Original article

Two-dimensional gel proteome analysis of honeybee, *Apis mellifera*, worker red-eye pupa hemolymph

Tomas ERBAN[1], Dagmar PETROVA[1], Karel HARANT[4], Petr L. JEDELSKY[3,4,5],
Dalibor TITERA[2,6]

[1]Department of Pest Control of Stored Products and Food Safety, Laboratory of Proteomics, Crop Research Institute, Drnovska 507/73, Prague 6-Ruzyne, CZ16106, Czech Republic
[2]Bee Research Institute at Dol, Libcice nad Vltavou, Czech Republic
[3]Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic
[4]Service Laboratories of the Biology Section, Laboratory of Mass Spectrometry, Faculty of Science, Charles University in Prague, Prague, Czech Republic
[5]Department of Cell Biology, Faculty of Science, Charles University in Prague, Prague, Czech Republic
[6]Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague 6-Suchdol, Czech Republic

Abstract – *Apis mellifera* Linnaeus is a holometabolous insect that undergoes complete metamorphosis in its nonfeeding pupal stage before transitioning to the adult stage. Its pupal stages are classifiable by the unique color pigmentation of its compound eyes and thorax; notably, there is a red-eye stage involving an unpigmented body that has a relatively short duration and is easy to recognize. The aim of the current study was to create a proteomic reference map of the worker red-eye pupa hemolymph. Hemolymph was collected from dorsal vessels using glass capillary tubes and was examined using pI 3–10 two-dimensional gel electrophoresis (2DE; 10 and 14 %) and matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF protein identification. This experimental approach allowed us to identify 129 different proteins organized into orthologous groups. Overall, the predominant category was post-translational modifications, protein turnover and chaperones (23.3 % of the identified proteins). In addition, we identified proteins in the non-orthologous groups of olfaction (2.3 % of the identified proteins) and storage hexamerins (3.1 % of the identified proteins). Quantitatively, the major protein isoforms that were accurately identified via 10 % 2DE were four forms of storage hexamerin: the 110, 70a, 70b, and 70c forms. The most abundant enzymes identified were short-chain dehydrogenases/reductases with pivotal developmental roles in ecdysteroidogenesis and a sigma class glutathione-S-transferase that most likely serves as a major protectant against the by-products of oxidative stress. Many of the identified proteins are known to be involved in the mechanisms of metamorphosis. All of the identified proteins are useful as markers for future comparative physiological and developmental studies.

Honeybee / pupa / hemolymph / metamorphosis / proteome / hexamerin

1. INTRODUCTION

The sequenced and annotated genome (Honeybee Genome Sequencing Consortium 2006) of the honeybee, *Apis mellifera* Linnaeus, allows for large-scale mass spectrometry (MS)-based proteomic studies to be performed on this species. Similar to vertebrate blood, the proteins in insect hemolymph provide important information on physiology and the immune system and reflect the health of the organism (Levy et al. 2004; Chan et al. 2006; Randolt et al. 2008; Bogaerts et al. 2009). Insect

Corresponding author: T. Erban,
arachnid@centrum.cz
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hemolymph is a body fluid that is mainly composed of water, inorganic salts, carbohydrates (mainly trehalose), proteins, hormones, lipids, free amino acids, and macrophage-like cells (hemocytes). The hemolymph mediates chemical exchanges between tissues and organs and serves as a water reserve (Leta et al. 1996; Lavine and Strand 2002; Hrassnigg et al. 2003; Chan et al. 2006; Burmester and Hankeln 2007). Because honeybees are social insects and the protein composition of the hemolymph varies between developmental stages, hemolymph proteins can be used to investigate caste differences and development (Danty et al. 1998; Chan et al. 2006; Chan and Foster 2008; Bogaerts et al. 2009; Erban et al. 2013). Proteomic studies investigating the role of hemolymph proteins in honeybee physiology remain rare. Honeybee hemolymph has been previously examined using one-dimensional gel electrophoresis (1DE) in adults and/or larvae (Chan et al. 2006; Randolt et al. 2008; Chan and Foster 2008), while two-dimensional gel electrophoresis (2DE) and a gel-free system (2DLC) have been used to evaluate summer adults (Bogaerts et al. 2009), and 2DE was used to study summer de-queened adults (Cardoen et al. 2011), worker and queen larvae (Li et al. 2010b) and winter workers (Erban et al. 2013). N-terminal sequencing was used with 1DE to identify the hemolymph hexamerins (Danty et al. 1998). However, the pupal hemolymph proteome has not yet been studied with MS or 2DE proteomic techniques.

The pupal stage is a nonfeeding, immobile, reconstruction stage when larval tissues are broken down and rebuilt. The honeybee begins its pupal stage when the larva is sealed into its cell after the final molting (Gullan and Cranston 2005). Although there are caste differences in the duration and size of the pupal stage (Rembold et al. 1980), honeybee pupal stages are classifiable by the color pigmentations of their compound eyes and thorax. The distinguished pupal eye colorations are white, pink, red, and brown (Duay et al. 2003). The red-eye stage is sometimes omitted when describing the pupal stage in the literature (Michelette and Soares 1993; Barchuk et al. 2002; Piulachs et al. 2003), and a dark pink (Michelette and Soares 1993) or red/brown (Rembold et al. 1980) color is instead recognized and denoted. However, the pink/red compound eye stage is

characterized by a nonpigmented (unmelanized) body (Rembold et al. 1980; Michelette and Soares 1993; Barchuk et al. 2002; Piulachs et al. 2003; Duay et al. 2003). The relatively easy recognition and short duration of the red-eye stage makes it useful for studying the physiological and developmental oddities that can be caused by entomopathogens, such as viruses, bacteria, *Nosema*, or the hemolymph-sucking mite, *Varroa*.

In this study, we aimed to establish a 2DE map of the *A. mellifera* worker red-eye pupal stage. We present an overview of the proteins present in the hemolymph to offer insight into the red-eye pupal developmental stage. The resulting map will be useful for future studies on pupal development or pupal diseases.

2. MATERIALS AND METHODS

2.1. Biological samples

The worker red-eye pupas used in this study were collected from an *A. mellifera carnica* colony at the Bee Research Institute at Dol in the Czech Republic. A monitored honeybee colony was selected. Hemolymph was collected from the dorsal vessels after puncture of the abdominal intersegmental membrane between the fourth and fifth tergites. Approximately 2 µL of transparent hemolymph was collected from each individual. The hemolymph was transferred from the glass capillary tube to a microcentrifuge tube. The collected samples were stored on ice during collection.

2.2. Two-dimensional gel electrophoresis

A total of 20 µL of pooled hemolymph from eight to ten individuals was used for each 2DE run. We did not process the hemolymph before analysis and the samples were directly used for isoelectrofocusing (IEF). IEF was performed using an Ettan IPG Phor 3 instrument (GE Healthcare). IEF separation was performed in 13 cm ceramic strip holders using Immobiline™ DryStrips with a pH range of 3–10 (Cat. No. 17-6001-14, GE Healthcare). DeStreak™ rehydration solution (Cat. No. 18-1168-31; GE Healthcare), containing 0.5 % IPG buffer (Cat. No. 17-6000-87; GE Healthcare), was used for active rehydration. The separation procedure (total Vh 32600, duration 19 h) was as follows: (1) step, 30 V, 10 h

(active rehydration); (2) step 500 V, 500 Vh; (3) Grad 1,000 V, 800 Vh; (4) Grad 6,000 V, 15,000 Vh; and (5) step 6,000 V, 16,000 Vh. Immediately following IEF, the strips were equilibrated for 15 min in equilibration buffer containing dithiothreitol (DTT; Cat. No. 43817, Sigma-Aldrich) and for 15 min in buffer containing iodoacetamide (IAA; Cat. No. 57670, Sigma-Aldrich). The equilibrated strips were placed on a 14 or 10 % sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) 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/*bis*-acrylamide solution (Cat. No. A3699, Sigma-Aldrich). Electrophoresis was carried out under cooling at a constant voltage of 77 V for 30 min in an SE 600 Ruby electrophoresis instrument (GE Healthcare), after which the proteins were separated at a constant voltage of 300 V.

After electrophoresis, the gels were processed using the Coomassie staining method. The gels were fixed in fixing solution (40 % LC-MS grade methanol, 10 % ice acetic acid, and 50 % nanopure water) overnight. After removing the fixing solution, the gels were stained using 0.02 % PhastGel™ Blue R (Coomassie R 350 stain; Cat. No. 17-0518-01, GE Healthcare). Unused fixing solution was used for destaining. Finally, the results were visualized with the G:BOX documentation system (Syngene) in automatic capture mode.

2.3. MALDI TOF/TOF sample preparation

For matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF identification, spots (~0.5–1 mm inner diameter) were manually selected from the Coomassie-stained gels and placed into a 0.6 mL microtube. Spots were covered with 100 μL 50-mM ammonium bicarbonate (ABC) buffer in 50 % acetonitrile (ACN) with 50 mM DTT. All samples were subjected to sonication in an ultrasonic cleaning bath for 5 min. After 15 min, the supernatant was discarded and the gel was covered with 100 μL of 50 mM ABC/50 % ACN with 50 mM IAA and sonicated for 5 min. After 25 min, the supernatant was discarded and exchanged for 100 μL 50 mM ABC/50 % ACN with 50 mM DTT and sonicated for 5 min to remove any excess IAA. The supernatant was discarded and the samples were sonicated for 5 min in 100 μL of HPLC/MS-grade water. The water was discarded and samples were sonicated for another 5 min in 100 μL of ACN. The ACN was discarded, and the

microtubes with the samples were left open for a couple of minutes to allow the rest of the ACN to evaporate. Then, 5 ng of trypsin (Cat. No. v5111, Promega) in 10 μL of 50 mM ABC were added to the gel. The samples were incubated at 37 °C overnight. Trifluoroacetic acid (TFA) and ACN were added for a final concentration of 1 % TFA and 30 % ACN. The samples were sonicated for 10 min, and a 0.5 μL drop was transferred onto MALDI targets and allowed to dry. The dried droplets were covered with a 0.5 μL drop of α-cyano-4-hydroxycinnamic acid (Cat. No. 70990, Sigma-Aldrich) solution (10 mg/mL in 50 % ACN) and allowed to dry.

2.4. MALDI TOF/TOF protein identification

The MS spectra were acquired in the range of 700–4,000 m/z using a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems/MDS Sciex) equipped with an Nd:YAG laser (355 nm, firing rate of 200 Hz). Ten strongest precursors were selected for MS/MS analysis. Peak lists were generated from the MS spectra using a 4000 Series Explorer V 3.5.3 (AB Sciex) without smoothing. Peaks with a local signal-to-noise ratio greater than 5 were selected automatically. The spectra were then subjected to searches against the nonredundant National Center for Biotechnology Information (NCBI) 20130221 database (23,214,025 sequences; 7,977,717,942 residues) using MASCOT 2.2 (Matrix Science). The database search criteria were as follows: enzyme, trypsin; taxonomy, Insecta (749851 sequences); fixed modification, carbamidomethylation (C); variable modifications, deamidated (NQ) and methionine oxidation (M); protein mass, unrestricted; peptide mass tolerance, ±125 ppm; fragment mass tolerance, ±0.3 Da; and one missed cleavage allowed. Hits that were scored and a $p < 0.05$ was considered significant. The protein score was $-10 * \mathrm{Log}(p)$, where p is the probability that the observed match is a random event. Protein scores were derived from ion scores as a nonprobabilistic basis for ranking protein hits.

2.5. Protein classification

For protein classification, the considered results were entered into the NCBI protein (http://www.ncbi.nlm.nih.gov/protein/), UniProt (http://www.uniprot.org/), eggNOG (http://eggnog.embl.de/), and STRING (http://string-db.org/) databases to find

clusters of predicted orthologs. The proteins were assigned based on function according to the combined search results (obtained KOGs, COGs, or NOGs) and assembled into orthologous groups based on NCBI COGs (http://www.ncbi.nlm.nih.gov/COG/). Pie charts were generated using OriginPro 8 software (v8.0891, OriginLab).

3. RESULTS

A 2DE proteomic profile of honeybee worker red-eye pupa hemolymph was obtained for the first time. Significant MALDI TOF/TOF results were protein scores greater than 71. Representative images of the 2DE Coomassie-stained 14 and 10 % SDS-PAGE pI 3–10 IEF gels are presented in Figures 1A,B and 2, respectively; see the Electronic supplementary material (ESM) Tables 1AB and 2AB for the list of identified proteins with details of identification and characterization. The patterns obtained from technical replicates of the 2DE gels were almost identical, and we observed distinctive separation of proteins of higher molecular weights (such as hexamerins), which was due to their high content in the hemolymph sample. The application of 10 % SDS-PAGE separation in the second dimension of 2DE greatly improved the separation of the higher molecular mass protein spots, and we were able to easily distinguish four hexamerin forms in the hemolymph. The 10 and 14 % 2DE assays allowed for significance to be assigned to 277 and 109 protein spots, respectively. In total, 129 different proteins were identified. The proteins were grouped and functionally categorized into 20 categories (Figure 3). Overall, the most abundant category was post-translational modification, protein turnover, and chaperones (23.3 %). However, some proteins were not found to be members of orthologous groups such as three members of the olfaction (2.3 %) and four members of the hexamerin–storage protein (3.1 %) categories. In addition, some proteins belonged to more than one category and were assigned to a multiple function (4.7 %) category. Many identified proteins were previously identified in hemolymph of adult summer workers (Bogaerts et al. 2009), winter adult workers (Erban et al. 2013), and larvae (Chan et al. 2006; Randolt et al. 2008; Chan and Foster 2008). Herein, the most important proteins and protein groups are discussed.

4. DISCUSSION

4.1. Hexamerin–storage protein

The hexamerins were found to be the most abundant hemolymph proteins. Hexamerins are highly expressed because they are principally storage proteins that serve as a source of energy and amino acids in nonfeeding periods and when the demand for amino acids is high. Hexamerins are synthesized mainly by the fat body during larval development and belong to a family of proteins that includes hemocyanins, which lack an oxygen transport function and prophenoloxidases. Hexamerins may also function as arylphorin–receptor proteins and juvenile hormone (JH)-binding proteins (Burmester et al. 1998; Burmester 2002; Martins et al. 2008, 2010). In the 2DE analyses, we successfully identified protein products that were expressed by all four hexamerin genes identified in the honeybee genome: Hex70a, Hex70b, Hex70c, and Hex110 (Martins et al. 2010). The 2DE profile revealed the distribution of hexamerin expression in the hemolymph, indicating that in the red-eye pupa these proteins rank as follows: hexamerin 110 (BAI82214), the most abundant; 70a (NP_001104234; ABR45904) and 70b (NP_001011600), which are present in moderate amounts; and 70c (ABR45905), the least abundant. Notably, four spots corresponding to a high molecular weight hexamerin 70a were identified in all of the 2DE gels that were analyzed. The presence of hexamerin spots smaller than the theoretical MW (70–110 kDa) may be due to the post-translational cleavage of hexamerin (Burmester and Scheller 1999). Spots smaller than the theoretical MW were identified in 2DE analyses of both the hemocytes and the fat body of *Sarcophaga bullata* (Masova et al. 2010). Hemocyte localization to the hemolymph was suggested in winter honeybee hemolymph (Erban et al. 2013).

Studies on hexamerin expression conducted in different developmental stages, castes, and tissues have revealed distinct structural organizations and expression patterns, suggesting that hexamerins have specialized functions in honey bee physiology (Cunha et al. 2005; Martins et al. 2010). Hexamerin 110, which has different isoforms with sizes of ~110
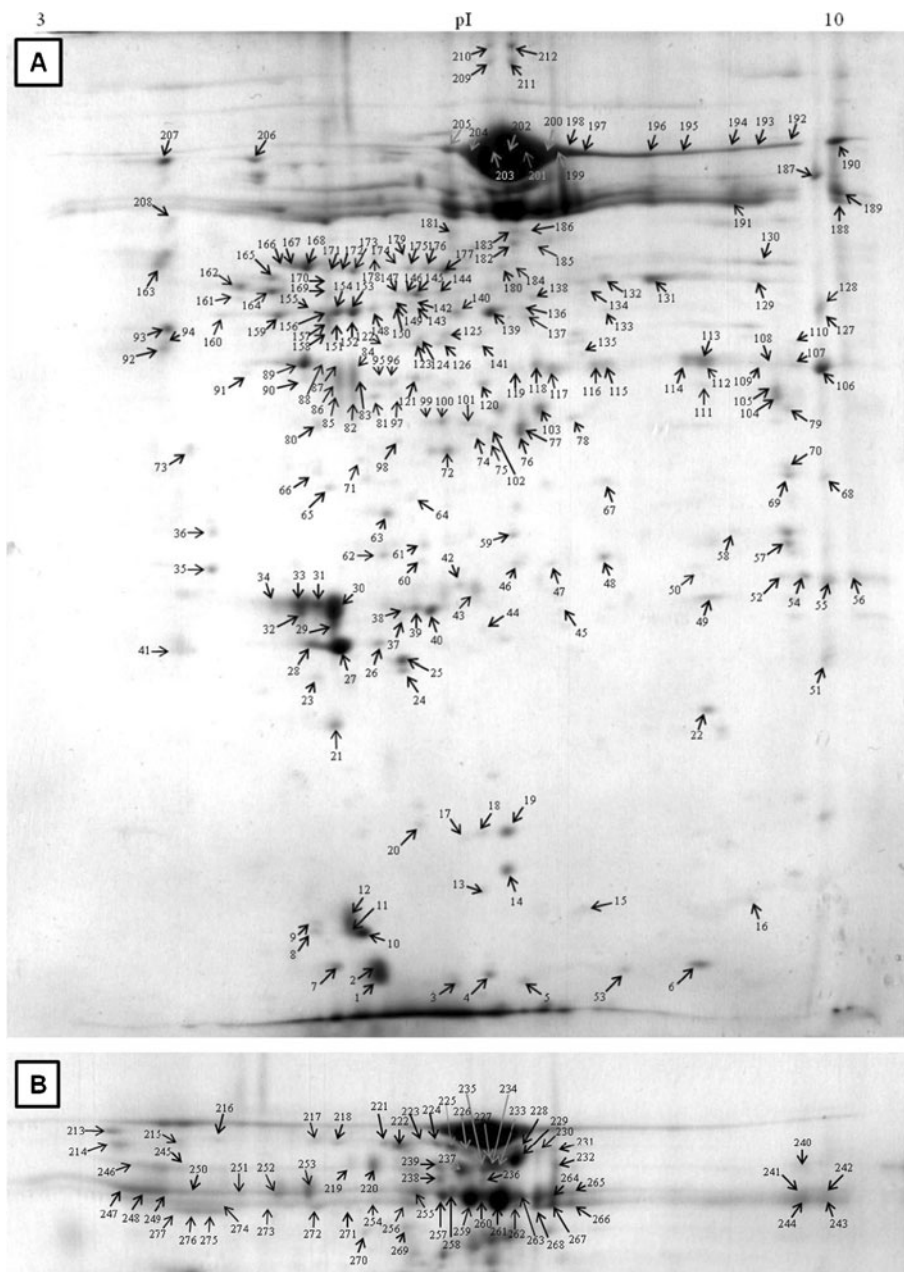
Figure 1 Proteome map of the *A. mellifera* worker red-eye pupa hemolymph. A 2DE separation was performed on IPG gel strips (pI 3–10) followed by 14 % SDS-PAGE. Protein spots identified in an entire 2DE gel (**A**) and protein spots identified in a region of hexamerins with better separation (**B**). See the ESM Table 1AB for the list of identified proteins. See the ESM Figure 1 for the figures lacking assigned identified spots (four repeats)
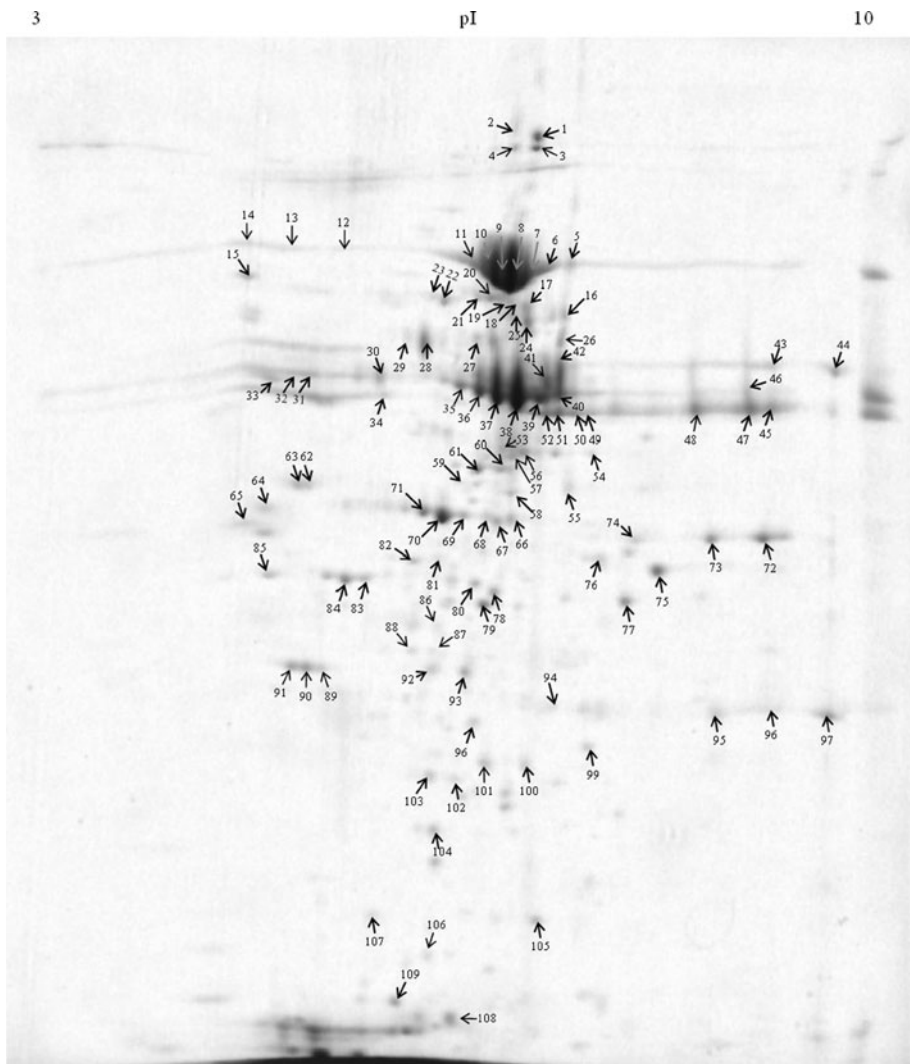
Figure 2 Proteome map of *A. mellifera* worker red-eye pupa hemolymph. A 2DE separation was performed on IPG gel strips (pI 3–10) followed by 10 % SDS-PAGE. See the ESM Table 2AB for the list of identified proteins. See ESM Figure 2 for the figures lacking assigned identified spots (two repeats)

and ∼80 kDa, decreases steadily in the hemolymph from the larval through the pupal stage, and its decrease is associated with cuticle pigmentation. The protein nearly disappears before the end of metamorphosis (Danty et al. 1998; Cunha et al. 2005; Bitondi et al. 2006). Hexamerins 70b, 70c, and 70a are present in the pupal hemolymph continuously until emergence. However, 70c and

70b disappear in drones before the transition to the adult stage, whereas 70c and 70b disappear from

Figure 3 Pie chart representing the identified functional categories of the of the *A. mellifera* worker red-eye pupa hemolymph proteins; 129 different proteins are included
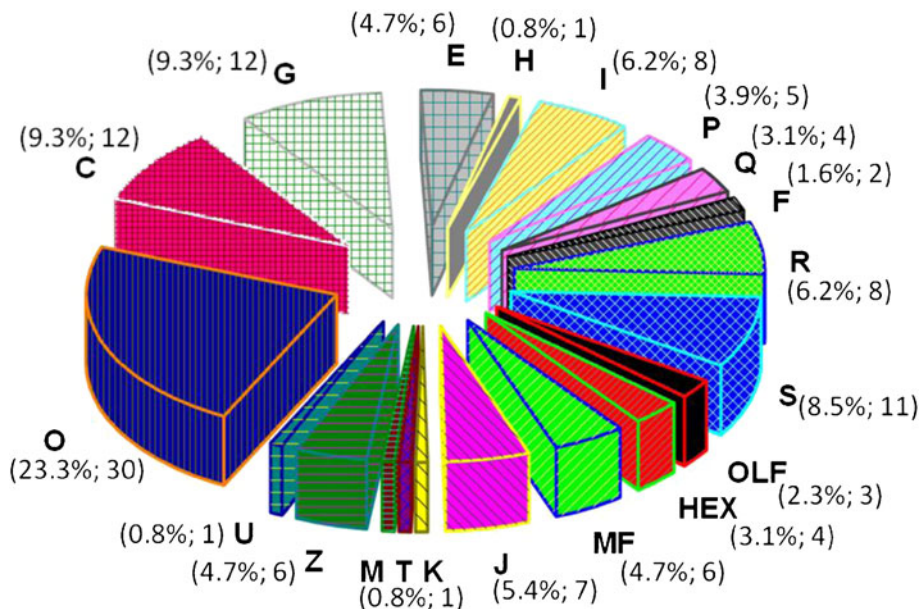
Others
- ▮ OLF - Olfaction
- ▮ HEX - Hexamerin - storage protein
- ▮ MF - Multiple function

Information storage and processing
- ▮ J - Translation, ribosomal structure and biogenesis
- ▮ K - Transcription

Cellular processes and signaling
- ▮ T - Signal transduction mechanisms
- ▮ M - Cell wall/membrane/envelope biogenesis
- ▮ Z - Cytoskeleton
- ▮ U - Intracellular trafficking, secretion, and vesicular transport
- ▮ O - Posttranslational modification, protein turnover, chaperones

Metabolism
- ▮ C - Energy production and conversion
- ▮ G - Carbohydrate transport and metabolism
- ▮ E - Amino acid transport and metabolism
- ▮ H - Coenzyme transport and metabolism
- ▮ I - Lipid transport and metabolism
- ▮ P - Inorganic ion transport and metabolism
- ▮ Q - Secondary metabolites biosynthesis, transport and catabolism
- ▮ F - Nucleotide transport and metabolism

Poorly characterized
- ▮ R - General function prediction only
- ▮ S - Function unknown

workers after the transition to the adult stage. Only hexamerin 70a persists in adult workers (Danty et al. 1998; Cunha et al. 2005). Transcripts of hexamerin 110, 70a, and 70b have been detected in developing ovaries and testes; 110 is highly transcribed in the ovaries of egg-laying queens (Martins et al. 2010). An overexpression of six forms of 110 from the early to middle stages of the pupae head was observed (Zheng et al. 2011). The immunolocalization of hexamerin 70a revealed a tissue-specific role in developing and maturing ovarioles and testioles (Martins et al. 2011). This observation implies that hexamerin 110 is a storage protein that plays a role in honeybee metamorphosis and in amino acid acquisition in eggs, whereas hexamerins 70b and 70c play roles after emergence and 70a remains functional in adults.

4.2. Olfaction–odorant binding and chemosensory proteins

Olfaction plays crucial role in insect reproduction and in recognition of food odors. However, in highly social species, such as *A. mellifera*, olfaction is not only used to recognize airborne molecules but to provide thousands of members of a colony with a sensory network (Foret and Maleszka 2006). Insect odorant-binding proteins (Obps), which are small (~13–20 kDa) proteins that greatly enhance the solubility of hydrophobic odorants, were identified from the chemosensory organs, and some of the Obps are thought to be antenna specific. They can be roughly classified as pheromone binding proteins and general odorant-binding proteins (Briand et al. 2002; Laughlin et al. 2008; Gu et al. 2011; Spinelli et al. 2012). Despite a long-term study, the physiological function of Obps is still not well understood (Zhou 2010).

Expression profiling has revealed that only 9 of 21 Obps in the *A. mellifera* genome are antenna specific, and the remaining genes are either ubiquitously expressed or are tightly regulated in non-olfactory specialized tissues or during specific developmental stages (Foret and Maleszka 2006). Recently, proteomic studies demonstrated differential protein expression in the antennae of the *A. mellifera* drone, worker, and queen (Fang et

al. 2012) as well as the drone and forager (Feng et al. 2011), and different olfactory mechanisms were identified in drones and workers of *A. mellifera ligustica* and *Apis cerana cerana* (Woltedji et al. 2012). Obp13, 14, 15, and chemosensory protein 3 were identified in the hemolymph of the *A. mellifera* summer worker (Bogaerts et al. 2009), and Obp14 was found in winter worker hemolymph (Erban et al. 2013).

During development, the Obps in the red-eye pupa hemolymph likely arises from Obp13, which is highly expressed in older larvae and throughout the pupal stages; from Obp14 and Obp15, which are found in larvae and are thought disappear after pupation; or from Obp10, which appears in pupae and reaches the highest level in the brain of newly emerged bees before declining in older bees (Foret and Maleszka 2006). We identified two of these Obps, Obp13 (NP_001035314) and Obp14 (NP_001035313), similarly to Zheng et al. (2011) who identified Obp13 and 14 expression during *A. mellifera* pupa head development (Zheng et al. 2011). Thus, our results demonstrated that Obp14 does not disappear after pupation and is a relatively abundant pupal protein.

Small chemosensory proteins (CSPs) are similar to Obps and share some of their characteristics; however, they belong to a distinct evolutionary lineage (Foret et al. 2007). In this study, we identified chemosensory protein 3 precursor (CSP3; NP_001011583 or CAJ01448 with same sequence). Briand et al. (2002) observed that this protein is a brood pheromone carrier in *A. mellifera* (Briand et al. 2002). Foret et al. (2007) found CSP3 in late larvae but no detectable expression during the initial stages of pupal development (24 h); however, the CSP3 transcripts were again detectable in red-eye pupae, and its level gradually increased until the head became dark. This result suggested that CSP3 plays a role in cuticle maturation and its binding properties are not limited only to brood pheromone recognition but can transport hydrophobic cuticular hydrocarbons. Thus, it was concluded that CSPs are multifunctional, context-dependent proteins involved in diverse cellular processes ranging from embryonic development to chemosensory signal transduction, and some CSPs may function in cuticle synthesis, consistent with their

evolutionary origins in arthropods (Foret et al. 2007).

The function of Obps and CSPs in hemolymph is not clear; however, due to the facts mentioned above and the binding capacity of these proteins for hydrophobic compounds, it seems possible that they function as transport proteins in the hemolymph.

4.3. Lipid transport and metabolism (I category)

Fatty acid-binding proteins (FABPs; NP_001011630) and FABP-like proteins (NP_001011636) are lipid chaperones that facilitate the transport of lipids. FABPs are abundantly expressed 14–15 kDa proteins that are present across a wide range of both invertebrate and vertebrate species. FABPs bind reversibly to hydrophobic ligands with high affinity, such as saturated and unsaturated long-chain fatty acids and other lipophilic substances, including eicosanoids and retinoids (Furuhashi and Hotamisligil 2008). In addition to being involved in metabolic pathways, FABPs are linked to inflammatory pathways (Makowski and Hotamisligil 2004). Honeybee FABPs are thought to be connected to the insulin/insulin-like signaling network, which is conserved among animals and is central to growth and development (Wolschin et al. 2011). FABPs were found to be continuously highly expressed in honeybee worker larvae but showed decreased expression in adults (Randolt et al. 2008), suggesting age-dependent decreases in the worker hemolymph. FABP (clone 1BH4 in reference Evans and Wheeler (1999)) is notable for its potentially differential expression between castes, as it is expressed in worker but not queen larvae (Evans and Wheeler 1999). More recently, differences in the expression of FABPs have been found in the drone, worker, and queen antennal proteomes (Fang et al. 2012).

Lipophorins are the principal lipoproteins in the insect hemolymph that transport fats and other hydrophobic compounds (Robbs et al. 1985). Apolipophorin III-like (NP_001107670) is a proto-typical, exchangeable apolipoprotein found in many insect species that functions in the adult life stage to transport diacylglycerol from the fat body lipid storage depot to flight muscles (Wang et al. 2002).

ApoLp-III is a multifunctional insect protein that can stimulate antimicrobial peptide production in insect hemolymph, enhance phagocytosis in insect blood cells (hemocytes), and may act as a pattern recognition molecule for multiple microbial and parasitic invaders (Whitten et al. 2004). The ApoLp-III transcripts of Africanized honeybees were found to be strongly downregulated after bacterial infection (Lourenco et al. 2009). We identified another lipoprotein, apolipophorins-like (XP_003249876), which was present in a number of isoforms and/or subunits and is also likely restricted to typical insect lipid transport functions.

The key enzymes involved in fatty acid beta-oxidation are 3-ketoacyl-CoA thiolase-like (XP_391843), lambda crystalline-like a 3-hydroxyacyl-CoA dehydrogenase (NP_001129207), medium-chain acyl-CoA dehydrogenase (XP_392111), and enoyl-CoA hydratase (XP_001123353). Beta-oxidation is typically localized to the mitochondria and/or peroxisomes, and acetyl-CoA, the entry molecule of the citric acid cycle, is generated by breaking down fatty acids (Kunau et al. 1995).

4.4. Carbohydrate transport and metabolism (G category)

α-Glucosidase (NP_001035349) contains the α-amylase catalytic domain found in maltase and an α,α-phosphotrehalase domain (Marchler-Bauer et al. 2013). Therefore, α-glucosidase is related to the metabolism of maltose and/or trehalose, which is the main hemolymph sugar in most insects (Thompson 2003).

The chitinase-like protein Idgf4-like (XP_396769; XP_001120887) belongs to the family of imaginal disk growth factors (Idgfs), which are structurally related to chitinases, but an amino acid substitution in their active site abrogates their hydrolase activity. Idgfs are secreted and transported to target tissues via the hemolymph. In *Drosophila*, in cooperation with insulin, Idgfs stimulate the proliferation, polarization, and motility of imaginal disk cells (Kawamura et al. 1999). Idgf4 is considered a developmental protein in *A. mellifera* larva (Li et al. 2010a, b).

The beta-*N*-acetylhexosaminidase subunit beta-like (β-GlcNAcase; XP_001122538) protein is a chitinolytic enzyme that plays an important role in metamorphosis because it degrades chitin by hydrolyzing *N*-acetylated chitooligosaccharides to *N*-acetylglucosamine (Nagamatsu et al. 1995). A proteomic analysis identified β-GlcNAcase as a protein involved in larval to pupal metamorphosis in *Bombyx mori* (Hou et al. 2010).

The enolase-like (ENO; XP_625056), fructose-biphosphate aldolase-like (FbaB; XP_623342), glyceraldehyde 3-phosphate dehydrogenase (GADPH; XP_393605), phosphoglycerate mutase 2-like (XP_625114), and triosephosphate isomerase (TPI; NP_001090623) enzymes are generally connected to glycolysis pathways, within which most reverse steps constitute gluconeogenesis pathways. However, these enzymes can also play divergent roles. ENOs exhibit tissue-specific isoenzymes that can be expressed by alpha, beta, and gamma genes (Tracy and Hedges 2000). In addition to its essential metabolic function in glycolysis, recent studies have revealed new multifunctional roles for ENO, which include affecting transcriptional regulation (Kim and Dang 2005), functioning as a heat shock protein (Aaronson et al. 1995), and binding to polynucleotides and cytoskeletal proteins (al-Giery and Brewer 1992). ENO has been shown to exist extracellularly (Falabella et al. 2009) and is associated with the *Aphidius ervi* extracellular fatty acid-binding protein (Falabella et al. 2005). FbaB catalyzes the reversible aldol condensation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate to fructose-1,6-bisphosphate during glycolysis and gluconeogenesis (Marchler-Bauer et al. 2013). Notably, FbaB and ENO have been found to be more abundantly expressed in the hemolymph of immune-challenged *Drosophila melanogaster* larvae (Vierstraete et al. 2004). GADPH was first identified as a glycolytic dehydrogenase that oxidizes glucose into CO_2 and H_2O, producing energy; however, more recent research has shown that GAPDH is a multifunctional protein with a structural role in many fundamental cellular pathways (Kim and Dang 2005; Sirover 2011).

These novel functions include protein binding, induction of apoptosis, maintenance of DNA integrity, tRNA export, transcriptional and post-transcriptional gene regulation, vesicular transport and receptor-mediated cell signaling (Sirover 2011). An extracellular isoform of GADPH was also found to inhibit cell spreading (Yamaji et al. 2005). TPI is not only essential in glycolysis and gluconeogenesis but is also involved in fatty acid biosynthesis and the pentose phosphate shunt (Hollan et al. 1995).

Phosphoglucomutase (PGM1; XP_395366) is essential in both glycogenolysis and the opposite step, glycogenesis. PGM1 catalyzes the bidirectional interconversion of glucose-1-phosphate and glucose-6-phosphate (G-6-P) via a glucose-1,6-diphosphate intermediate (Marchler-Bauer et al. 2013). In insects, G-6-P can enter the glycolysis or trehalose synthesis pathway (Meyer-Fernandes et al. 2001) or can be used for chitin synthesis (Muthukrishnan et al. 2012), which is connected to glutamine synthetase (see Section 4.6 below). PGM1 expression has been detected in the hemolymph in all stages in *B. mori*, although it was found to be weakly expressed relative to other tissues (Staykova 2008).

Hydroxypyruvate isomerase (EFN70011 *Camponotus floridanus*) interconverts aldoses and ketoses and participates in glyoxylate and dicarboxylate metabolism. Hydroxypyruvate isomerase has also been identified in *B. mori* hemolymph (Hou et al. 2010). In addition, 6-phosphogluconate dehydrogenase, decarboxylating (XP_625090), and glucose-6-phosphate 1-dehydrogenase (XP_001121185), which both participate in the pentose phosphate pathway and serve as a major source of reducing power and metabolic intermediates for biosynthetic processes (Kruger and von Schaewen 2003), were identified.

4.5. Energy production and conversion (C category)

Two dehydrogenases, retinal dehydrogenase 1-like (RALDH1; XP_392104) and an aldehyde

dehydrogenase (XP_623084), contain a region for catalyzing the oxidation of retinal (retinaldehyde) to retinoic acid, which is an important morphogen in growth and development in the chordate/vertebrate lineage, but with no evidence of function in invertebrates (Luo et al. 2006; Marletaz et al. 2006). Therefore, it is necessary to isolate and characterize these enzymes to prove that RALDH1 or another enzyme function in *A. mellifera* pupa.

Malate dehydrogenases (MDHs; XP_392478 and XP_394487) are key enzymes involved in the biosynthesis of lipids due to their ability to produce NADPH. Recent research indicates that MDH functions go beyond the basic metabolism and address their potential role in cell differentiation and ontogenic development (Danis and Farkas 2009). Insect MDHs are regulated by both JH and ecdysone during development. In *D. melanogaster*, MDH activity is low at the beginning of postembryonic development and gradually increases in larva, culminating in the first half of the last larval instar; then, MDH activity decreases during the pupal stage and finally increasing again with age in adults (Farkas and Knopp 1998). An MDH analysis of individuals throughout development in *A. mellifera* indicated identical MDH isozyme patterns for larvae and adults, while in pupae, the isoenzyme pattern was more complex; an additional pupal-specific expression of a MDH or an epigenetic modification of the isozymes was suggested (Contel et al. 1977).

Arginine kinase (AK; NP_001011603) constitutes part of eight known phosphagen systems, each of which consists of a phosphagen that serves as a reservoir of phosphate-bond energy and a corresponding phosphagen kinase. In general, phosphagen kinase reactions function in temporal ATP buffering, regulating inorganic phosphate levels (which affects glycogenolysis and proton buffering) and intracellular energy transport. AKs are functionally analogous to the more thoroughly studied creatine kinases, which are involved in the only phosphagen system found in vertebrates (Ellington 2001). Expression of AKs was found to be hormonally regulated during *D. melanogaster* development, notably in

prepupa by 20-hydroxyecdysone (James and Collier 1990).

Other proteins belonging to the C category included the electron transfer flavoprotein subunit beta-like (XP_393789), ATP synthase subunits beta (XP_624156) and alpha (XP_392639), v-type proton ATPase subunit B-like (XP_624112), isocitrate dehydrogenase [NADP] (IDH; XP_623673), pyruvate dehydrogenase E1 component subunit beta (NP_001229442), and 4-hydroxybutyrate coenzyme A transferase-like (XP_003696005 *Apis florea*) proteins. Notably, IDH has been identified as a component of the supramolecular JH-binding protein complex in *Galleria melonella* (Zalewska et al. 2011) and is associated with the MDH part of the tricarboxylate cycle in basic metabolism (Danis and Farkas 2009).

4.6. Amino acid transport and metabolism (E category)

Spermidine synthase (XP_001120306) synthesizes polyamine spermidine. Polyamines have been implicated in physiological and developmental processes in many organisms, including insects (Wu et al. 2010). The high polyamine and spermidine concentrations observed during periods of intravitelline nuclear division, cell formation, and cell proliferation suggest the involvement of polyamines in continued embryonic growth (Callaerts et al. 1992). The involvement of spermidine in metamorphosis has been demonstrated in *Tenebrio molitor* (Besson et al. 1986).

Glutamine synthetase (GlnS; NP_001164445) is essential in the metabolism of nitrogen as it catalyzes the condensation of glutamate and ammonia to form glutamine. GlnS is also involved in chitin synthesis (see Section 4.4 above) because glutamate is a by-product of chitin synthesis in the epidermis, where glucosamine-phosphate isomerase transfers an amino group from glutamine to fructose-6-phosphate to produce glucosamine (which is then acetylated to form *N*-acetylglucosamine) and glutamate (Yarema et al. 2000). The processing of L-glutamate, which was retrieved from the molting

fluid by GlnS, was observed in the pupal midgut of *Calpodes ethlius* (Yarema et al. 2000).

Beta-ureidopropionase-like (BU; XP_392773), also known as β-alanine synthase, catalyzes the third and final step of reductive pyrimidine degradation (Wasternack 1980). In addition, BU serves as a major endogenous β-alanine source. The structure of BU has been characterized in *D. melanogaster* (Lundgren et al. 2008). In insects, β-alanine is used with dopamine in insect cuticle tanning (sclerotization and pigmentation; Arakane et al. 2009). Therefore, BU may play a crucial role in cuticle sclerotization and the initiation of pigmentation through β-alanine production during metamorphosis in the honeybee pupa.

Neprilysin 2 (XP_393860) is a member of the M13 peptidase family. The insect M13 peptidases have been found to be associated with metamorphosis, specifically with insect imaginal disks (Zhao et al. 2001; Wilson et al. 2002). Cytosolic nonspecific dipeptidase-like (XP_395851) is a metalloexopeptidase that contains a region for M20 cytosolic nonspecific dipeptidases, including anserinase and serum carnosinase (Marchler-Bauer et al. 2013). Finally, the last member identified in the E category was kynurenine–oxoglutarate transaminase 3 (EGI61832 *Acromyrmex echinatior*).

4.7. Post-translational modification, protein turnover, chaperones (O category)

Numerous protein groups in the O category were heat shock proteins (HSPs) and heat shock cognates (HSCs). HSPs are generally grouped into several different families, including the chaperonin family (HSP110, 100, 90, 70, 60, and 40) and the small heat shock (sHSP)/α-crystallin (AC) proteins (Liang and MacRae 1997; Ranford et al. 2000). We identified HSP90 (NP_001153536) and endoplasmin-like (XP_395614) with an HSP90 region (Marchler-Bauer et al. 2013), HSP60 (XP_392899), a 10-kDa sHSP (XP_624910), and an AC (XP_392405); however, HSP70 was not identified. Three HSCs, an HSC3 (NP_001153524), an HSC4 (NP_001153522), and an HSC5 (NP_001153520), were recognized. HSPs70 and HSCs70 (HSC3-5) are similar chaperones that assist

in distinct cellular functions (Goldfarb et al. 2006). HSP expression levels were originally found to increase under exposure to heat shock or other stressors. These proteins are synthesized constitutively and mediate many cellular processes by influencing and supporting a higher order of protein structure (Liang and MacRae 1997; Bukau and Horwich 1998). In addition, they are potent immunogens, and their secreted forms can interact with a variety of cell types to activate key cellular activities, such as the synthesis of cytokines and adhesion proteins (Ranford et al. 2000). HSP90 has essential roles in eukaryotic cells in the folding and activation of a range of proteins involved in cell cycle regulation, steroid hormone responsiveness, and signal transduction (Prodromou et al. 1997). HSPs10 are known to promote substrates bound to HSP60 (Ranford et al. 2000) and the cooperation of HSP60 and HSP70 can mediate the native folding of proteins in an ATP-dependent manner (Liang and MacRae 1997; Bukau and Horwich 1998). ACs are known to be major proteins found in the eye lens, but they are also detected in many other cell types (Horwitz 2009). In addition, we identified the epsilon subunit of the t-complex protein (XP_003400274 *Bombus terrestris*), which belongs to the chaperonin family for which cytoskeletal proteins, tubulins, and actins appear to be the primary substrates (Coghlin et al. 2006).

Glutathione-S-transferases (GSTs; XP_624662; NP_001153742; XP_003689632 *A. florea*), peroxiredoxins (XP_003249289; NP_001164444) and thioredoxin reductase (NP_001171497) are enzymes connected to the antioxidative defense system (AOS) that eliminates reactive oxygen species (ROS) generated as by-products of aerobic metabolism (Corona and Robinson 2006). XP_624662 and NP_001153742 GSTs were sigma class GSTs with a high affinity for the products of lipid peroxidation (Singh et al. 2001). The strong spots observed for XP_624662 indicate that this protein plays a major role in the pupa hemolymph during metamorphosis. Because XP_624662 in earlier studies was also identified in honeybee hemolymph in summer (Bogaerts et al. 2009) and winter adults (Erban et al. 2013), worker and queen larvae (Li et al. 2010b) venom (Peiren et al. 2008), royal jelly (Li et al. 2008), and developing

hypopharyngeal glands (Feng et al. 2009; Li et al. 2010a), it is apparent that this enzyme is ubiquitous throughout the entire life cycle of the honeybee.

l(2)37Cc-like (XP_391959) is a member of the prohibitin family that has been found to be involved in cuticle formation and catecholamine metabolism and is required for larval metabolism and the progression of the larva to the pupal stage in *Drosophila* (Eveleth and Marsh 1986). Additionally, 14-3-3 protein epsilon (14-3-3ε) (XP_392479; NP_001091764 *B. mori*) and 14-3-3 protein zeta-like (14-3-3ζ; Leonardo) (XP_003692803 *A. florea*) are both essential during development for regulating the cell cycle in *Drosophila* (Su et al. 2001; Acevedo et al. 2007). It has been demonstrated that 14-3-3ε serves as a central modulator of the activity of the transcription factor FoxO in *Drosophila* in vivo, thus regulating growth, apoptosis and longevity (Nielsen et al. 2008). Lysozomal aspartic protease (XP_392857) is similar to cathepsin D (NP_001037351 *B. mori*, identity 64 %) in that it has been found to be critically involved in programmed cell death during *B. mori* metamorphosis (Gui et al. 2006). l(2)37Cc, 14-3-3ε, 14-3-3ζ and cathepsin D have all been identified as the important proteins involved in honeybee larval development (Li et al. 2010b); thus, developmental function of these proteins is obvious also in pupa.

Furthermore, we identified disulfide-isomerases (XP_623831; XP_623282), which catalyze thiol-disulfide exchange reactions and exhibit chaperone activity (Hatahet and Ruddock 2009), and peptidyl-prolyl *cis-trans* isomerases (NP_001229473; XP_624498; XP_397224), which interconvert the *cis* and *trans* isomers of peptide bonds with the amino acid proline and can act as molecular timers in many biological and pathological processes (Lu et al. 2007). Additionally, we found components of the 26S (XP_623053) and the 20S proteasomes (XP_394993; XP_393468; XP_003697298) (Coux et al. 1996), which are responsible for the degradation of proteins (Coux et al. 1996); the t-complex protein 1 subunit epsilon (XP_393315), which belongs to the TCP-1 chaperonin family

(Marchler-Bauer et al. 2013); and stress-induced-phosphoprotein 1-like (XP_003486756 *Bombus impatiens*).

4.8. Inorganic ion transport and metabolism (P category)

Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1; NP_001171498) and catalase (NP_001171540) are the primary components of the AOS that are necessary for the maintenance of redox homeostasis. SOD1 and catalase act in tandem and eliminate ROS. SOD converts superoxide anion radicals to H_2O_2 and catalase breaks down H_2O_2 into water and oxygen, which eliminates the production of highly reactive hydroxyl free radicals (Orr and Sohal 1994).

Ferritins are characterized by high capacities for iron storage and are composed of two types of 24 small polypeptide chains. One subunit type (XP_624044) is responsible for the ferroxidase activity of these subunits and is essential for the incorporation of iron into the ferritin shell. The second ferritin subunit (XP_624076) typifies these subunits. Most insect ferritins are secreted, which is in contrast to the predominantly cytosolic vertebrate ferritins and plant ferritins, which are found in plastids (Dunkov and Georgieva 2006). Voltage-dependent anion-selective channels (XP_623725) are a type of porin ion channel involved in the regulation of the metabolite flow across the mitochondria (Komarov et al. 2004).

4.9. Secondary metabolites biosynthesis, transport and catabolism (Q category)

Short-chain dehydrogenase/reductase (SDR; NP_001011620), which was identified in multiple isoforms, exhibits great functional diversity. NP_001011620 is identical to NCBI record AAP45005, which has been reported to be a target of the ecdysone response during *A. mellifera* caste development (Guidugli et al. 2004). This SDR (clone 52A9 in Evans and Wheeler (1999)) was previously found to be worker-specific (Evans and Wheeler 1999). A crucial developmental role of SDRs in ecdysteroidogenesis has been reported in

several insect species (Niwa et al. 2010). The enzyme 3-hydroxyacyl-CoA dehydrogenase type-2-like (HADH2) (XP_001120471) was later identified as 17-hydroxysteroid dehydrogenase 10 (HSD10) (Marchler-Bauer et al. 2013). HSD10/HADH2 is a member of the SDR family and shows a broad spectrum of enzymatic activities towards steroids and other compounds, such as prostaglandins, retinoids and fatty acid derivatives (Shafqat et al. 2003).

Fumarylacetoacetase (XP_624590) is involved in the tyrosine catabolism pathway (Marchler-Bauer et al. 2013). Sorbitol dehydrogenase-like isoform 2 (SDH; XP_392401) is the second enzyme in the polyol pathway and catalyzes the conversion of sorbitol to fructose (Marchler-Bauer et al. 2013).

4.10. General function prediction only (R category)

Juvenile hormone esterase (JHE; NP_001011563/ XP_392274) is identical to NCBI protein records AAU81605 (in Mackert et al. (2008)) and BAC54130 (in Kamikouchi et al. (2004)). JHEs constitute the major metabolic route for JH production, which controls this stage of insect development. Thus, JHEs function as regulators of honeybee castes, behavior, and development (Kamikouchi et al. 2004; Mackert et al. 2008).

Aldose reductase-like (AR; XP_624353) is associated with SDH (see above XP_392401), as it is the first enzyme in the polyol pathway and catalyzes the reduction of glucose to sorbitol (Marchler-Bauer et al. 2013). Alcohol dehydrogenase [NADP+] A-like (XP_624401), similar to AR, belongs to the aldo/ keto reductase protein family, which has a primary purpose to reduce aldehydes and ketones to primary and secondary alcohols (Marchler-Bauer et al. 2013).

Cytosol aminopeptidase-like (XP_003249720) is a member of the M17 family that includes leucyl aminopeptidase (Marchler-Bauer et al. 2013). The phosphotriesterase-related protein-like (XP_395159) protein is a member of the amidohydrolase superfamily and has a sequence that is highly similar to phosphotriesterase but shows little or no esterase activity (Wang et al. 2011), while phosphatidylethanolamine-binding protein homolog F40A3.3-like (XP_392060)

belongs to the phosphatidylethanolamine-binding protein family, which has members that have different substrates and oligomerization states (Marchler-Bauer et al. 2013). The last members of the R category were arylsulfatase J-like (XP_624454) and hypothetical protein TcasGA2_TC004196 (EFA12567 *Tribolium castaneum*), which has a region for pepsin-like aspartate proteases from retroviruses (Marchler-Bauer et al. 2013).

4.11. Other categories

Seven members were classified as belonging to the multiple function category due to presenting more than one assigned function: calumenin (XP_624357; categories T and U); slit homolog 2 protein (XP_003249857) (categories W and T); hypothetical protein LOC409786 (XP_393280) (categories T and Z); *trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase-like (XP_623497; categories G and Q); uncharacterized oxidoreductase yrbE (XP_624408; categories C and Q); *n*-acetylneuraminate lyase (XP_001120250; categories E and M), which assists in the metabolism of sialic acids (Maru et al. 1998); and *trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase-like (XP_623497; categories C and Q), which is connected to the synthesis of catechol, an important function in the construction of the insect skeleton (Kramer et al. 2001).

In the translation, ribosomal structure and biogenesis (J) category, we identified ribonuclease UK114 (XP_003251902), eukaryotic initiation factor 4A (XP_623285), hypothetical protein LOC551211 (XP_623682), tyrosyl-tRNA synthetase (XP_397348), elongation factor 1-alpha (NP_001014993), N(2),N(2)-dimethylguanosine tRNA methyltransferase (XP_003249791), and a 40S ribosomal protein, SA (XP_393965).

The proteins assigned to the function unknown (S) category include transferrin 1 (NP_001011572), which is the best-studied insect transferrin. This multifunctional glycoprotein functions to reduce oxidative stress and enhance the duration of infections, in addition to its canonical role of iron delivery (as reviewed by Geiser and Winzerling (2012)). Transferrin expression in *A. mellifera* has been reported to be controlled by ecdysteroids (do

Nascimento et al. 2004). Other proteins in the S category were an ester hydrolase C11orf54 homolog (XP_394859), endochitinase-like (XP_003402985 *B. terrestris*), hypothetical protein (ADD51172), hypothetical protein LOC727503 (XP_001123213), hypothetical protein LOC412149 (XP_395613), hypothetical protein LOC726722 (XP_001122443), hypothetical protein LOC100578640 (XP_003250458), hypothetical protein LOC100746991 (XP_392060 *B. impatiens*), hypothetical protein LOC100647123 (XP_003401909 *B. terrestris*) and hypothetical protein LOC725960 (XP_001121746).

Six proteins were categorized as belonging to the cytoskeleton (Z) category. Actin-related protein 1 (NP_001172074), actin, clone 205-like (XP_003251465), tubulin beta-1 chain (XP_392313), and tubulin beta-3 chain-like (XP_394469) are cytoskeletal proteins that are likely found in hemolymph cells. Actin is detectable in hemolymph hemocytes (Matova and Anderson 2010), and tubulin was also previously identified in honeybee hemolymph (Chan et al. 2006). Profilin (NP_001011626) and a cofilin/actin-depolymerizing factor homolog (XP_001120072) are proteins that bind to actin and affect the structure of the cytoskeleton (Marchler-Bauer et al. 2013).

Other proteins with one or a few members in a category were also identified. Slit homolog 2 protein-like (XP_001120937), which is required for normal neural development (Holmes et al. 1998), belongs to the signal transduction mechanisms (T) category. Annexin B9-like (XP_395944) belongs in the intracellular trafficking, secretion, and vesicular transport (U) category and is a Ca^{2+}-dependent phospholipid-binding protein that demonstrates down-regulated expression in cells that are destined to undergo apoptosis but not in those that survive pupal metamorphosis in *B. mori* (Kaneko et al. 2006). A pyridoxal kinase-like (XP_625042) protein, assigned to the coenzyme transport and metabolism (H) category, plays a key role in the synthesis of the active coenzyme pyridoxal-5′-phosphate (Marchler-Bauer et al. 2013). Nucleoside diphosphate kinase (XP_393351), which catalyzes the phosphorylation of nucleoside diphosphates to form triphos-

phates (Schneider et al. 2001), and deoxyribose-phosphate aldolase-like (XP_624372), which catalyzes the aldol reaction between two aldehydes (Marchler-Bauer et al. 2013), both belong to the nucleotide transport and metabolism (F) category. A member of the transcription (K) category was pterin-4-alpha-carbinolamine dehydratase (XP_395077). Finally, in the cell wall/membrane/envelope biogenesis (M) category, we identified an apolipoprotein D-like (XP_623787), which belongs to the lipocalin family and functions as transporters for small hydrophobic molecules, such as lipids, steroid hormones, bilins, and retinoids (Marchler-Bauer et al. 2013).

5. CONCLUSIONS

We have generated a 2DE protein profile of *A. mellifera* worker red-eye pupa hemolymph. A total of 129 different proteins were identified and organized into orthologous groups. One of the key accomplishments of our study was that we were able to detect the distribution of four hexamerins, which are the major storage proteins in *A. mellifera*, within the 2DE map. Thus, future studies that involve examining the consumption of hexamerin 110, 70a, 70b, and 70c during development may benefit from our results. All of the identified proteins will be useful as markers for future comparative physiological and developmental studies. The generated 2DE map can also be used to investigate *V. destructor* physiology and host–parasite interactions because the pupal hemolymph serves as the sole food source for this major honeybee pest.

Analyse protéomique par gel bidimensionnel de l'hémolymphe des pupes (stade 'yeux rouges') de l'ouvrière d'abeille, *Apis mellifera*

Pupe / abeille / hémolymphe / métamorphose / protéome / hexamérine

Proteomanalyse der Hämolymphe rotäugiger Arbeiterinnenpuppen mittels zweidimensionaler Gele

Honigbienenpuppe / Hämolymphe / Metamorphose / Proteom / Hexamerin

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