Glycosyl hydrolase 18*	Predicted: chitinase-like protein Idgf4-like
52	160425209	261	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
53	58585086	358	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
54	58585086	188	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
55	58585086	230	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
56	58585086	451	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
57	58585196	324	80,443	NP_001011627.1	PPO	*Tyrosinase*	Phenoloxidase subunit A3
58	58585196	267	80,443	NP_001011627.1	PPO	*Tyrosinase*	Phenoloxidase subunit A3
59	58585196	280	80,443	NP_001011627.1	PPO	*Tyrosinase*	Phenoloxidase subunit A3
60	58585104	472	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
61	160425209	224	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
62	58585086	274	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1 precursor
63	160425209	190	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
64	149939403	348	81,549	ABR45904.1	Hex70a	*Hemocyanin*	Hexamerin
65	160425209	97	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
66	58585196	388	80,443	NP_001011627.1	PPO	*Tyrosinase*	Phenoloxidase subunit A3
67	110758758	119	376,750	XP_392490.4	Rfabg	*Apolipoprotein*	Predicted: apolipophorins isoform 1
68	110758758	159	376,750	XP_392490.4	Rfabg	*Apolipoprotein*	Predicted: apolipophorins isoform 1
69	160425209	215	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
70	66514614	514	48,996	XP_396769.2	LOC413324	*Glycosyl hydrolase 18*	Predicted: chitinase-like protein Idgf4-like
71	66507096	312	30,915	XP_397526.2	LOC408807	*Nogo receptor*	Predicted: reticulon-4 receptor-like
72	66507096	281	30,915	XP_397526.2	LOC408807	*Nogo receptor*	Predicted: reticulon-4 receptor-like
73	110758534	401	46,444	XP_623150.3	SPH42	*Peptidase S1*	Predicted: serine proteinase stubble isoform 1
74	110758534	265	46,444	XP_623150.3	SPH42	*Peptidase S1*	Predicted: serine proteinase stubble isoform 1
75	66514614	336	48,996	XP_396769.2	LOC413324	*Glycosyl hydrolase 18*	Predicted: chitinase-like protein Idgf4-like
76	66514614	367	48,996	XP_396769.2	LOC413324	*Glycosyl hydrolase 18*	Predicted: chitinase-like protein Idgf4-like

Table I (continued).

Spot no.	GI NCBI	Best score	Best mass	Latest accession no.	Description according to latest accession no.		
					Gene	Protein family	Protein description according to accession no.
77	110758758	134	376,750	XP_392490.4	Rfabg	*Apolipoprotein*	Predicted: apolipophorins isoform 1
78	58585086	206	80,033	NP_001011572.1	Tsf1	*Transferrin*	Transferrin 1
79	160425209	113	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
80	160425209	204	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
81	66514614	311	48,996	XP_396769.2	LOC413324	*Glycosyl hydrolase 18*	Predicted: chitinase-like protein Idgf4-like
82	160425209	176	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
83	160425209	147	81,522	NP_001104234.1	Hex70a	*Hemocyanin*	Hexamerin 70a precursor
84	94158822	366	15,590	NP_001035313.1	Obp14	*Odorant binding*	Odorant binding protein 14
85	94158822	116	15,590	NP_001035313.1	Obp14	*Odorant binding*	Odorant binding protein 14
86	94158822	139	15,590	NP_001035313.1	Obp14	*Odorant binding*	Odorant binding protein 14
87	110758758	209	376,750	XP_392490.4	Rfabg	*Apolipoprotein*	Predicted: apolipophorins isoform 1
88	66550509	246	26,112	XP_624977.1	LOC552600	*GILT*	Predicted: gamma-interferon-inducible lysosomal thiol reductase-like
89	58585104	118	202,117	NP_001011578.1	Vg	*Apolipoprotein*	Vitellogenin precursor
90	66534655	113	17,689	XP_624662.2	LOC552283	*Sigma (GSTs)*	Predicted: glutathione *S*-transferase-like
91	58585196	76	80,443	NP_001011627.1	PPO	*Tyrosinase*	Phenoloxidase subunit A3
92	110758758	318	376,750	XP_392490.4	Rfabg	*Apolipoprotein*	Predicted: apolipophorins isoform 1
93	110758758	136	376,750	XP_392490.4	Rfabg	*Apolipoprotein*	Predicted: apolipophorins isoform 1

used by both invertebrates and vertebrates in egg maturation and embryo development (Finn et al. 2009; Arrese and Soulages 2010). In insects, vitellogenins are produced by the fat body, from which they are secreted to the hemolymph (Tufail and Takeda 2008; Arrese and Soulages 2010). In honeybees, it is the major hemolymph protein (30–50 % of total) in queens, hive bees, and wintering workers (Amdam et al. 2003). However, in foragers, vitellogenin is expressed at low levels and is linked to increased JH titers (Amdam et al. 2003; Piulachs et al. 2003; Guidugli et al. 2005). Moreover, vitellogenin-derived proteins can constitute up to 25–60 % dry weight of HPGs of summer hive bees and winter bees (Fluri et al. 1982). Although vitellogenin function is commonly associated with reproduction and storage, it also has a lipid carrier function (Wheeler and Kawooya 1990; Mann et al. 1999; Piulachs et al. 2003). Vitellogenin is also involved in protection from oxidative stress; large amounts of vitellogenin extend the life of honeybees, thus affecting honeybee longevity (Amdam and Omholt 2002; Seehuus et al. 2006). Vitellogenin is associated with immune defense in both invertebrates and non-mammalian vertebrates (Zhang et al. 2011). The link between vitellogenin level, JH titer, zinc level, and immune function was demonstrated in honeybee workers (Amdam et al. 2004). Thus, the increased level of hemolymph vitellogenin in the winter honeybees should correspond to their resistance to diseases. The results from the 2DGE analyses of winter honeybee hemolymph could be used to study the correlation between hemolymph vitellogenin levels and the longevity and health of honeybees. Because the vitellogenin level of winter honeybees has been proposed as a possible predictive determinant for CCD (Dainat et al. 2012), we believe that future studies related to CCD could benefit from the results of this paper.

4.2. Hexamerin—sole presence of hexamerin 70a in the winter honeybee hemolymph

Hexamerins are another group of relatively high-abundance proteins that we identified in the hemolymph of winter honeybees. Typical hexamerins are large oligomers of approximately 500 kDa in their native form. They are typically composed of six similar or identical subunits of 75–90 kDa each. Hexamerins belong to the class of hemolymph proteins originally described as storage proteins that serve as a source of energy and amino acids in non-feeding periods. They belong to a family of proteins that includes hemocyanins, prophenoloxidases, and arylphorin receptor proteins. Although arthropod hemocyanins are involved in oxygen transport, the analogous insect proteins, hexamerins, have lost the ability to bind Cu^{2+} ions and transport oxygen (Burmester et al. 1998; Burmester 2002; Martins et al. 2008, 2010).

Four hexamerin subunits exist in the honeybee genome: 70a, 70b, 70c, and 110. However, protein expression studies using honeybees at different developmental stages and tissues reveal functional distinctions (Danty et al. 1998; Cunha et al. 2005). Of the four hexamerins present in honeybee larvae, hexamerin 70a is present in adults, suggesting a distinct developmental pattern (Danty et al. 1998; Cunha et al. 2005; Bogaerts et al. 2009). Hexamerin 70a is classified as an arylphorin because more than 15 % of its amino acids are aromatic and it serves as an amino acid source during metamorphosis. Hexamerin 70a plays a role in ovary differentiation and testis maturation and functioning. However, because hexamerin 70a is detected in developing worker honeybees, queens, and drone gonads, its reproductive role differs from that of vitellogenin. Additionally, it is not detected in eggs or embryos (Martins et al. 2008, 2010). Martins et al. (2011) confirmed that hexamerin 70a plays role in cell proliferation, a function previously described for arylphorins. The amino acids derived from hexamerin 70a hydrolysis are essential in cuticle formation (Martins et al. 2011). The identification of hexamerin 70a in the hemolymph of the winter honeybee confirms that it is functional in winter workers. Although we identified hexamerins that are expressed only by the Hex70a gene, multiple isoforms of different MWs and

isoelectric points (pIs) were identified by 2DGE. The presence of spots smaller than 70 kDa (Figure 1) suggests fragmentation of hexamerin 70a in the hemolymph. This can be due to insect hexamerins undergoing posttranslational cleavages (Burmester and Scheller 1999). Hexamerin spots smaller than theoretical MW were identified in 2DGE of both hemocytes and fat body of *Sarcophaga bullata* (Masova et al. 2010). Thus, we suggest that the fragmented hexamerin 70a can be localized to hemolymph hemocytes of winter honeybee. Hexamerin was present in low abundance compared to vitellogenin, suggesting that it is not the primary storage protein in overwintering honeybees.

4.3. Transferrin 1

Transferrin 1 is the most studied insect transferrin and is similar to mammalian transferrin (Geiser and Winzerling 2012). The predicted ~75 kDa size from sequence analysis of the *A. mellifera* transferrin 1 (Geiser and Winzerling 2012) was verified, although multiple isoforms with different MWs and pIs were present in the 2DGE. Transferrins are iron-binding glycoproteins that control the level of free iron in biological fluids in vertebrates and insects, including honeybees (do Nascimento et al. 2004; Dunkov and Georgieva 2006). Insect transferrins are multifunctional glycoproteins, and multiple functions have been investigated. In addition to iron delivery, transferrin functions to reduce oxidative stress and to increase survival in infected insects (as reviewed in Geiser and Winzerling (2012)). An important role for transferrin in longevity has been hypothesized based on the evolutionary change in its function from transport to immune response (do Nascimento et al. 2004). The function of transferrins may be similar to mammalian lactoferrin (do Nascimento et al. 2004; Geiser and Winzerling 2012). Due to the similarity between the functions of transferrin 1 and vitellogenin, it is apparent that both proteins play important roles in preventing infection by foreign factors that can negatively affect longevity. In winter honeybee hemolymph, vitellogenin likely simulates the defense function of transferrin and the quantitative level of both of these proteins is indicative of immune tolerance.

4.4. Enzymes

Albeit frequent, enzymes were among the low-abundance proteins identified in the analyzed spots. It is important to note that enzymes, especially those with detoxification potency, can be localized to hemolymph hemocytes. Hemolymph hemocytes are invertebrate phagocytes and play important roles in the insect immune system (Lavine and Strand 2002). Therefore, we did not separate them from the hemolymph. α-Glucosidase (NP_001035349) is involved in carbohydrate metabolism and has the α-amylase catalytic domain found in maltase and an α,α-phosphotrehalase domain (Marchler-Bauer et al. 2011). Because trehalose is the main hemolymph sugar in most insects (Thompson 2003), the identified α-glucosidase could relate primarily to trehalose metabolism. Glutathione *S*-transferase (GST) functions as a detoxification agent and as an antioxidant defense against oxidative stress (Weirich et al. 2002). Identified GST (XP_624662) is a member of the Sigma class GSTs that generally show low levels of activity with the typical GST substrates, but have a high affinity for the lipid peroxidation product 4-hydroxynonenal. Their localization in metabolically active tissues of flies, such as the flight muscles, has been suggested to be instrumental in protecting these tissues from by-products of oxidative stress (Singh et al. 2001). Earlier proteomic studies also identified XP_624662 in the hemolymph of summer honeybees (Bogaerts et al. 2009), workers and queen larvae (Li et al. 2010), venom (Peiren et al. 2008), and royal jelly (Li et al. 2008). Thus, it is apparent that this enzyme is ubiquitous in the life cycle of the honeybee. The presence of esterase E4-like protein (XP_393293) suggests esterase and/or lipase activity. Esterases function as hymenopteran caste regulators, and ester hydrolysis by esterases is thought to be the major metabolic

route for JH degradation in most insects (Mackert et al. 2008). Decreased levels of JH increase longevity (Herman and Tatar 2001). In honeybees, JH titers vary seasonally, with winter honeybees having lower JH levels than summer honeybees (Fluri et al. 1977, 1982; Huang and Robinson 1995). We suggest that XP_393293 could act similarly to juvenile hormone esterase (AAU81605) (Mackert et al. 2008), thereby preventing elevated levels of JH in winter honeybee workers. The chitinase-like protein Idgf4-like belongs to the family of imaginal disc growth factors (Idgfs). Idgfs are structurally related to chitinases, but an amino acid substitution in their active site prevents hydrolase activity. Idgfs are secreted into the hemolymph and transported to target tissues via the hemolymph, and with insulin cooperation, they stimulate the proliferation, polarization, and motility of imaginal disc cells in *Drosophila* (Kawamura et al. 1999). Hypothetical protein LOC413792 is classified as the metalloenzyme S-ribosylhomocysteinase (LuxS) that catalyzes the non-redox cleavage of thioether bonds (Pei and Zhu 2004). Proteins identified as gamma-interferon-inducible lysosomal thiol reductase-like (GILT-like) may be involved in reducing protein disulfide bonds and the immune response (Arunachalam et al. 2000).

In addition, we identified phenoloxidase (PO) subunit A3. PO is a copper-containing enzyme that controls melanization, thereby affecting immune reactions. The inactive PO form, a zymogen, is a prophenoloxidase. It is found in hemolymph, hemocytes, and cuticle where it is activated by serine protease protein-activating factors (pPO-a) (Lourenco et al. 2005; Chan et al. 2006; Bogaerts et al. 2009). A serine proteinase, stubble isoform 1, identified by 2DGE could function as the pPO-a. The dynamics of PO levels have sex- and caste-specific characteristics. PO levels are highest in adults. PO activity reaches a plateau within the first week of adult worker life. PO levels continuously increase with age in queens and reach levels twice as high as those found in workers (Randolt et al. 2008; Schmid et al. 2008). The relatively high-abundance spots in the 2DGE of the winter honeybee suggest that PO levels are significant in long-lived worker bees and are similar to the levels in queens. This result supports other findings that PO levels correlate with differences in longevity, which has significance for colony survival (Randolt et al. 2008). In contrast with the results from Bogaerts et al. (2009), we were not able identify bacteria/peptidoglycan recognition proteins that were suggested to be the trigger for the activation of the prophenoloxidase cascade in the hemolymph of summer honeybees (Bogaerts et al. 2009).

4.5. Other proteins

Various odorant binding proteins (Obps) that are involved in olfaction are present in honeybee hemolymph. While Obp13, 14, and 15 were identified in summer honeybee hemolymph (Bogaerts et al. 2009), the only Obp that we identified in winter honeybee hemolymph was Obp14. This finding indicates a dominant role of Obp14 in winter honeybees. Other identified proteins were apolipophorins isoform 1, reticulon-4 receptor-like (RTN4), and toll-like receptor 13-like isoform 1 (TLR13). Lipophorins are the principal lipoproteins of the insect hemolymph and transport fats and other hydrophobic compounds (Robbs et al. 1985). Members of TLRs are responsible for the recognition of structures typical of pathogenic molecules and are essential to fight infection in *Drosophila* (Tanji and Ip 2005). RTN4s are also known as NOGO proteins. They are important regulators of cell motility and growth, i.e., developing neurons or blood vessels (Schwab 2010).

4.6. Methodical note

The methodical approach adopted in this work was based on gel-based 2DGE followed by MS identification. The gel-based method has the advantage that the identified proteins are marked to a map with unique pI and MW and are available for reference in the future. However, although we demonstrated that our 2DGE had a distinct pattern and contained different

major proteins than the earlier described 2DGE of summer honeybee by Bogaerts et al. (2009), our data are directly not comparable. The core limitation is that data from different labs are not comparable because they can use different sample collection and preparation and/or separation techniques. Some particular differences with the mentioned study (Bogaerts et al. 2009) are the following: (a) we used a pool of winter worker bees versus random summer workers that should differ greatly in castes, such as foragers or nurse bees; (b) we performed collection of hemolymph directly from the dorsal vessel versus droplet collection by gentle squeezing after removing the wings; (c) we did not process the hemolymph before analysis versus sonicating and desalting; (d) we used different separation methods; and (e) our evaluation of MS data was based on significance evaluation versus percentage of sequence coverage. Because *A. mellifera* are classified to different subspecies (Franck et al. 1998), the selection of the subtype used for the analysis could affect the results. We used subtype *A. mellifera mellifera* while Bogaerts et al. (2009) used *A. mellifera carnica.*

4.7. Conclusion

A hemolymph protein profile of winter honeybee workers has been generated. A series of immune- and longevity-related proteins were identified and discussed. However, the number of identified proteins was limited due to dominance of vitellogenin. Depletion of vitellogenin can increase signals of the lower-abundance proteins in the 2DGE. Future studies related to the physiology of overwintering bees, including their health, immunity, longevity, and nutrition, will benefit from these results. Because overwintering is critical to the life of honeybee colonies in temperate zones and because of the elevated loss of managed honeybee colonies in recent years, we believe that these results can provide a foundation for studies related to CCD. Future studies are needed that directly contrast winter castes with summer castes to identify pathways that may confer longevity.

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Analyse protéomique par électrophorèse bidimensionnelle de l'hémolymphe des ouvrières d'hiver de l'abeille *Apis mellifera*

***Apis mellifera* / hémolymphe / vitellogénine / protéomique / longévité**

Zweidimensionale Proteomanalyse der Hämolymphe von Winterbienen der Honigbiene *Apis mellifera*

***Apis mellifera* / Hämolymphe / Winterbiene / Vitellogenin / Proteomik / Lebensdauer**

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