

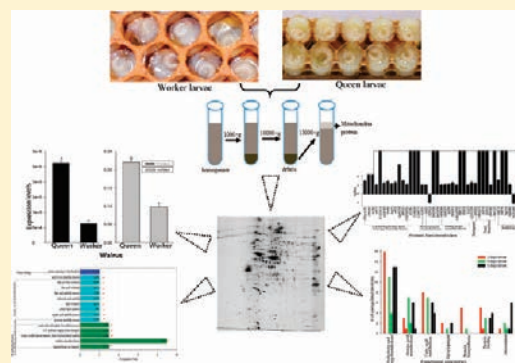
Mitochondrial Proteins Differential Expression during Honeybee (*Apis mellifera* L.) Queen and Worker Larvae Caste Determination

Desalegn Begna, Yu Fang, Mao Feng, and Jianke Li*

Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture/Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing, China

S Supporting Information

ABSTRACT: Despite their similar genetic makeup, honeybee (*A. mellifera*) queens and workers show alternative morphologies driven by nutritional difference during the larval stage. Although much research have been done to investigate the causes of honeybee caste polymorphism, information at subcellular protein levels is limited. We analyzed queen- and worker-destined larvae mitochondrial proteome at three early developmental stages using combinations of differential centrifugation, two-dimensional electrophoresis, mass spectrometry, bioinformatics, and quantitative real time PCR. In total, 67, 69, and 97 protein spots were reproducibly identified as mitochondrial proteins at 72, 96, and 120 h, respectively. There were significant qualitative and quantitative protein expression differences between the two castes at three developmental stages. In general, the queen-destined larvae up-regulated large proportions of proteins at all of the developmental stages and, in particular, 95% at 72 h. An overwhelming majority of the queen larvae up-regulated proteins were physiometabolic-enriched proteins (metabolism of carbohydrate and energy, amino acid, and fatty acid) and involved in protein folding, and this was further verified by functional enrichment and biological interaction network analyses as a direct link with metabolic rates and cellular responses to hormones. Although wide-ranging mitochondrial proteomes participate to shape the metabolic, physiologic, and anatomic differences between the two castes at 72 h, physiometabolic-enriched proteins were found as the major modulators of the profound marking of this caste differentiation. Owing to nutritional difference, prospective queen larvae showed enhanced growth, and this was manifested through the overexpression of metabolic enzymes. Differently from similar studies targeting the causes of honeybee caste polymorphism, this subcellular level study provides an in-depth insight into mitochondrial proteins-mediated caste polymorphism and greatly improves protein coverage involved during honeybee caste determination. Hence, it is a major step forward in the analysis of the fundamental causes of honeybee caste pathway decision and greatly contributes to the knowledge of honeybee biology. In particular, the consistency between the 22 proteins and mRNA expressions provides us important target genes for the reverse genetic analysis of caste pathway modulation through RNA interference.



KEYWORDS: honeybee, pathways, polymorphism, proteome, mitochondria

1. INTRODUCTION

A honeybee (*Apis mellifera* L.) colony is a highly organized insect society consisting of three castes: a single queen, thousands of workers, and a few males (drones). Despite their similar genetic makeup, the female queen is large in size and specializes in reproduction, whereas workers are small and engage in colony-maintaining activities.^{1–3} Their life spans also vary, with the queen living for 1–2 years⁴ and the workers living only 6–7 weeks.⁵ The size, function, behavior, morphology, and metabolism differences between the two castes have been well studied.^{1,6,7} In particular, the morphological, physiological, and biochemical characterization of honeybee caste differences has been addressed by a number of researchers.^{8,9} Differences in metabolic enzymes associated with metabolic rates in cellular responses to hormones¹⁰ and the effects of environmental actions, mainly early larvae food quantity and quality on the developmental switch between queens and workers, have also

been well documented.^{3,4,7,11} It is established that a larva younger than 3.5 days can develop into either a queen or a worker depending on the quality and quantity of its nutrition (royal jelly or worker jelly).^{12,13} Microarray analyses indicated nutritionally driven gene differential expressions between the developing queens and workers at early larval stage.^{10,14} Similarly, the role of the endocrine system, mainly the juvenile hormone (JH), in caste differentiation and behavioral development has been well studied.^{3,6,14} The investigations on the causes of honeybee caste polymorphism have been also treated through total or global protein differential expressions,^{14–18} but information at the subcellular level however remains scarce, with the exception of a RNA-differential display set of expression analyses that isolated 3 mitochondrial genes differences between 4.5-day-old queens

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and workers larvae.¹⁵ Nevertheless, a protein situated in a particular subcellular site is an important principle of the functional organization of the cell, and the knowledge of the proteins provides a clue to their possible roles in manipulating the life of an organism. Particularly, the mitochondrion is one of the most complex and multifunctional organelles in eukaryotic subcellular cells that carries out a wide variety of biochemical processes such as energy production and is a place where transcription and translation take place.^{19–21} Therefore, comparing the growing honeybee queen and worker larvae mitochondrial proteome could provide insight into mitochondrial proteins associated with regulatory mechanism of caste differentiation. Therefore, with the opportunity of honeybee genome sequence completion,²² rapid expansion of proteomic research approaches, advanced mass spectrometry techniques, bioinformatics, and high-resolution two-dimensional electrophoresis (2-DE), we provide large-scale identification of differentially expressed mitochondrial proteomes during honeybee queens and workers larvae caste determination with greater accuracy than ever before.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

Urea, Tris-base, sodium dodecyl sulfate (SDS), sodium bicarbonate ((NH₄)HCO₃), dithiothreitol (DTT), iodoacetamide, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Biolyte from Bio-Rad (Hercules, CA, USA), acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate (AP), *N,N,N',N'*-tetramethylethylenediamine (TEMED), 3-[(3-cholamidopropyl)-dimethylammonio]-propane sulfonate (CHAPS), glycerol, bromophenol blue, Coomassie Brilliant Blue (CBB) G-250, α -cyano-4-hydroxycinnamic acid (CHCA) were from Bruker Daltonics (Billerica, Mass. USA). Trypsin was from from Roche (Modified, Sequencing grade, Roche, Mannheim, Germany), and trifluoroacetic acid (TFA) and acetonitrile were from J. T. Baker (Phillipsburg, NJ, USA). Other chemicals used but not specified here are noted with their sources in the text.

2.2. Biological Samples

Larvae of workers and queens from *Apis mellifera ligustica* colonies were collected at 72 h (3 days), 96 h (4 days), and 120 (5 days) h from 5 bee colonies established in the apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Science, according to the method previously described.¹⁸ Briefly, the exact age of the collected larvae was guaranteed by confining the queen into a single wax comb frame containing worker cells for 5 h with a cage made of a queen excluder, through which the workers but not the queen could pass. Subsequently, the queen was removed and the eggs laid in the frame were maintained in the honeybee colony for further development. Soon after the eggs hatched, some of the young larvae were transferred from the worker cells to the queen cell cups in a queen rearing frame and put into the queenless colony from which subsequent queen larvae were sampled. However, the sample of worker larvae was collected directly from the worker cells. From each caste and at each developmental stage 300 larvae were collected and frozen at -80°C until use. Biological replicates were independently carried out 3 times.

2.3. Protein Extraction and Two-Dimensional Gel Electrophoresis (2-DE)

Mitochondrial fractions of the collected larval were obtained according to a method previously described²³ with minor

modifications. Briefly, the collected larvae samples were washed with ice-cold phosphate buffered saline (PBS) and laid on ice-cold tissue paper to absorb the PBS buffer. The total protein extraction was done by finely mincing and homogenizing 50 larvae from each caste and each developmental stage in ice-cold lysis buffer 1 (LB1: 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF) using a tight fitting Teflon pestle with a ratio of 5 mL LB1/1 g larvae. (All subsequent steps were performed at 4°C or on ice). The homogenate was centrifuged in a benchtop centrifuge at $1,000 \times g$ for 15 min, and the supernatant was recovered as the source of crude mitochondria. Subsequently, the mitochondria pellets were obtained from the crude mitochondria protein by differential centrifugation at $10,000 \times g$ for 15 min, and the resultant pellets was washed twice in LB1 and served as the mitochondria fraction. Preparations of mitochondria fraction samples from 3, 4, and 5 day queen and worker larvae were obtained, and mitochondrial proteins were isolated by incubating the isolated mitochondria in hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1 mM DTT, 1 mM PMSF) for 30 min on ice. The suspension was sonicated briefly, and the debris was removed by centrifugation at $13,000 \times g$ for 30 min. The supernatant was stored at -80°C until use. Protein concentration was determined according to the Bradford method²⁴ using BSA as the standard, and the absorption was measured at 595 nm (Beckman, spectrophotometer DU800).

Protein samples (1000 μg) from each larval sample with 3 biological replicates were suspended in lysis buffer 2 (LB2: 8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT, 2% Biolyte, pH 3–10) and then mixed with rehydration buffer (8 M urea, 2% CHAPS, 0.001% bromophenol blue, 45 mM DTT, 0.2% Biolyte pH 3–10). The mixture was loaded onto a 17 cm immobilized pH gradient (IPG, pH 3–10, linear, Bio-Rad) strip. Isoelectric focusing (IEF) was performed at 18°C according to manufacturer's instructions (Protean IEF Cell, Bio-Rad). Before SDS-PAGE, the IPG strips were first equilibrated for 15 min in equilibration buffer 1 [6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2% DTT] and then continued in equilibration buffer 2 [6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2.5% iodoacetamide] for another 15 min. Then the strips were transferred to SDS polyacrylamide gel, 12% T separating gel (1.00 mm). Second dimension electrophoresis, SDS-PAGE, was performed in a Protean II Xi Cell (Bio-Rad) at 25 mA/gel for 6 h.

2.4. Image Acquisition and Statistic Analysis

Gels were fixed overnight in 50% (v/v) ethanol with 10% (v/v) acetic acid, washed in water, and stained with Flamingo fluorescent dye (Bio-Rad) for image analysis and then further dyed with CBB G-250 to visualize spots for MS analysis. 2-DE gel images from 3 independent biological replicates were digitized with Image Scanner III (GE Healthcare) at 16 bit and 300 dpi resolution. Image filtration, background subtraction, spot detection, spot matching, and quantitative intensity (all of the pixels making up the spot) analysis were performed using PDQuest software (ver. 8.0.1, Bio-Rad). All gels were matched with one of the selected reference gels. The match analysis was performed in automatic mode, and further manual editing was performed to correct the mismatched and unmatched spots. The expression level of a given protein spot was calculated on the basis of the volume of the spot. To compare spot quantities between gels accurately, the spot volumes were normalized as a percentage of

the total volume of all of the spots in the gel. Means and standard deviations from the triplicate experiments were calculated, and the statistical significance of the expression level of the protein and mRNA at differential time-point was assessed with one-way ANOVA (SPSS version 16.0, SPSS Inc.). A Duncan's multiple range test was used to compare the difference between means of the expression level at 3 time-points. At least 2-fold changes with a probability of $p < 0.05$ were considered to be statistically significant.

2.5. Tryptic Digestion and Protein Identification by MS

The differentially expressed proteins spots were manually excised from CBB stained gels. This material was digested with trypsin (Roche, cat. no. 11418025001) for MS analysis according to our previous method.²⁵ The digested protein spots were identified by liquid chromatography–chip/electrospray ionization–quadrupole time-of-flight–mass spectrometry (LC–Chip–ESI–QTOF–MS) (QTOF G6520, Agilent Technologies), equipped with capillary pump G1382A, nano pump G2225A, autosampler G1377D, and Chip Cube G4240A. The LC–Chip used (Agilent Technologies) consisted of a Zorbax 300SB–C18 enrichment column (40 nL, 5 μ m) and a Zorbax 300SB–C18 analytical column (75 μ m \times 43 mm, 5 μ m). Loading flow rate was 4 μ L/min and loading mobile phase was water with 0.1% formic acid. Elution from the analytical column was performed by a binary solvent mixture composed of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The following gradient program was used: from 3% to 8% B in 1 min, from 8% to 40% B in 5 min, from 40% to 85% B in 1 min, and 85% B for 1 min. Chip flow rate was 300 nL/min. MS conditions were positive ion mode; V_{cap} 1,900 V; drying gas flow rate 5 L/min; drying gas temperature 350 $^{\circ}$ C; fragment voltage 175 V; skimmer voltage 65 V; reference masses m/z 149.02332 and 1221.02332. The digested samples were diluted in 20 μ L of water with 0.1% formic acid and centrifuged for 5 min at 10,000 $\times g$, and 8 μ L of the upper solution was injected. Spectra were calibrated by mass reference standard purine and HP-0921 (121.050873, 922.009798, Agilent Technologies). Tandem mass spectra were retrieved using the MassHunter software (Version B. 02. 01, Agilent Technologies). Before MS/MS data searching, peak-list was generated by Mascot Distiller software (Version 3. 2. 1. 0, Matrix Science). MS/MS data were searched against Mascot 2.2 (Matrix Science) applied to NCBI nr (release date, June 26, 2008). Search parameters were as follows: carboxymethyl (C) and oxidation (M) were selected as variable modifications and no fixed modification was selected. The other parameters used were taxonomy, all entries; enzyme, trypsin; missed cleavages, 1; peptide tolerance, ± 1.2 Da, MS/MS tolerance, ± 0.6 Da. The searches were against 6,649,798 sequences and 2,279,950,795 residues in the database. When the identified peptides matched to multiple members of a protein family or a protein appeared under the same name and accession number, the match was made in terms of higher Mascot score, the putative function and differential patterns of protein spots on 2-DE gels. Protein identifications were accepted if they established probability greater than 95% and contained at least 2 identified peptides having maximum peptide coverage.

2.6. Protein Subcellular Prediction and Classification

FASTA sequences of the identified proteins obtained from Uniprot (<http://www.uniprot.org/fasta>) were applied in the predictor software to predict the subcellular locations of the

proteins. For a given protein to be designated as mitochondrial location, it should be qualified at least by one or combinations of bioinformatics tools. Mitochondrial sublocations of the proteins were predicted by five bioinformatics tools SubLoc v1.0 (<http://www.bioinfo.tsinghua.edu.cn/Sub-Loc/>), MitoProtII (<http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter/>), TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) PSORT-II (<http://psort.hgc.jp/form2.html>), and Predotar (<http://urgi.versailles.inra.fr/predotar/predotar.html>). To outline the prediction, PSORT II and Sub-Loc were used in the winner-takes-all mode without setting a specificity cutoff for targeting. TargetP (mTP), MitoProtII, and Predotar predict proteins as mitochondria-based on a probability cutoff of ≥ 0.50 (Jiang et al., 2005). Subsequently, the identified proteins were classified into functional categories after searching against the Uniprot database (<http://www.uniprot.org/>) in combinations with the search results from flybase (<http://flybase.org/>) and KEGG (<http://www.genome.jp/kegg/kegg2.html>) based on available literatures.

2.7. Functional Enrichment and Biological Network Analysis

After obtaining the list of the differentially expressed proteins at 72, 96, and 120 h of the queen and the worker larval sample from the LC–Chip–ESI–QTOF–MS analysis, proteins of interests were generated from this list. To enrich the identified proteins to specific functional terms, the protein list was analyzed by CluoGo software by applying to the Drosophila database downloaded from the Gene Ontology database (release date, January 10, 2011).²⁶ Ontology was selected as a biological process, and the enrichment analysis was done by right-side hypergeometric statistical testing, with the probability value adjusted by Bonferroni. Moreover, in order to establish the possible biological interaction network (BIN) among them, proteins of interest were further analyzed by Pathway Studio software (<http://www.ariadnegenomics.com>). Briefly, the protein list was blasted against the *Drosophila* database that was implemented with the functional relationships of protein molecules supported by the scientific literature. The filter, with shortest paths among selected entities, was applied, and the data analysis information received was narrowed down to our protein list of interest, namely, proteins whose involvement in regulatory functions had been observed. Each link was built with evidence from at least 3 publications. Protein entities that belong to different functional groups were represented by different shapes according to the default settings of the software as shown in the figure.

2.8. Validation of Identified Proteins by Quantitative Real-time PCR (qRT-PCR)

Total RNAs preparation and reverse transcription were performed using TRIzol reagent and a RNA PCR Kit (Takara Bio), according to the manufacturer's instructions. Twenty-four differentially expressed proteins from 7 major functional groups (carbohydrate metabolism and energy production, fatty acid, amino acid, protein biosynthesis, protein folding, development, and antioxidant) were selected for qRT-PCR analysis, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference (Supplementary Table S1). Real-time PCR was conducted using an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). PCR was performed in a 25- μ L reaction system containing 1 μ L cDNA, 5 pmol forward and reverse primers, 12.5 μ L SYBR Green Supermix (Bio-Rad), and water. Fold-change was calculated using the $2^{-\Delta\Delta C_t}$ method.²⁷

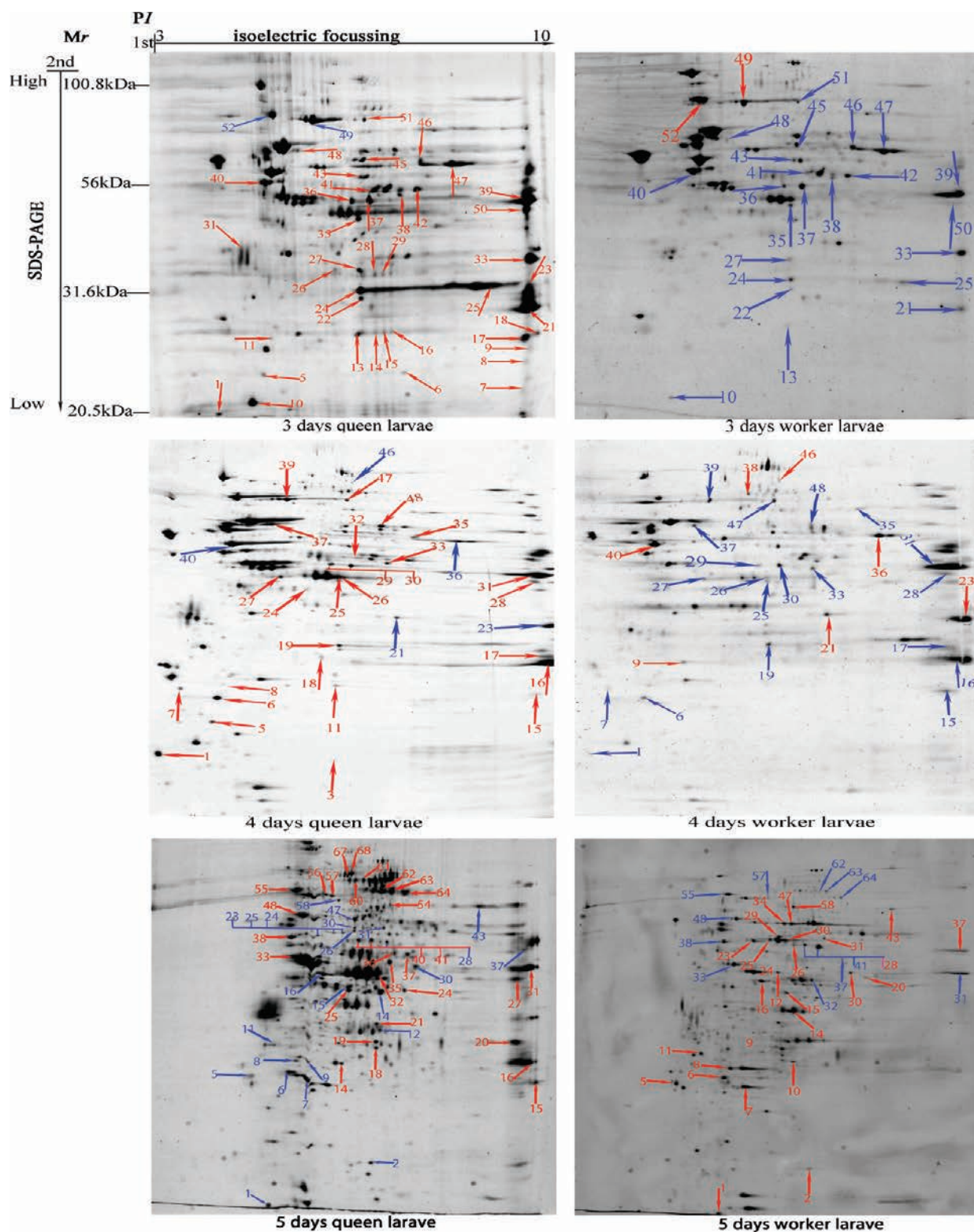


Figure 1. 2-DE gel analyses of honeybee queen and worker larval mitochondrial proteome at 72 (3 days), 96 (4 days), and 120 (5 days) h. Proteins were separated on IPG gel strips (17 cm, 3–10 L) with 1000 μ g sample loaded, followed by SDS-PAGE on a vertical slab gel (12.5%), and detected by Coomassie Brilliant Blue G-250. Differentially expression proteins spots of known identity are marked with color codes, red indicating up-regulated and blue indicating down-regulated in each caste larvae.

Each sample was analyzed independently and processed in triplicate. The values from the 3 independently obtained results were analyzed using ANOVA and means compared

using Duncan's Parametric Test (SAS, version 6.12, SAS Institute). A probability $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Identification of Differentially Expressed Proteins

We detected approximately 448, 519, and 533 protein spots on 2-DE gels at 72, 96, and 120 h, respectively, when queen and worker larvae proteins were considered together. Among them, 121, 134, and 191 protein spots showed differential regulation (>2 -fold, $p < 0.05$) at 72, 96, and 120 h, respectively. Subsequently, 97, 102, and 147 protein spots were identified as *A. mellifera* origin in both castes larvae at 72, 96, and 120 h, respectively. Furthermore, 67, 69, and 97 protein spots were reproducibly identified as mitochondrial proteins at 72, 96, and 120 h, respectively (Figure 1). The remaining unidentified differential protein spots on 2-DE images could be attributed to an abundance too low to produce enough spectra or the search scores in the databases not reaching more than 95% to produce unambiguous results. Similarly, the identified proteins were not all *A. mellifera* mitochondrial source proteins due to a common drawback of differential centrifugation enrichment processes.²⁸ As a result, on the average 62% of the identified proteins were predicted as mitochondrial, with the others belonging to other subcellular organelles and membrane.

3.2. Qualitative Comparisons of Differentially Expressed Proteins

Qualitative comparison of the two castes intended larvae would help to understand the type and number of the differentially expressed proteins in each caste at each developmental stage. From the total 67 differentially expressed proteins at 72 h, 42 (62.7%) and 25 (37.3%) were in the prospective queen and the worker larvae, respectively (Figure 2). Similarly, from the 69 differentially expressed proteins at 96 h, 37 (53.6%) were in the queen and 36 (46.4%) in the worker intended larvae. Also, at the 120 h, the queen- and the worker-destined larvae differentially expressed 56 (57.7%) and 41 (42.3%) from the total 97 proteins, respectively (Figure 2). Considering the number of differentially expressed proteins at all of the developmental stages, the queen-intended larvae on the average expressed 58% and the worker 42%.

Generally, at all developmental stages (except at 120 h) differentially expressed proteins in both castes larvae were categorized into 7 functional classes, and each varied with number and species of proteins they contained. These functional classes were carbohydrate and energy metabolism, amino acid metabolism, fatty acid metabolism, development, protein biosynthesis, protein folding, and antioxidation (Table 1). Carbohydrate and energy metabolism proteins were the most represented forms at all of the developmental stages in both castes larvae, but largely in the queen-intended larvae (Figure 3).

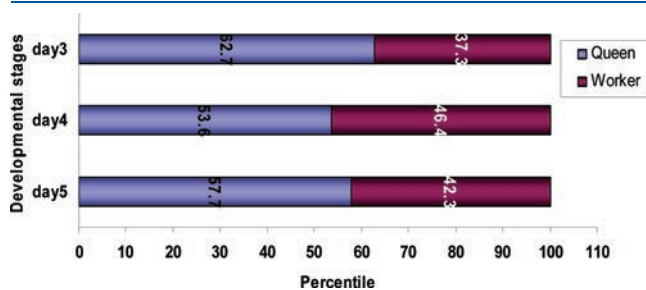


Figure 2. Compared percentage of differentially expressed proteins in honeybee queen and worker intended larvae at 72, 96, and 120 h developmental stages.

Likewise, at all of the developmental stages (except at 96 h), the queen-intended larvae overexpressed more proteins as amino acid, fatty acid metabolism, protein biosynthesis, and antioxidation proteins compared with the worker larvae (Figure 3). However, proteins related to amino acid metabolism were slightly higher in the worker larvae at 96 h and were equal in relation to development related proteins at 72 h (Figure 3).

In addition to the differentially expressed protein numbers, the two groups of larvae were contrasted on the basis of the up-regulated protein numbers. To do so, proteins that were uniquely expressed in each caste were considered as up-regulated, and the same protein species that were differently positioned on the same gel were considered as an independent isoform. Accordingly, 42, 39, and 59 proteins were up-regulated in both larvae groups at the 72, 96, and 120 h developmental stages, respectively. Of the 42 up-regulated proteins at 72 h, 40 (95.2%) were in the prospective queen larvae and 2 (4.8%) in the worker larvae. Surprisingly, the queen-intended larvae exclusively up-regulated all of the proteins involved in carbohydrate and energy metabolism, fatty acid metabolism, development, protein biosynthesis, and antioxidation and additionally up-regulated proteins from amino acid metabolism and protein folding as compared with the 72 h data (Figure 4). Likewise, from the total 39 proteins up-regulated at 96 h, 32 were up-regulated by the queen and 7 by the worker larvae. Also, from the total 59 proteins up-regulated at 120 h, 31 were by the queen and 28 by the worker-destined larvae (Figure 4).

3.3. Quantitative Comparisons of Differentially Expressed Proteins

To estimate the biological significance in detail and to obtain better understanding of the biological relationship, the levels of protein expression between the two castes intended larvae were analyzed using ANOVA log ratio ($|\log_2 R| \geq 1$). The p -values for the differentially expressed proteins were calculated as the ratio of the protein abundance (queen/worker larvae) and protein spots with $p \leq 0.05$ were considered (Figure 5). Also, vertical log ratios were computed for all of the up-regulated proteins in both castes larvae. Accordingly, the calculated results indicated high expressional intensity difference between all proteins that were up-regulated in both castes larvae at 3 developmental stages (Figure 5). To this fact, among the 42 differentially expressed proteins at 72 h, the expressional intensities of 17 were with >10 fold changes in queen larvae (Figure 5a).

Similarly, at 96 h, there were still a large number of proteins expressed at high levels (32 out of 39) in the queen-intended larvae as compared with the same age of worker larvae (Figure 5b). In particular, the expressional intensities of 7 proteins were increased >10 -fold in the queen-intended larvae, and there were 3 proteins related to carbohydrate metabolism and energy production, *etf* (spot 18), *CI-23kD* (spot 9), and *CG3609-PA isoform 2* (spot 24); 1 to fatty acid metabolism, *MCAD* (spot 32); 1 to protein biosynthesis, *mrpL12* (spot 5); 1 to protein folding, *Roe1* (spot 11); and 1 to antioxidant protein, *Sod* (spot 3). In addition, *Gapdh2* (spot 28), *CI-23kD* (spot 8), *etf* (spot 18), *MCAD* (spot 32), *Mrp12* (spot 5), *GrpE* (spot 11), and *SOD* (spot 3) showed high expressional intensity as a sign of prolongation from the previous day in the queen larvae (Figure 5b). At 72 h, the worker intended larvae expressed only 2 proteins with high intensities, 1 from amino acid metabolism (*CG9514*, spot 38) and 1 from antioxidation (*SDR*, spot 9) (Figure 5b). Interestingly, *wal* (spot 26) that expressed >10 -fold

Table 1. Differentially Expressed Mitochondrial Proteins during Development of Honeybee (*Apis mellifera* L.) Queen- and Worker-Intended Larvae at 72, 96, and 120 h^a

| exptl pI /M _r (kDa) | theor pI /M _r (kDa) | sequence coverage (%) | matched/ searched | score | protein name | accession no. | 72 h (day 3) | | | 96 h (day 4) | | | 120 h (day 5) | | |
|------------------------------------|-----------------------------------|--------------------------|----------------------|-------|--|------------------|--------------|-----------------------------|---------|--------------|-----------------------------|---------|---------------|-----------------------------|---------|
| | | | | | | | spot no. | log ₂ R (+/-) | P-value | spot no. | log ₂ R (+/-) | P-value | spot no. | log ₂ R (+/-) | P-value |
| Carbohydrate and Energy Metabolism | | | | | | | | | | | | | | | |
| 5.71/24.7 | 5/20.3 | 61 | 13/193 | 429 | ATP synthase D chain, mitochondrial [EC 3.6.3.14] | gi 48098315 | 10 | 3.0 | 0.028 | 6 | 2.1 | 0 | | | |
| 8.79/25.27 | 9.73/22.7 | 72 | 19/172 | 783 | ATP synthase O subunit, mitochondrial precursor (ATPO) [EC 3.6.3.14] | gi 66504360 | 18 | 10.0 | | 15 | 2.4 | 0 | | | |
| 6.23/35.31 | 8.49/35.6 | 48 | 17/156 | 724 | valrus (val) | gi 66526470 | 26 | 10.0 | | 21 | -2.2 | 0.002 | 21 | 10 | |
| 6.41/35.47 | 8.49/35.6 | 51 | 24/175 | 1070 | valrus (val) | gi 66526470 | 27 | 2.1 | 0.007 | | | | 12 | -2.03 | 0.003 |
| 5.3/20.5 | 5.09/17.6 | 54 | 10/186 | 382 | lethal (1) G0230 [EC 3.6.3.14] | gi 66517317 | 1 | 10.0 | | 1 | 2.9 | 0.003 | 1 | -2.05 | 0 |
| 5.69/55.97 | 5.34/53.9 | 51 | 29/233 | 1260 | ubiquinol-cytochrome-c reductase UQCRC (EC 1.10.2.2) | gi 66500205 | 40 | 2.5 | 0.001 | | | | | | |
| 7.08/64.93 | 9/59.8 | 20 | 10/212 | 359 | bellwether (blw) [EC 3.6.3.14] | gi 48100966 | 46 | 2.9 | 0.027 | 35 | 2.1 | 0.005 | | | |
| 7.84/62.65 | 9/59.8 | 46 | 35/260 | 1516 | bellwether (blw) [EC 3.6.3.14] | gi 48100966 | 47 | 2.3 | 0 | 36 | -2.0 | 0.036 | | | |
| 6.41/30.21 | 6.46/27.7 | 36 | 9/174 | 255 | electron transfer flavoprotein (ETF) | gi 66520256 | 22 | 2.7 | 0.001 | 18 | 10 | | | | |
| 8.92/47.57 | 8.11/36 | 22 | 10/1112 | 216 | glyceraldehyde-3-phosphate dehydrogenase 2 (Gapdh2) (EC 1.2.1.13) | gi 48142692 | 50 | 6.7 | 0.032 | 28 | 5.7 | 0 | 27 | 10 | |
| 8.78/37.03 | 9.33/35.95 | 57 | 25/208 | 1376 | mitochondrial malate dehydrogenase precursor (Mdhm) (CG5889) [EC 1.1.1.37] | gi 66513092 | 33 | 3.1 | 0 | 23 | -2.1 | 0 | 20 | 10 | |
| 6.41/47.06 | 7.99/45 | 41 | 24/210 | 889 | lethal (1) G0334 [EC 1.2.4.1] | gi 110765081 | 35 | 2.2 | 0.025 | | | | | | |
| 9.04/23.26 | 9.28/19.4 | 24 | 4/161 | 219 | NADH-ubiquinone oxidoreductase 18 kDa subunit, mitochondrial precursor (ND23) [EC 1.6.5.3] | gi 110773175 | 8 | 10.0 | | | | | | | |
| 9/24.2 | 9.41/24.9 | 30 | 8/169 | 309 | NADH-ubiquinone oxidoreductase, 20 kD subunit (CI-20kD) (EC 1.6.5.3 1.6.99.3) | gi 66512193 | 9 | 10.0 | | | | | | | |
| 5.74/25.44 | 5.69/23.3 | 18 | 3/225 | 143 | NADH:ubiquinone reductase 23 kD subunit precursor (CI-23kD) | gi 66524760 | 11 | 10.0 | | 8 | 10.0 | | | | |
| 6.48/65.08 | 7.55/55.11 | 48 | 33/235 | 1333 | dihydropyridyl dehydrogenase (DLD), EC 1.8.1.4] | gi 66526646 | 45 | 2.0 | 0.005 | | | | | | |
| 6.41/47.06 | 7.99/45 | 41 | 23/218 | 889 | lethal (1) G0334 [(1) G0334]) [EC 1.2.4.1] | gi 110765081 | | | | 25 | 2.1 | 0.006 | | | |
| 6.32/29.63 | 5.66/40 | 15 | 5/1002 | 108 | arginine kinase (argk) [EC 2.7.3.3] | gi 58585146 | | | | 27 | 2.2 | 0.038 | 16 | -2.04 | 0.001 |
| 4.79/30.68 | 5.25/55 | 31 | 29/1325 | 732 | ATP synthase (ATPA) [EC 3.6.3.14] | gi 110762902 | | | | 40 | -2.1 | 0.001 | 38 | 2.15 | 0.001 |
| 4.9/48 | 9.33/35.95 | 57 | 25/208 | 1376 | mitochondrial malate dehydrogenase precursor (Mdh1), (CG5362) [EC 1.1.1.37] | gi 66513092 | | | | 33 | 2.0 | 0.001 | 24 | 10 | |
| 6.69/29.34 | 6.07/36.9 | 15 | 8/1290 | 253 | myo-inositol 2-dehydrogenase isoform 2 (CG3609-PA isoform 2) [EC 1.1.1.18] | gi 66530373 | | | | 23 | 10.0 | | 15 | -2 | 0 |
| 6.79/30.61 | 5.51/40 | 39 | 19/1304 | 517 | enolase (eno) (EC 4.2.1.11) | gi 110761968 | | | | 40 | 10 | | 40 | 10 | |
| 6.96/30.62 | 5.51/40 | 40 | 19/1260 | 444 | enolase (eno) (EC 4.2.1.11) | gi 110761968 | | | | 41 | 2.01 | 0 | 41 | 2.01 | 0 |
| 6.6/32.4 | 5.97/97.3 | 36 | 37/1338 | 94 | glycogen phosphorylase isoform 2 (GlyP) [EC 2.4.1.1] | gi 66557932 | | | | 67 | 10 | | 67 | 10 | |
| 6.7/32.4 | 5.97/97.3 | 41 | 41/1337 | 161 | glycogen phosphorylase isoform 2 GlyP [EC 2.4.1.1] | gi 66557932 | | | | 68 | 10 | | 68 | 10 | |
| 7.16/30.56 | 5.51/40 | 19 | 8/1176 | 224 | enolase (eno), (EC 4.2.1.11) | gi 110761968 | | | | 28 | -2.33 | 0.015 | 28 | -2.33 | 0.015 |

Table 1. Continued

| exptl pI /M _r (kDa) | theor pI /M _r (kDa) | sequence coverage (%) | matched/ searched | score | protein name | accession no. | 72 h (day 3) | | | 96 h (day 4) | | | 120 h (day 5) | | |
|-----------------------------------|-----------------------------------|--------------------------|----------------------|-------|--|------------------|--------------|-----------------------------|---------|--------------|-----------------------------|---------|---------------|-----------------------------|---------|
| | | | | | | | spot no. | log ₂ R (+/-) | P-value | spot no. | log ₂ R (+/-) | P-value | spot no. | log ₂ R (+/-) | P-value |
| 6.6/27.87 | 6.46/27.7 | 56 | 33/1191 | 843 | electron transfer flavoprotein beta subunit (etfB) | gi 66S20256 | | | | | | 10 | -10 | | |
| 7.44/30.21 | 7.98/46 | 7 | 6/1103 | 146 | alcohol dehydrogenase, iron containing, 1 (Fe-ADH) [EC 1.1.1.1] | gi 66S17100 | | | | | | 37 | 2.1 | 0.009 | |
| 7.21/31.59 | 7.26/67 | 5 | 3/1275 | 73 | electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO), [EC 1.5.5.1] | gi 66S40209 | | | | | | 54 | 10 | | |
| 7.03/27.82 | 6.46/27.7 | 56 | 33/1191 | 843 | electron transfer flavoprotein beta subunit (etfB) | gi 66S20256 | | | | | | 18 | 10 | | |
| 6.46/30.81 | 6.69/56 | 26 | 13/1161 | 171 | Aldehyde dehydrogenase (aldh) [EC 1.2.1.3] | gi 66S30423 | | | | | | 25 | -2.38 | 0.02 | |
| 6.59/30.81 | 6.69/56 | 23 | 17/1260 | 507 | aldehyde dehydrogenase (aldh) [EC 1.2.1.3] | gi 66S30423 | | | | | | 24 | -4.72 | 0 | |
| 6.74/30.96 | 6.69/56 | 24 | 10/1169 | 174 | aldehyde dehydrogenase (aldh) [EC 1.2.1.3] | gi 66S30423 | | | | | | 26 | -2.7 | 0.012 | |
| 6.46/30.81 | 6.69/56 | 26 | 13/1161 | 171 | aldehyde dehydrogenase (aldh) [EC 1.2.1.3] | gi 66S30423 | | | | | | 27 | -10 | | |
| 6.25/30.73 | 5.25/45 | 12 | 6/1193 | 270 | aldehyde dehydrogenase (NAD+) [EC 1.2.1.3] | gi 66S26635 | | | | | | 23 | -2.33 | 0 | |
| 6.73/30.87 | 6.0/54 | 18 | 9/1065 | 145 | dihydropyrimidine dehydrogenase (CG7430) [EC 1.8.1.4] | gi 66S55742 | | | | | | 30 | -2 | 0.001 | |
| 7.12/30.97 | 7.55/54.5 | 6 | 3/1163 | 78 | dihydropyrimidine dehydrogenase (CG7430) [EC 1.8.1.4] | gi 66S26646 | | | | | | 31 | -2.14 | 0 | |
| 6.65/29.31 | 6.07/36.9 | 15 | 8/1290 | 253 | myo-inositol 2-dehydrogenase isoform 2 [EC 1.1.1.18] Amino Acid Metabolism | gi 66S30373 | | | | | | 25 | 10 | | |
| 7.28/55.1 | 8.5/47.7 | 50 | 26/185 | 1066 | ornithine aminotransferase precursor (Oat) [EC 2.6.1.13] | gi 66S24972 | 42 | 2.0 | 0.025 | | | | | | |
| 4.83/31.98 | 7.62/67.4 | 25 | 19/1274 | 357 | choline dehydrogenase (CHDH) [EC 1.1.99.1] | gi 110751363 | 52 | -2.1 | 0.002 | 38 | 2.3 | 0 | 43 | -2.45 | 0.011 |
| 6.63/34.49 | 9.22/30 | 17 | 4/195 | 193 | fumarylacetoacetate hydrolase domain containing 2A (fahd2A) [EC 3.-.-.] | gi 66S25858 | 28 | 10.0 | | | | | | | |
| 6.75/34.54 | 9.22/30 | 14 | 5/180 | 212 | fumarylacetoacetate hydrolase domain containing 2A (fahd2A) [EC 3.-.-.] | gi 66S25858 | 29 | 10.0 | | | | | | | |
| 6.4/64.1 | 7.62/67.4 | 25 | 19/1274 | 357 | choline dehydrogenase (CHDH) [EC 1.1.99.1] | gi 110751363 | | | | 41 | 2.1 | 0 | 62 | 4.46 | 0.002 |
| 4.9/64.2 | 6.58/70 | 24 | 29/1323 | 654 | choline dehydrogenase (CHDH) [EC 1.1.99.1] | gi 110749126 | | | | 43 | 2.0 | 0.001 | 55 | 2.19 | 0.013 |
| 4.9/64.3 | 6.58/70 | 41 | 49/1353 | 1060 | choline dehydrogenase (CHDH) [EC 1.1.99.1] | gi 110749126 | | | | 44 | 2.3 | 0.027 | 64 | 2.47 | 0.026 |
| 4.9/64.1 | 6.58/70 | 38 | 30/1397 | 584 | choline dehydrogenase [EC 1.1.99.1] | gi 110749126 | | | | 45 | 2.0 | 0.004 | | | |
| 4.9/67.2 | 6.54/67.2 | 7 | 7/1279 | 173 | choline-cytochrome c reductase (CG9S14)[EC 1.1.99.1] | gi 110749123 | | | | 46 | -3.7 | 0.001 | | | |
| 7.23/31.96 | 6.58/70 | 41 | 49/1353 | 1060 | choline dehydrogenase (CHDH) (CG9S14) [EC 1.1.99.1] | gi 110749126 | | | | 47 | 2.0 | 0.025 | 63 | 3.27 | 0.032 |
| 6.75/32.26 | 6.54/67.2 | 7 | 7/1279 | 173 | choline-cytochrome c reductase (CG9S14) (CHD) [EC 1.1.99.1] | gi 110749123 | | | | 38 | -10.0 | | 60 | 10 | |
| 4.88/54.97 | 6.54/67.2 | 10 | 10/1357 | 297 | choline-cytochrome c reductase [EC 1.1.99.1] | gi 110749123 | | | | 48 | 2.2 | 0.001 | 61 | 10 | |

Table 1. Continued

| exptl pI /M _r (kDa) | theor pI /M _r (kDa) | sequence coverage (%) | matched/ searched | score | protein name | accession no. | 72 h (day 3) | | 96 h (day 4) | | 120 h (day 5) | | | | |
|-----------------------------------|-----------------------------------|--------------------------|----------------------|-------|--|------------------|--------------|-----------------------------|--------------|-------------|-----------------------------|---------|-------------|-----------------------------|---------|
| | | | | | | | spot no. | log ₂ R (+/-) | P-value | spot no. | log ₂ R (+/-) | P-value | spot no. | log ₂ R (+/-) | P-value |
| Fatty acid metabolism | | | | | | | | | | | | | | | |
| 6.36/52.02 | 8.51/46.8 | 57 | 41/205 | 1565 | medium-chain specific acyl-CoA dehydrogenase, mitochondrial (MCAD) [EC 1.3.99.3] | gi 66499429 | 36 | 2.1 | 0.002 | 29 | 2.1 | 0.008 | 18 | -2.02 | 0 |
| 6.57/52.01 | 8.51/46.83 | 11 | 4/171 | 129 | medium-chain specific acyl-CoA dehydrogenase, mitochondrial (MCAD) [EC 1.3.99.3] | gi 66499429 | 37 | 2.1 | 0.013 | 30 | 2.1 | 0.007 | 35 | 10 | |
| 9.02/50.06 | 8.89/42.7 | 41 | 25/201 | 1032 | yippee interacting protein 2 (yip2) [EC 2.3.1.16] | gi 48097100 | 39 | 2.2 | 0.005 | 31 | 2.1 | 0 | 31 | 2.1 | 0.002 |
| 6.64/56.29 | 8.51/46.83 | 11 | 4/171 | 129 | medium-chain specific acyl-CoA dehydrogenase, mitochondrial (MCAD) [EC 1.3.99.3] | gi 66499429 | 41 | 2.6 | 0.013 | 32 | 10.0 | | 20 | -10 | |
| 6.44/59.97 | 6.9/22.9 | 32 | 17/194 | 739 | 4-hydroxybutyrate CoA-transferase (4-HB-CoAT) [EC 1.1.1.61] | gi 66531425 | 43 | 2.0 | 0.002 | | | | | | |
| 8.67/25.18 | 8.84/20.6 | 51 | 18/208 | 594 | phosphatidylethanolamine-binding protein (PEBP) | gi 66524882 | 17 | 10.0 | | | | | | | |
| 8.99/31.22 | 8.89/30.2 | 25 | 8/148 | 163 | succinate dehydrogenase B (SdhB) [EC 1.3.5.1] | gi 66505914 | 23 | 10.0 | | 17 | 2.4 | 0.014 | | | |
| 9.1/27 | 9.07/27 | 27 | 9/152 | 264 | 3-hydroxyacyl-CoA dehydrogenase type-2 (Type II HADH), Scully protein (Scu) [EC 1.1.1.35] | gi 110760701 | 21 | 2.1 | 0 | 16 | 3.7 | 0.013 | 15 | 10 | |
| 7.04/29.63 | 6.56/35.8 | 37 | 18/1584 | 505 | lambda crystallin-like protein (Cry11) [EC 1.1.1.45] | gi 209180477 | 26 | | | 26 | 3.1 | 0.011 | 32 | 3.06 | 0.005 |
| 4.86/30.1 | 8.89/42.7 | 41 | 25/201 | 1032 | yippee interacting protein 2 (yip2) (EC 2.3.1.16) | gi 48097100 | | | | | | | 33 | 2.24 | 0 |
| 8.84/27.48 | 9.07/27 | 16 | 11/1159 | 240 | 3-hydroxyacyl-coa-dehydrogenase-1 (Type I HADH), Scully protein (Scu) [EC 1.1.1.35] | gi 110760701 | | | | | | | 16 | 10 | |
| 9/29.8 | 8.89/42.7 | 41 | 25/201 | 1032 | yippee interacting protein 2 (yip2) (EC 2.3.1.16) | gi 48097100 | | | | | | | 37 | -2.35 | 0.001 |
| 7.09/29.2 | 6.56/35.8 | 37 | 18/1584 | 505 | lambda crystallin-like protein (Cry11) (EC 1.1.1.45) | gi 209180477 | | | | | | | 14 | -2.22 | 0 |
| Development | | | | | | | | | | | | | | | |
| 6.41/31.61 | 6.54/29.99 | 66 | 24/215 | 1434 | lethal (2) 37Cc | gi 48097857 | 24 | 2.7 | 0 | 19 | 2.0 | 0.016 | 19 | 10 | |
| 8.07/31.64 | 8.25/30.6 | 61 | 18/161 | 882 | porin (porin) | gi 66521459 | 25 | 2.4 | 0.011 | | | | | | |
| 7.3/30.2 | 6.43/11.2 | 4 | 4/1119 | 92 | elongation factor Tu mitochondrial (EfTuM) | gi 66518848 | | | | | | | 36 | 10 | |
| Protein Biosynthesis | | | | | | | | | | | | | | | |
| 9.54/22.02 | 9.13/19.5 | 24 | 5/172 | 164 | probable mitochondrial 28S ribosomal protein S25 (mRpS25) | gi 48102586 | 7 | 10.0 | | | | | | | |
| 6.71/25.76 | 9.88/21.6 | 31 | 6/164 | 264 | 60S ribosomal protein L9 (rpL9) | gi 66565444 | 14 | 10.0 | | | | | | | |
| 6.78/25.86 | 9.88/21.6 | 42 | 8/128 | 440 | 60S ribosomal protein L9 (rpL9) | gi 66565444 | 15 | 10.0 | | | | | | | |
| 7.04/54.52 | 8.16/53 | 43 | 24/202 | 854 | elongation factor Tu mitochondrial (efTuM) | gi 66518848 | 38 | 2.1 | 0.006 | | | | | | |
| 5.7/24.7 | 6.11/19.8 | 23 | 5/166 | 247 | mitochondrial ribosomal protein L12 (MrpL12) | gi 66499627 | 5 | 10.0 | | 5 | 10 | | | | |
| Protein Folding | | | | | | | | | | | | | | | |
| 6.38/25.78 | 7.82/26.6 | 38 | 11/167 | 522 | Roe1 (GrpE) | gi 66525522 | 13 | 3.2 | 0.031 | 11 | 10 | | | | |
| 6.84/25.82 | 7.82/26.6 | 32 | 9/130 | 257 | Roe1 (GrpE) | gi 66525522 | 16 | 10.0 | | | | | | | |
| 5.5/35.87 | 8.49/79.5 | 44 | 33/199 | 1575 | Trap1 | gi 66511337 | 31 | 10.0 | | | | | | | |

Table 1. Continued

| exptl pI/M_r (kDa) | theor pI/M_r (kDa) | sequence coverage (%) | matched/ searched | score | protein name | accession no. | 72 h (day 3) | | | 96 h (day 4) | | | 120 h (day 5) | | |
|-------------------------|-------------------------|--------------------------|----------------------|-------|--|------------------|--------------|---------------------|---------|--------------|---------------------|---------|---------------|---------------------|---------|
| | | | | | | | spot no. | $\log_2 R$ (+/-) | P-value | spot no. | $\log_2 R$ (+/-) | P-value | spot no. | $\log_2 R$ (+/-) | P-value |
| 5.91/67.98 | 5.64/60.5 | 28 | 18/222 | 638 | 60 kDa heat shock protein, mitochondrial precursor (Hsp60) | gi 66547450 | 48 | 2.1 | 0.03 | 37 | 2.0 | 0.001 | 11 | -2.41 | 0.039 |
| 6.04/81.13 | 6.38/75.64 | 43 | 54/224 | 2025 | heat shock protein cognate 5 (Hsc70-5) | gi 229892203 | 49 | -2.3 | 0 | 39 | 2.0 | 0.003 | 56 | 10 | |
| 6.51/81.87 | 8.49/79.5 | 44 | 33/199 | 1575 | Trap1 | gi 66511337 | 51 | 2.3 | 0 | 42 | 2.0 | 0.011 | | | |
| 6.44/31.81 | 6.38/75.4 | 37 | 31/1408 | 196 | heat shock protein cognate 5 (Hsc70-5) | gi 229892203 | | | | | | | 57 | 2.27 | 0.001 |
| 6.52/31.72 | 6.38/75.4 | 23 | 17/1192 | 60 | heat shock protein cognate 5 (Hsc70-5) | gi 229892203 | | | | | | | 38 | -2.03 | 0.003 |
| 6.77/30.75 | 5.64/60.3 | 23 | 13/347 | 432 | 60 kDa heat shock protein, mitochondrial precursor (Hsp60) | gi 66547450 | | | | | | | 47 | -2.14 | 0.001 |
| 6.77/30.75 | 5.64/60.3 | 45 | 54/1414 | 1718 | 60 kDa heat shock protein, mitochondrial precursor (Hsp60) | gi 66547450 | | | | | | | 48 | 2.16 | 0.002 |
| 4.87/64.32 | 5.64/60.3 | 45 | 54/1414 | 1718 | 60 kDa heat shock protein, mitochondrial precursor (Hsp60) | gi 66547450 | | | | | | | 34 | -10 | |
| 6.94/22.9 | 6.21/15.8 | 53 | 13/1315 | 398 | superoxide dismutase (SOD) | gi 66513527 | 6 | 10.0 | | 3 | 10.0 | | 2 | -2.06 | 0 |
| 8.4/26.6 | 5.24/27.6 | 30 | 10/1086 | 376 | short-chain dehydrogenase/reductase (SDR) [EC 1.1.1.100] | gi 58585184 | | | | 7 | 2.0 | 0.015 | 6 | -2.1 | 0.001 |
| 4.82/27.52 | 5.24/27.6 | 28 | 8/1238 | 210 | short-chain dehydrogenase/reductase (SDR) [EC 1.1.1.100] | gi 58585184 | | | | 9 | -10.0 | | 5 | -2.08 | 0 |
| 4.82/27.52 | 5.24/27.6 | 28 | 8/1238 | 210 | short-chain dehydrogenase/reductase (SDR) [EC 1.1.1.100] | gi 58585184 | | | | | | | 8 | -2.85 | 0.102 |
| 6.56/27.45 | 5.88/25.2 | 36 | 17/1094 | 285 | peroxiredoxin (prx2540) (EC 1.1.1.1.15) | gi 283436152 | | | | | | | 14 | 10 | |
| 6.18/1927 | 5.65/21.9 | 50 | 12/1102 | 310 | peroxiredoxin (prx2540) (EC 1.1.1.1.15) | gi 66548188 | | | | | | | 7 | -2.13 | 0 |
| 4.84/27.52 | 5.24/27.6 | 28 | 8/1238 | 210 | short-chain dehydrogenase/reductase (SDR) [EC 1.1.1.100] | gi 58585184 | | | | | | | 9 | -3.28 | 0.006 |

^a Spot no. corresponds to the number of protein spots in Figure 1. Theoretical molecular weight (M_r) and isoelectric point (pI) of the identified proteins were retrieved from the protein database of NCBInr. Experimental M_r and pI were calculated with PDQuest Software and internal standard molecular mass markers. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Matched peptide is the number of pairing an experimental fragmentation spectrum to a theoretical segment of protein, and searched is the total searched peptide. Mascot score is search against the database NCBInr. Protein name is given when proteins were identified by LC-CHIP-ESI-QTOF-MS. The taxonomy is *Apis mellifera* L. Accession number is the unique number given to mark the entry of a protein in the database NCBInr. The ratios (R) of the protein abundance (queen larvae/worker larvae) were transformed and proteins with $|\log_2 R| \geq 1$ and a p -value $P \leq 0.05$ were considered as differentially expressed proteins. The log ratios of uniquely expressed proteins in each caste were limited to 10 and the others with (+/-), where + designates queen and - worker.

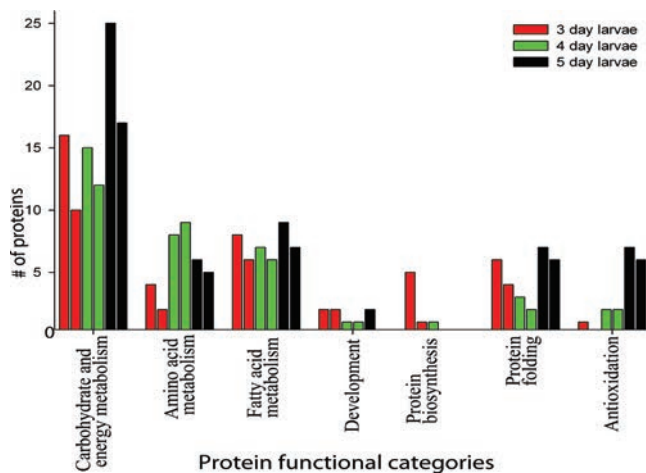


Figure 3. Functional annotation and distributions of number of differentially expressed mitochondrial proteins identified from honeybee queen and worker larvae at 72 h (3 days), 96 h (4 days), and 120 h (5 days) of developmental stages, where the first bar from each color represents proteins from the queen and the second bar proteins from the worker larvae.

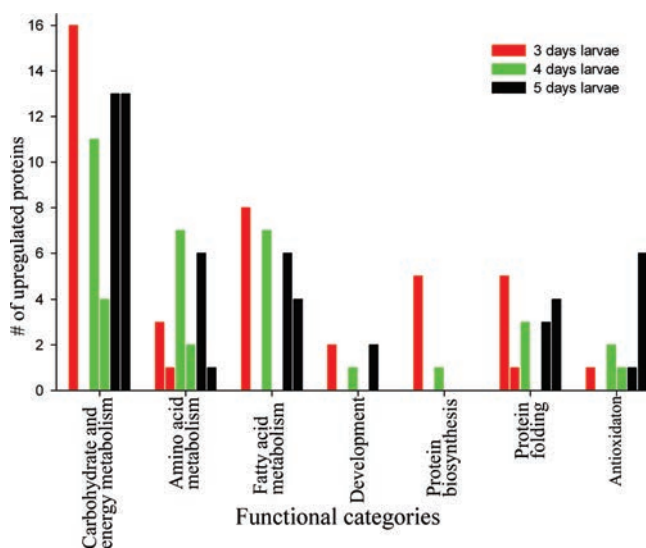


Figure 4. Compared number of up-regulated proteins between the honeybee queen and worker intended larvae at 72 h (3 days), 96 h (4 days), and 120 h (5 days) developmental stages, where the first bar from each color code represents proteins from the “Queen” and the second the “Worker” larvae.

in the queen larvae at 72 h had changed and expressed ≥ 2 -fold in worker larvae this time (wal, spot 21). Similarly, blw (spot 36) and Mdhm (spot 23) had altered their directions and highly expressed in the worker larvae. In contrast, CHDH (spot 38) and Hsc70-5 (spot 39) that were intensively expressed in the worker larvae at 72 h had intensively expressed in the queen larvae, whereas SDR (spot 9) continued with >10 -fold change in worker larvae as it was at 72 h (Figure 5a, b).

At 120 h, the two castes larvae greatly differed in the number of proteins with high levels of expression in which the queen-intended larvae expressed 19 proteins >10 -fold and its counterpart worker larvae only 4 (Figure 5c). Interestingly, the queen larvae expressed

wal (spot 21) >10 -fold, similar to 72 h. More interestingly, Gapdh2 (spot 27) observed queen caste biases and expressed ≥ 10 -fold, this time also in the queen-intended larvae as follow-up of previous days (Figure 5c).

3.4. Functional Enrichment

Enrichment analysis of the 41 differentially expressed proteins at the 72 h developmental stage showed that 3 major functional groups were significantly enriched, i.e., carbohydrate metabolism and energy production, fatty acid metabolism, and protein folding. With oxidative phosphorylation as a the leading term (a term with statistically highly significant or with lowest *P*-value), proteins involved in carbohydrate and energy metabolism were the most overrepresented, comprising $>51.2\%$ of the enriched proteome and consisted of ATPsyn-d (spot 10), UQCRC (spot 40), etf (spot 22), CI-20kD (spot 9), ND23 (spot 8), SdhB (spot 23), blw (spot 46), I(1)G0230 (spot 1), and wal (spot 26). Similarly, with fatty acid β -oxidation as leading term, fatty acid functional group was the second highly enriched group and contained MCAD (spots 36, 37, and 41) and yip2 (spot 39), whereas, the proteins with folding functions were enriched by Hsp60 and Roe1 (Figure 6).

The analysis of the 63 enriched proteins at the 96 h showed 3 groups that included carbohydrate metabolism and energy production, fatty acid metabolism, and protein folding (Supplementary Figure S1). The leading terms for carbohydrate metabolism and energy production was oxidative phosphorylation that included ATPsyn-d (spot 6), etf (spot 18), CI-23kD (spot 9), SdhB (spot 17), blw (spot 35 and 36), I(1)G0230 (spot 25), and wal (spot 21), whereas the leading term for the fatty acid metabolism was monocarboxylic acid metabolic process (Supplementary Figure S1), and enrichment to protein folding was through the participations of Hsp60 and Roe1 proteins. Still proteins involved in carbohydrate and energy metabolism were the most enriched.

However, at 120 h, most of the 45 up-regulated proteins showed significant enrichments to 2 major functional groups, that is, carbohydrate metabolism and energy production and fatty acid metabolism. There were two leading terms for the carbohydrate and energy metabolism, i.e., the glycolysis and the energy coupled proton transport, down electrochemical gradient (Supplementary Figure S2), whereas the leading term for fatty acid metabolism was monocarboxylic acid metabolic process. Generally, the enrichment analysis of this time also showed that the carbohydrate and energy metabolism group was the most enriched protein set (Supplementary Figure S2).

3.5. Biological Network Analysis

In a living cell, proteins function together in the context of networks through protein–protein interactions, modifications, and regulation of expression relationships.³⁰ Therefore, the biochemical relationship of the proteins involved in the development of the two castes intended larvae was estimated by constructing BINs using Pathway Studio software and presented in a color-coded legend next to the map (Figure 7). The biological association and interaction networks of the altered mitochondrial proteins indicated that most of these proteins (17% or 59%) were involved in carbohydrate and energy metabolism. The remaining components were associated with fatty acid metabolism (3% or 10%), protein folding (3% or 10%), development (3% or 10%), protein biosynthesis (2% or 7%), and antioxidant activities (1% or 3%). Proteins with carbohydrate and

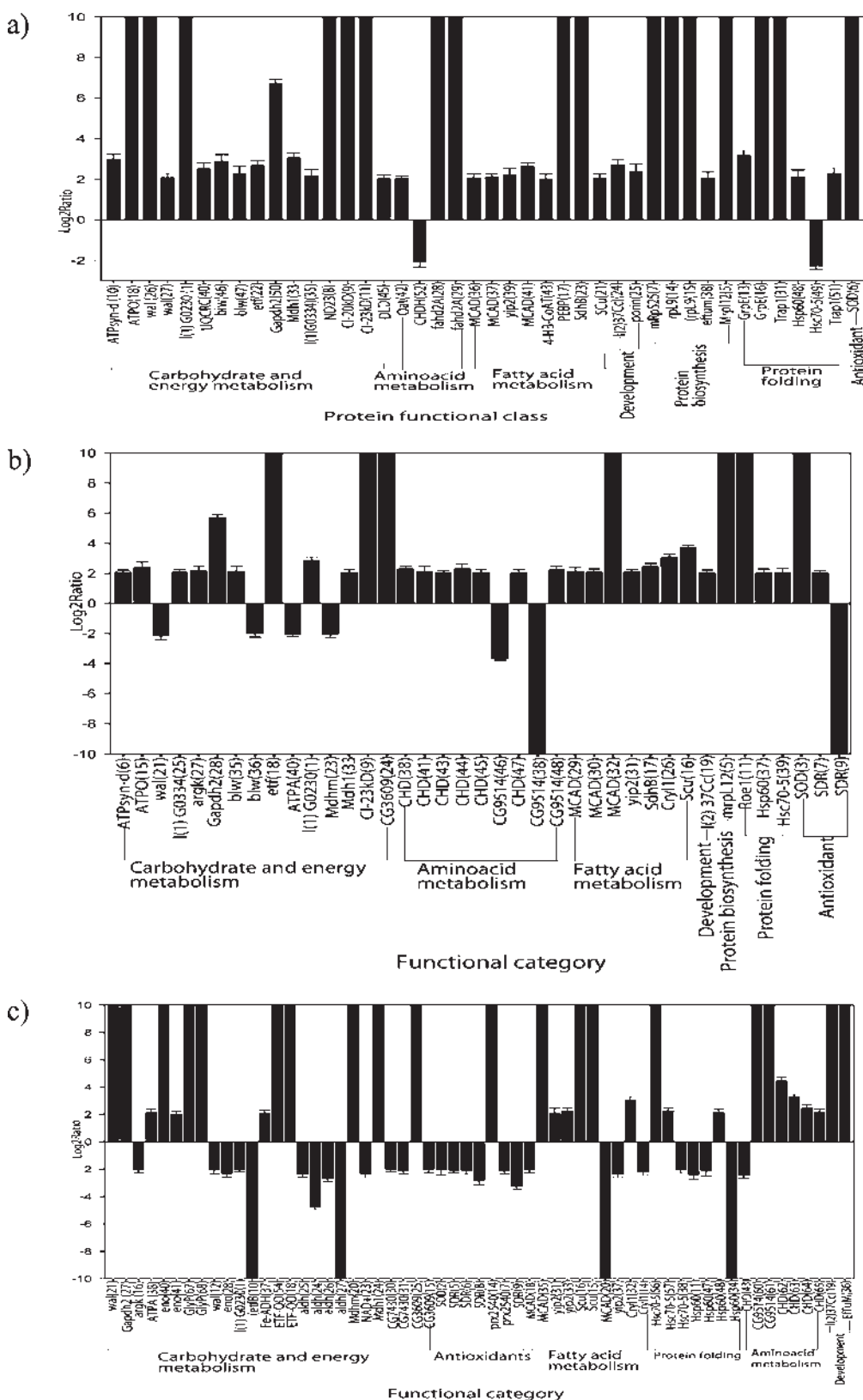


Figure 5. Quantitative expression analysis of differentially expressed proteins between the two castes of intended larvae at each development stage during honeybee (*A. mellifera* L.) queen and worker larvae development. The ratios of the protein abundance (Queen/Worker larvae) were transformed, and the protein spots with $|\log_2 R| \geq 2$ and $p \leq 0.05$ were selected as the differentially expressed proteins. (a–c) Expressional difference on days 3, 4, and 5, respectively, and protein name (in abbreviation) with corresponding protein numbers in the parentheses as in the 2DE map as well as Table 1, where + direction indicates “queen” expression over “worker”, and – indicates values for the “worker” over the “queen” larvae. The $|\log_2 R|$ of the uniquely expressed proteins were limited to 10.

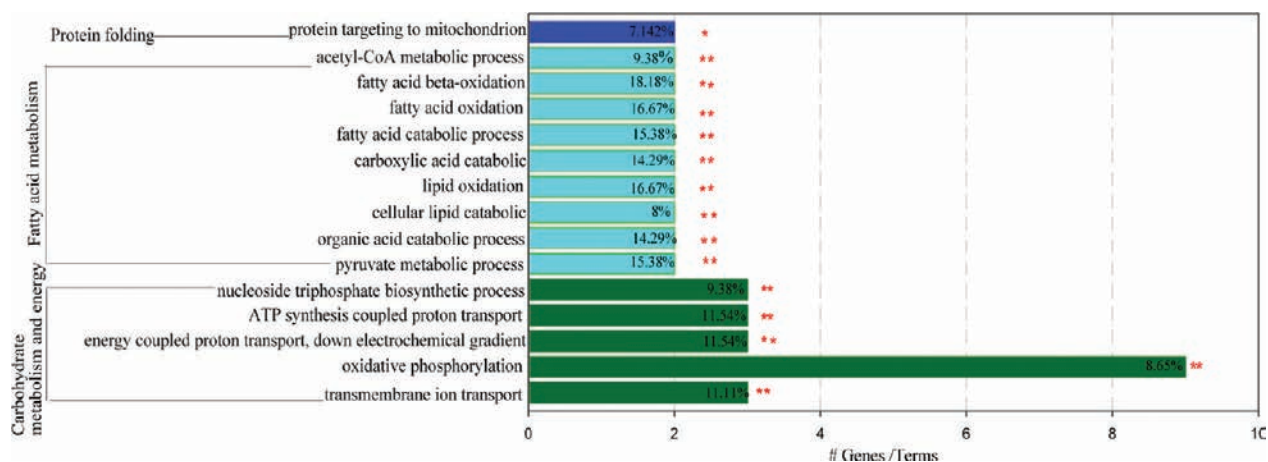


Figure 6. Enrichment analysis of identified mitochondrial proteins from developing honeybee (*A. mellifera* L.) queen and worker larvae at 72 h (3 days) by ClueGO software. * $p \leq 0.05$ and ** $p \leq 0.01$ level of significance.

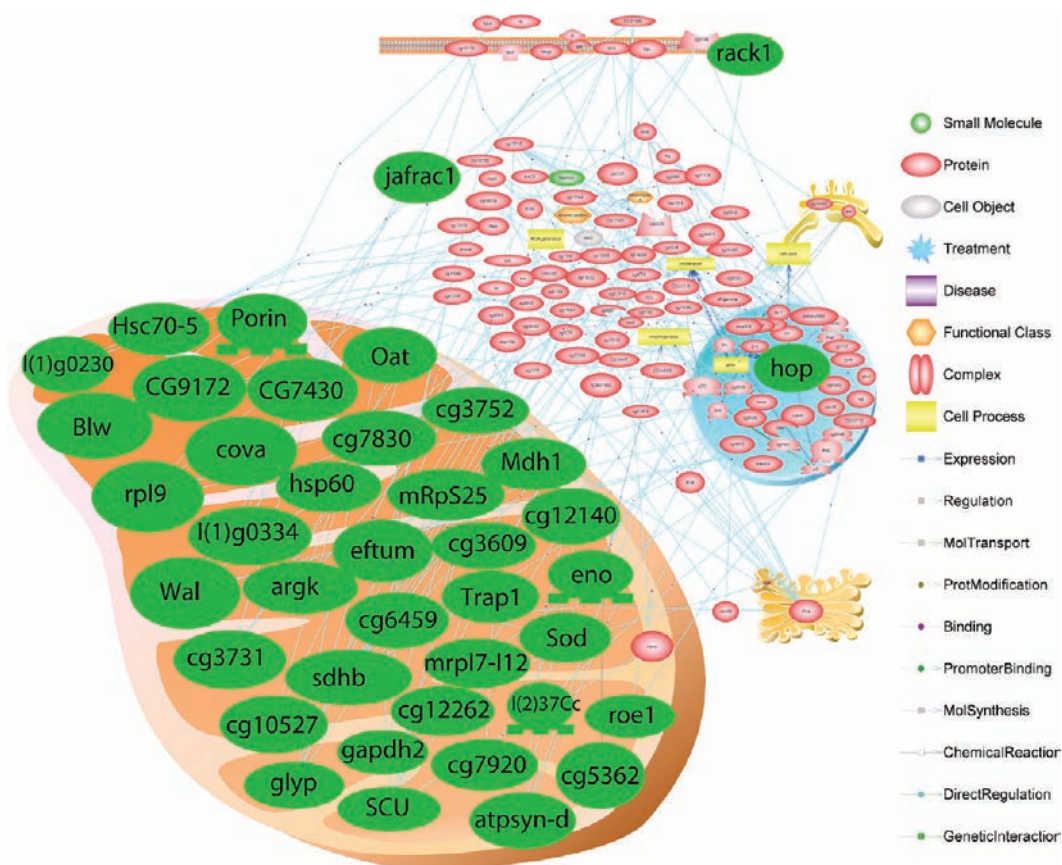


Figure 7. Biological interaction networks generated for developing honeybee queen and worker mitochondrial proteins through Pathway Studio software. Those highlighted in green color and imposed over the mitochondrial shape represent the key node proteins identified in this study. Protein entities that belong to distinct functional groups are represented as different shapes according to the default settings of the software as described in the legend.

energy metabolism functions involved in the BIN were Mdh, UQCRC, Gapdh2, Sdhb, l(1)G0334, blw, ATPsyn-d, eno, wal, glyp, argk, ald, etfb, l(1)G0230, CG7430, ETF-QO, and CI-20kD. Protein folding was connected to the network through 3 proteins, namely, Hsp60, Hsc70-5 and Roe1. Likewise, 3 proteins, namely, Scu, MCAD, and 4-HB-CoAT, were included in the network as fatty acid metabolism. Protein biosynthesis was

networked through l(2)37Cc, porin, and eftum, and the development proteins through, rpl9, and Mrpl12, as well as antioxidant protein by Sod. Even though all of the proteins were linked to the network, proteins such as hsp60, sod, lethal (2) 37Cc, Mdh1, UQCRC, and gapdh2 showed higher connectivity (≥ 25) in the mapped honeybee queen and worker larvae mitochondrial proteome networks (Figure 7).

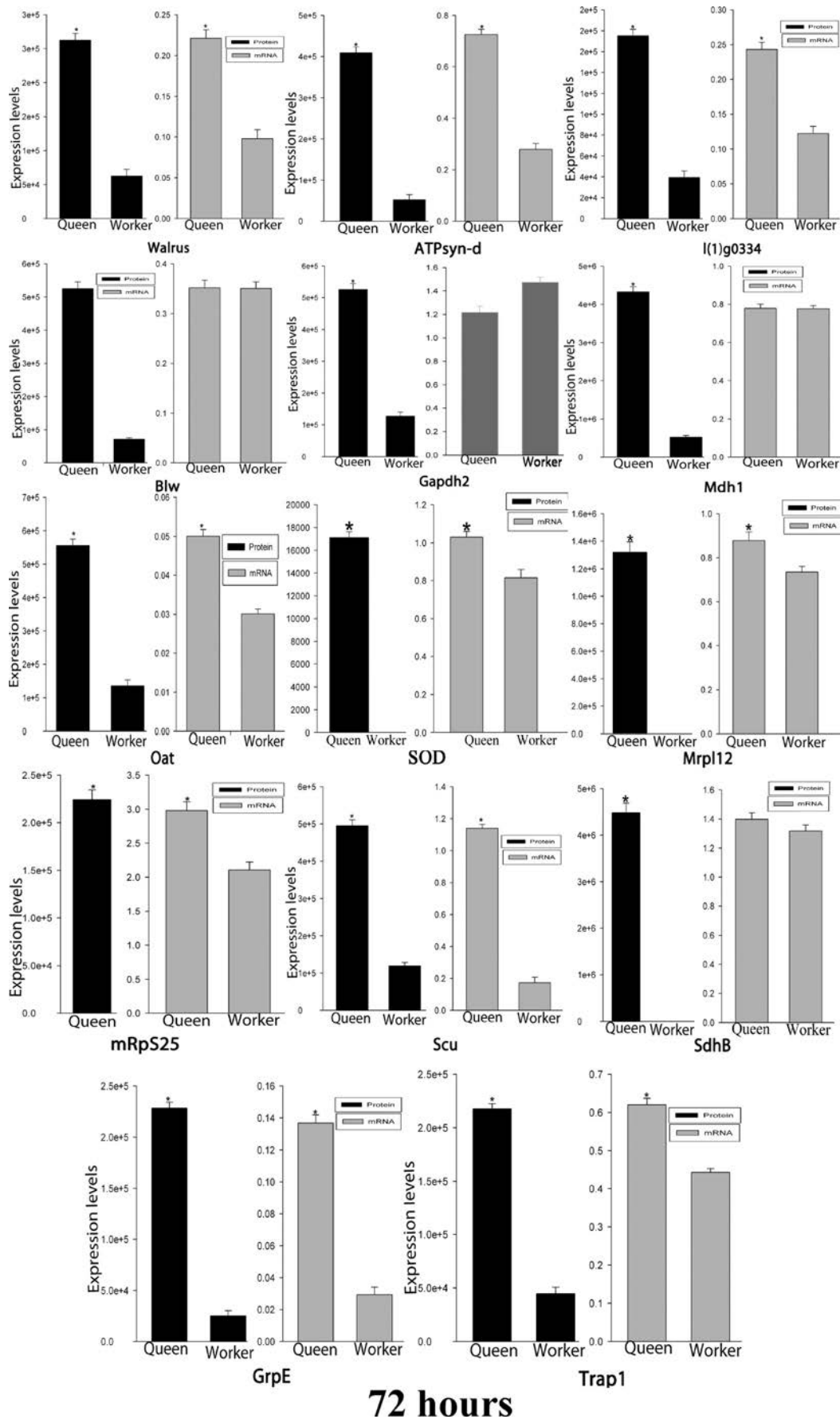


Figure 8. Continued

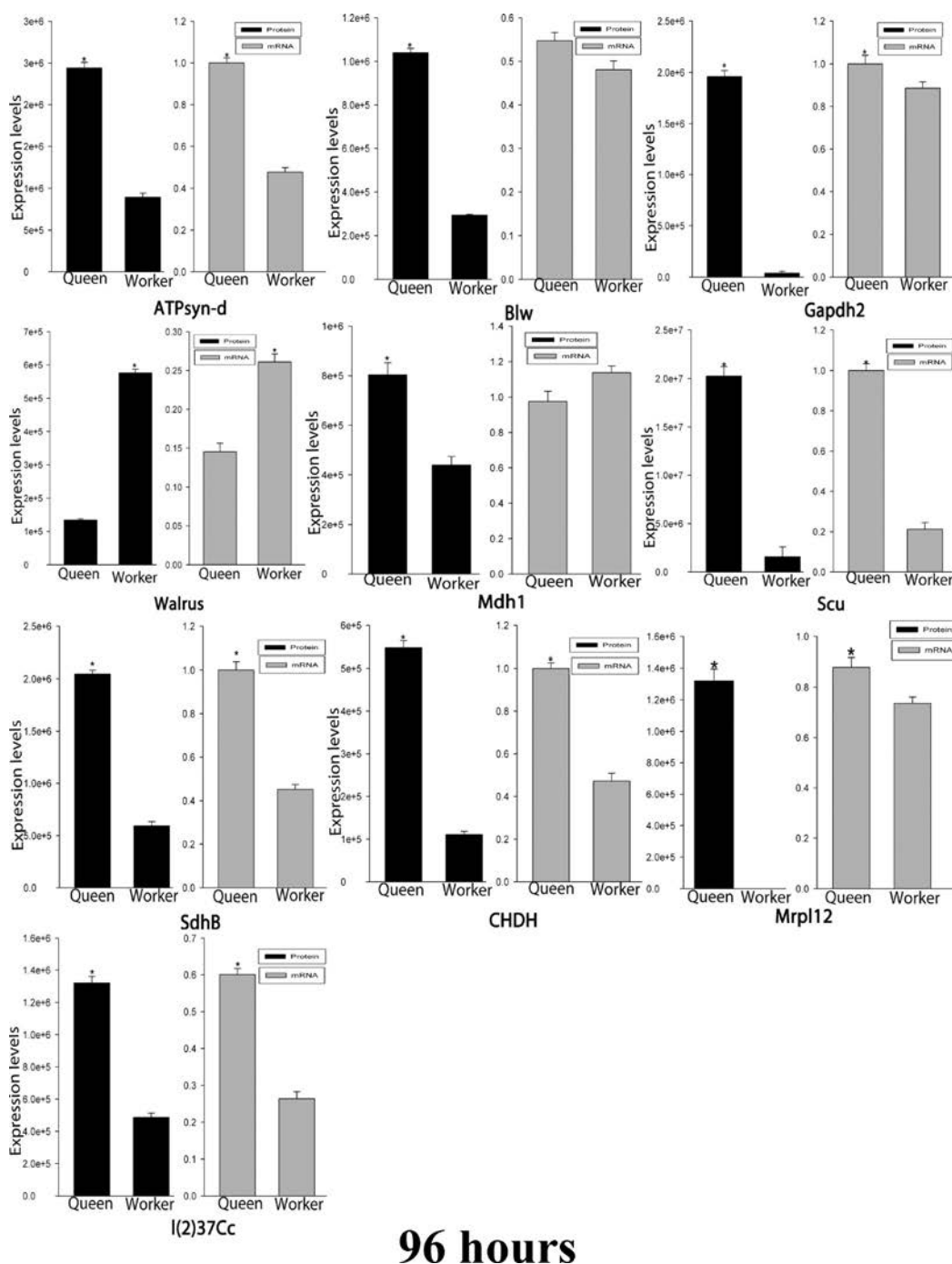


Figure 8. Validation of 24 differentially expressed proteins at mRNA level by quantitative real time PCR analysis during honeybee queen and worker larvae caste development at 72 and 96 h. Samples were normalized with the Gapdh gene as the control housekeeping gene. Error bars represent the standard deviation.

3.6. Validation of Differentially Expressed Proteins

As protein differential expressions between the two castes intended larvae were very conspicuous from the early ages, 24 interesting proteins were selected from 72 and 96 h developmental stages, and mRNA expressions were detected using qRT-PCR. Hence, the mRNA expressions of 22 proteins were consistent with protein expressions as confirmation of proteome data. Among the 22 proteins that have shown consistent protein and mRNA expressions, 14 were in 72 h larvae and all of them (except

Gapdh2 that showed inconsistency) showed increased consistencies in queen larvae (Figure 8). Likewise, at 96 h, the mRNA validation results of 10 proteins further confirmed protein differential expression consistencies in the two larvae (except wal, which has increased in worker larvae and Mdh1 that showed inconsistency with protein expression) with all increased in queen-intended larvae (Figure 8). The inconsistency between protein and mRNA expressions for Gapdh2 at 72 h and Mdh1 at 96 h of developmental stages might be due to the lack of a direct

relationship between protein and mRNA expression or lack of synchronization.

4. DISCUSSION

Despite over a century of studies on the causes of honeybee caste polyphenisms, information at the subcellular protein levels remains limited. To gain further insights, we analyzed the mitochondrial proteome of developing honeybee queen- and worker-intended larvae at 3 developmental stages (72, 96, and 120 h). The existences of large differences in protein expressions between the two castes intended larvae at all of the different developmental stages suggest strong directional selection pressures owing to the quantity and quality of nutrition provided across the developmental stages.

The analyses of the BIN and functional enrichment identified metabolic proteins as those present in highest amounts. This suggests proteins with metabolic enhancing activities generally appear to have significant roles in the process of caste determination. Interestingly, from the overwhelming majority of proteins up-regulated by the queen-intended larvae at 72 h (40 out of 42), the greater part (27 protein spots) are associated with carbohydrate metabolism and energy, amino acid, and fatty acid metabolism. This is in line with previous findings.^{9,10,14,31}

The queen-destined larvae overexpression of metabolic enzymes that are linked to metabolic rates and cellular responses to hormones appears to explain the enhanced growth rate during early larval development. The pathway decision marking as early as 72 h is in support of our previous finding using global differential proteins expression approach.¹⁸ However, this study has covered more proteins species and located them into the mitochondrial organelle. Moreover, detail clarifications on most of the proteins in the context of their mitochondrial roles in determining the pathway decisions of both castes intended larvae have been given at three developmental stages. At 72 h, the queen-intended larvae up-regulation of wide ranges of proteins with electron carrier molecular functions is to increase mitochondrial organelles and/or mitochondrial numbers to produce the ATP, which is required to enhance metabolism. For instance, a mitochondrial proton transport ATPsyn-d protein for ATP synthesis³² and walrus protein that helps the morphogenesis of malpighian tubule and ectodermal digestive tract and open tracheal system development process.³³ Likewise, both at 72 and 96 h, the queen larvae up-regulated blw and lethal (1) G0230 proteins, which participate in hydrogen-exporting ATPase activity and are involved in the larvae growth.^{32,34} Similarly, the queen larvae up-regulated the nuclear genes encoding proteins targeted to the mitochondrion, lethal (1) G0334, during the 72 h compared with 96 h, suggesting high demand for pyruvate enzymes that enhance pyruvate metabolic process³² for more ATP synthesis. Also, with glyceraldehyde-3-phosphate dehydrogenase activity and glycolysis process in mitochondria, Gapdh2 expressed at high levels in the thorax, head, and abdomen sections of *Drosophila*.³⁵ Concurrently, its overexpressions in the queen larvae throughout the developmental stages suggest its imperative roles in enhancing the metabolic rate.³⁵ However, the involvements of mitochondrial malate dehydrogenase precursor (Mdhm) and dihydrolipoyl dehydrogenase as TCA cycle enzymes is generally to play important roles in energy production.^{32,36} The queen larvae specific up-regulation of Mdhm at all of the developmental stages suggests its involvement to acquire the oxaloacetate (TCA intermediate) out of the mitochondria. In addition, the mitochondrial

electron transport chain proteins like, NADH-ubiquinone oxidoreductase (ND23, CI-20kD, and CI-23kD) and (UQCRC) have functions of NADH dehydrogenase (ubiquinone) activities in the process of mitochondrial respiratory chain complex assembly to avoid mitochondrial dysfunction due to defects of the NADH dehydrogenase complex.^{37,38} Hence, the unique expressions of ND23, CI-20kD, and CI-23kD and the up-regulation of the UQCRC in the queen-intended larvae at 72 h suggest their important functions in the mitochondrial electron transportations and protections. Likewise, the queen-intended larvae up-regulated an electron transport flavoprotein (etf) protein that produces ATP through an oxidative phosphorylation process³² at 72 and 96 h. On the contrary, the up-regulations of blw by the worker larvae at the 96 h probably suggests its performance as phagocytosis process in defending the larvae against pathogens and/or parasites.³⁹

Proteins as an input for the amino acid metabolism are needed for the metamorphosis process and for rapid larval growth¹⁰ and tissue building.¹⁴ Stimulated by food intake, high concentration of juvenile hormone (JH) in honeybee is associated with the time of highest respiratory rate and acts in the transcription of mitochondrial genes.¹⁵ To this reality, the 72 h queen larvae up-regulated many more amino acid metabolism proteins than the worker-destined larvae. This agrees with previous studies.^{10,18,40} The queen larvae expressed the JH-responsive amino acid metabolic enzyme fahd2A¹⁴ at 72 h in a caste-selective manner. This suggests the critical input of the protein to assist the glycolysis/gluconeogenesis normal process so it can take place during high respiratory conditions.⁴¹ In addition, the early stage (72 h) up-regulation of Oat by the queen larvae is in line with high expressional levels records in *Drosophila ananassae* and *Drosophila melanogaster* larvae.⁴² The function of Oat enzyme as enhancer of arginine biosynthesis is also documented in blowfly, silk moth, and *Drosophila* fat bodies with high elevation in female flies in relation to oogenesis.⁴² The queen-intended larvae also up-regulated a well-known nutrient choline dehydrogenase during 96 and 120 h. Reduction of choline dehydrogenase in the diet results in a phosphatidylcholine drop in larvae tissue, and its deficit can cause ethanol toxic susceptibility in *Drosophila*.⁴³ This probably suggests its vitality to defend the queen larvae against ethanol toxic effects that could be generated due to high metabolic rate during the organogenesis from mid to late developmental stages. On the other hand, its up-regulation in the worker larvae during early developmental stage (72 h) might suggest protecting the reduction of phosphatidylcholine in the larvae tissue.

Fatty acid metabolism proteins play important roles as an energy transporter from mitochondria to high energy demand areas for honeybee development.⁴⁴ The fact that the queen-intended larvae up-regulated all of the fatty acid metabolism proteins at 72 h further suggests the critical demand for proteins that transport energy from mitochondria to areas of high energy demand for effective development to take place. In particular, the mitochondria cells fatty acid β -oxidation catalyzing protein, MCAD,⁴⁵ which also participates in the process of carbon chain shortening,⁴⁶ was up-regulated in the queen larvae throughout the developmental stages. This protein was previously identified in the adult honeybee mandibular gland in a queen caste-selective manner as carbon chain shortening agent from the 17-hydroxyl stearic acid to 9-HAD, which is one of the major elements of queen mandibular pheromone.⁴⁶ However, even though its up-regulation in the queen-destined larvae at all of the developmental stages suggests its β -oxidation catalytic effect in the mitochondria cells to enhance energy productions for the

initiation and development of mandibular gland and its secretions, its up-regulation in the worker larvae at 120 h could activate reservation on its caste-selectiveness, which calls for further investigations. There is experimental evidence on the sequence similarities (~70%) and functional relationship between mammalian 17 β -hydroxysteroid dehydrogenases (HSD) 10 that controls mammalian hormone level and the protein Scu from *Drosophila*.⁴⁷ Scully plays important roles in fatty acid metabolism, and its knockout could lead to lethal phenotype difference during *Drosophila* embryonic and pupal development.⁴⁷ Accordingly, its queen-biased up-regulation at all of the developmental stages most likely suggests its action as HSD mediator in catabolic reactions of steroids to regulate queen larvae hormone to effect phenotypic difference between the two castes larvae. Besides, *yip2*, a protein with acetyl-CoA acyltransferase activity in the process of fatty acid β -oxidation, was up-regulated in the queen-intended larvae at all of the developmental stages, suggesting its principal contribution to the queen larvae brain development as in the *Drosophila*.⁴⁸ As well, the queen larvae uniquely expressed PEBP, which plays an important role in ATP and phosphatidylethanolamine binding, at 72 h and additionally up-regulated a multifunctional metabolic enzyme, *sdhb*, at 72 and 96 h. These further emphasize the largely participations of fatty acid metabolism enzymes in the mitochondrial activities of fast growing queen larvae to assist and speed up energy transport from mitochondria to high energy demanding areas, a result consistent with known physiological and anatomical differences between queen and worker larvae.

Depending on the growth status and physiological situations, larvae require proteins to regulate development and undergo transformation. Normally, reports from whole cells extract recognize more development proteins at 72 h in the worker than in the queen larvae.^{14,18} However, in this study, the queen-intended larvae up-regulated all of the development-related proteins at all of the developmental stages. This suggests the significance of subcellular level method in tagging proteins to their specific subcell through minimizing the possibilities of inclusions from other cells extracts. On the other hand, it reflects the enhanced growth rate of the queen compared with the worker larvae and development proteins have to be involved in realizing the phenomenon. Specifically, the up-regulation at 72 and 96 h of protein I(2)37Cc, which is recognized as key node protein in the adult honeybee worker hypopharyngeal gland,^{49,50} suggests high metabolism requirement of the queen-intended larvae from early embryos to mid developmental stages.^{18,51,52} Also, the 72 h queen larvae up-regulation of porin, which is a sucrose-specific transmembrane protein and is known in protecting mitochondrial morphological defects, signifies its probable role in assisting the normal remodeling process of mitochondria toward the development of healthy locomotive muscular systems.⁵³

Mitochondrial protein synthesis is one of the ways to increase higher mitochondrial activity.¹⁵ The overexpression of all biosynthesis proteins by the queen-intended larvae at 72 and 96 h suggests high metabolic rates of the queen-intended larvae that require more protein biosynthesis to increase either mitochondrial organelles or mitochondrial numbers to produce ATP. In particular, the up-regulation of a relatively high number of ribosomal proteins in the early age is associated with the queen larvae high metabolism and fast development and is in line with our previous finding for hypopharyngeal gland development of high royal jelly bees at early stages of development.¹⁸ In particular, at 72 h, the queen larvae uniquely expressed mRpS25 that partake in the process of translation,⁵⁴ rpl9 that performs

mitotic spindle elongation and organization,⁵⁵ mrpl12 that participate in transcriptional regulation,⁵⁶ and eftum that assists in translational elongation.⁵⁷ Hence, the queen larvae-biased expressions of mRpS25, mrpl12, and rpl9 in this study indicates the accumulation of mitochondrial transcripts as a result of more active mitochondria or a higher rate of biogenesis during the queen-determination process.¹⁵

Protein folding acts as molecular chaperone and facilitates nascent folding during early larval stage development of the honeybee.^{49,50} The queen larvae biased overexpressions of proteins folding at 72 and 96 h in this study suggest more molecular chaperone requirements in association with fast growth to facilitate the nascent protein folding and targeting. More specifically, the uniquely expressions of GrpE and Trap1 in the queen-intended larvae at 72 h suggests their imperative roles in easing nascent protein folding during the fast growth of the larvae. On the other hand, the 72 h worker larvae up-regulated only one protein folding known as Hsc70-5, indicating low metabolic activities and hence slow growth rate and small requirements of such proteins.

Antioxidant metabolites and enzymes work together to prevent cellular components from oxidative damages.^{58,59} The importance of antioxidant proteins in protecting cellular components from oxidative damages has been documented in honeybee embryo^{52,60} hypopharyngeal gland,^{49,50} sperm storage facilitation,⁶¹ and caste development.¹⁸ The high expression of antioxidant proteins in the early age of queen larvae agrees with the high level of antioxidant genes recorded for the younger queens.¹⁶ However, its expression at early stage in the queen larvae and at late stage in the worker larvae suggests high oxygen demand is based on the physiological condition and metabolic rate and antioxidant proteins have to be involved accordingly to protect the cellular components from oxidative damage.^{8,58} On the other hand, the expression of more antioxidant proteins by the queen larvae at 72 h is despite the existing record for its 2-fold expression by the same age of worker larvae.¹⁸ This discrepancy further emphasizes the significances of differential fractionation approach that helps to localize the information to specific subcellular organelles.⁶²

Furthermore, the enrichment analysis of the differentially expressed proteins at 72 and 96 h generally suggests 3 highly enriched functional terms in a similar mode, i.e., proteins as carbohydrate and energy metabolism, fatty acid metabolism and protein folding, while at 120 h, only 2 terms enriched, proteins as carbohydrate and energy and fatty acid metabolism. In general, carbohydrate and energy metabolism proteins were the most overrepresented followed by fatty acid metabolism proteins, suggesting higher respiratory rate in queen larvae that guides the larvae to follow queen developmental pathways. Similarly, the mapped BIN clearly demonstrates that carbohydrate metabolism and energy comprise about 69% in the BIN, which further ascertains the centrality of metabolic related proteins indicating the two castes intended larvae follow different developmental trajectories. Furthermore, the significantly consistent correlations between the protein and the mRNA expression status provide us important target proteins for the reverse genetic analysis of caste pathway modulation through RNA interference.

5. CONCLUSION

Understanding of proteins localized at the subcellular level is important to characterize the proteome and improves understanding of the protein functions. We applied combinations of differential centrifugation with biochemical fractionation techniques for the

enrichment of mitochondrial proteins with the large-scale identification of proteins by MS and analysis by bioinformatics during 3 developmental stages of honeybee queen and worker larvae. This spatial distribution of honeybee queen and worker larvae mitochondrial proteome indicates the two larvae are already on different developmental trajectories as early as 72 h and wide ranging proteins take part in shaping these metabolic, physiological, and anatomical differences. Many of the differently expressed proteins identified in this study linked to metabolic rates and cellular responses to hormones, consistent with physiological and anatomical differences between the queen and the worker larvae. The overexpression of metabolic enzymes by the prospective queen larvae shows the enhanced growth rate, and the large differences in the up-regulated proteins between the two larvae indicates that honeybee castes have faced strong directional selection pressures owing to nutritional changes. The queen-destined larvae involved a wide range of proteins contributing to metabolism of carbohydrate and energy, amino acid and fatty acid, protein biosynthesis, and protein folding to stimulate its active metabolism for fast growth through increased mitochondrial organelles. In particular, the critical role of carbohydrate and energy metabolism, fatty acid, and protein folding proteins in determining the larvae direction at the 72 h developmental stage was validated at the gene level. In this study we have achieved greatly improved coverage of the mitochondrial proteome and comprehensively provide a first overview on the mitochondrial protein profiles that are related to the honeybee queen and worker larvae caste determination process. Furthermore, this study emphasizes the value of the subcellular level approach and provides wide ranging information on caste polymorphism mediated by mitochondrial proteins, something that cannot be achieved by examining whole cell extracts on 2-DE. Hence, it is a major step forward in the analysis of the fundamental causes of honeybee caste-pathway decision process. In particular, visualizing proteins as interacting networks and confirming consistent correlation between protein and mRNA expressions status enables us to identify mitochondria located honeybee caste modulator proteins as early as 72 h of larval age. Thus, logically future proteome-based investigations on honeybee caste pathway decision proper time should consider less than 72 h of time points.

■ ASSOCIATED CONTENT

Supporting Information

Primer sequences used for qRT-PCR analysis of some of the differentially expressed mitochondrial proteins during the honeybee (*Apis mellifera* L.) larvae caste differentiation and enrichment analysis of identified mitochondrial proteins from developing honeybee (*A. mellifera* L.) queen and worker larvae at different developmental stages by ClueGO software. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

* Tel/Fax: +86 10 6259 1449. E-mail: apislijk@163.com.

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■ REFERENCES

- (1) Michener, C. D. *The Social Behavior of the Bees: A Comparative Study*; Belknap Press: Cambridge, 1974; Vol. 73.
- (2) Wilson, E. O., *Sociobiology: The New Synthesis*; Harvard University Press: Cambridge, 1975.
- (3) Robinson, G. E.; Fahrback, S. E.; Winston, M. L. Insect societies and the molecular biology of social behavior. *Bioessays* **1997**, *19* (12), 1099–1108.
- (4) Page, R. E., Jr.; Peng, C. Y. Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. *Exp. Gerontol.* **2001**, *36* (4–6), 695–711.
- (5) Amdam, G. V.; Page, R. E., Jr. Intergenerational transfers may have decoupled physiological and chronological age in a eusocial insect. *Ageing Res. Rev.* **2005**, *4* (3), 398–408.
- (6) Wheeler, D. E. Developmental and physiological determinants of caste in social Hymenoptera: evolutionary implications. *Am. Nat.* **1986**, *128* (1), 13–34.
- (7) West-Eberhard, M. J. *Developmental Plasticity and Evolution*; Oxford University Press: New York, 2003.
- (8) Seehuus, S. C.; Krekling, T.; Amdam, G. V. Cellular senescence in honey bee brain is largely independent of chronological age. *Exp. Gerontol.* **2006**, *41* (11), 1117–1125.
- (9) Cristino, A. S.; Nunes, F. M.; Lobo, C. H.; Bitondi, M. M.; Simoes, Z. L.; da Fontoura Costa, L.; Lattorff, H. M.; Moritz, R. F.; Evans, J. D.; Hartfelder, K. Caste development and reproduction: a genome-wide analysis of hallmarks of insect eusociality. *Insect Mol. Biol.* **2006**, *15* (5), 703–714.
- (10) Evans, J. D.; Wheeler, D. E. Expression profiles during honeybee caste determination. *Genome Biol.* **2001**, *2* (1), rearesearch0001.1–0001.6.
- (11) Rembold, H.; Kremer, J. P.; Ulrich, G. M. Characterization of postembryonic developmental stages of the female castes of the honey bee, *Apis mellifera* L. *Apidologie* **1980**, *11* (1), 29–38.
- (12) Weaver, N. Effects of larval age on dimorphic differentiation of the female honey bee. *Ann. Entomol. Soc. Am.* **1957**, *50* (3), 283–294.
- (13) Woyke, J. Correlations between the age at which honeybee brood was grafted, characteristics of the resultant queens, and results of insemination. *J. Apic. Res.* **1971**, *10* (1), 45–55.
- (14) Barchuk, A. R.; Cristino, A. S.; Kucharski, R.; Costa, L. F.; Simoes, Z. L.; Maleszka, R. Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera*. *BMC Dev. Biol.* **2007**, *7*, 70.
- (15) Corona, M.; Estrada, E.; Zurita, M. Differential expression of mitochondrial genes between queens and workers during caste determination in the honeybee *Apis mellifera*. *J. Exp. Biol.* **1999**, *202* (Pt 8), 929–938.
- (16) Corona, M.; Hughes, K. A.; Weaver, D. B.; Robinson, G. E. Gene expression patterns associated with queen honey bee longevity. *Mech. Ageing Dev.* **2005**, *126* (11), 1230–1238.
- (17) Chan, Q. W.; Howes, C. G.; Foster, L. J. Quantitative comparison of caste differences in honeybee hemolymph. *Mol. Cell. Proteomics* **2006**, *5* (12), 2252–2262.
- (18) Li, J.; Wu, J.; Begna Rundassa, D.; Song, F.; Zheng, A.; Fang, Y. Differential protein expression in honeybee (*Apis mellifera* L.) larvae: underlying caste differentiation. *PLoS One* **2010**, *5* (10), e13455.
- (19) Green, D. R.; Reed, J. C. Mitochondria and apoptosis. *Science* **1998**, *281* (5381), 1309–1312.
- (20) Finkel, T.; Holbrook, N. J. Oxidants, oxidative stress and the biology of ageing. *Nature* **2000**, *408* (6809), 239–247.
- (21) Henze, K.; Martin, W. Evolutionary biology: essence of mitochondria. *Nature* **2003**, *426* (6963), 127–128.
- (22) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* **2006**, *443* (7114), 931–949.
- (23) Lin, R. X.; Zhao, H. B.; Li, C. R.; Sun, Y. N.; Qian, X. H.; Wang, S. Q. Proteomic analysis of ionizing radiation-induced proteins at the subcellular level. *J. Proteome Res.* **2009**, *8* (1), 390–399.

- (24) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
- (25) Li, J.; Li, H.; Zhang, Z.; Pan, Y. Identification of the proteome complement of high royal jelly producing bees (*Apis mellifera*) during worker larval development. *Apidologie* **2007**, *38* (6), 545–557.
- (26) Bindea, G.; Mlecnik, B.; Hackl, H.; Charoentong, P.; Tosolini, M.; Kirilovsky, A.; Fridman, W. H.; Pagès, F.; Trajanoski, Z.; Galon, J. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* **2009**, *25* (8), 1091.
- (27) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods* **2001**, *25* (4), 402–408.
- (28) Andersen, J. S.; Mann, M. Organellar proteomics: turning inventories into insights. *EMBO Rep.* **2006**, *7* (9), 874–879.
- (29) Patton, W. F.; Fluorescence detection of glycoproteins in gels and on electrophoresis. *Curr. Protoc. Cell Biol.* **2002**, Chapter 6, Unit 6.8.
- (30) Phizicky, E. M.; Fields, S. Protein-protein interactions: methods for detection and analysis. *Microbiol. Rev.* **1995**, *59* (1), 94–123.
- (31) Evans, J. D.; Wheeler, D. E. Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera*. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (10), 5575–5580.
- (32) Sardiello, M.; Licciulli, F.; Catalano, D.; Attimonelli, M.; Caggese, C. MitoDrome: a database of *Drosophila melanogaster* nuclear genes encoding proteins targeted to the mitochondrion. *Nucleic Acids Res.* **2003**, *31* (1), 322–324.
- (33) Liu, X.; Kiss, I.; Lengyel, J. A. Identification of genes controlling malpighian tubule and other epithelial morphogenesis in *Drosophila melanogaster*. *Genetics* **1999**, *151* (2), 685–695.
- (34) Galloni, M.; Edgar, B. A. Cell-autonomous and non-autonomous growth-defective mutants of *Drosophila melanogaster*. *Development* **1999**, *126* (11), 2365–2375.
- (35) Sun, X. H.; Tso, J. Y.; Lis, J.; Wu, R. Differential regulation of the two glyceraldehyde-3-phosphate dehydrogenase genes during *Drosophila* development. *Mol. Cell. Biol.* **1988**, *8* (12), 5200–5205.
- (36) Steffan, J. S.; Minard, K. I.; McAlister-Henn, L. Expression and function of heterologous forms of malate dehydrogenase in yeast. *Arch. Biochem. Biophys.* **1992**, *293* (1), 93–102.
- (37) Rieske, J. S. Composition, structure, and function of complex III of the respiratory chain. *Biochim. Biophys. Acta* **1976**, *456* (2), 195–247.
- (38) Copeland, J. M.; Cho, J.; Lo, T., Jr.; Hur, J. H.; Bahadorani, S.; Arabyan, T.; Rabie, J.; Soh, J.; Walker, D. W. Extension of *Drosophila* life span by RNAi of the mitochondrial respiratory chain. *Curr. Biol.* **2009**, *19* (19), 1591–1598.
- (39) Stroschein-Stevenson, S. L.; Foley, E.; O'Farrell, P. H.; Johnson, A. D. Identification of *Drosophila* gene products required for phagocytosis of *Candida albicans*. *PLoS Biol.* **2006**, *4* (1), e4.
- (40) Lu, P.; Vogel, C.; Wang, R.; Yao, X.; Marcotte, E. M. Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nat. Biotechnol.* **2007**, *25* (1), 117–124.
- (41) Rachinsky, A.; Strambi, C.; Strambi, A.; Hartfelder, K. Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *Gen. Comp. Endocrinol.* **1990**, *79* (1), 31–38.
- (42) Yoshida, K. M.; Juni, N.; Hori, S. H. Molecular cloning and characterization of *Drosophila* ornithine aminotransferase gene. *Genes Genet. Syst.* **1997**, *72* (1), 9–17.
- (43) Tilghman, J. A.; Geer, B. W. The effects of a choline deficiency on the lipid composition and ethanol tolerance of *Drosophila melanogaster*. *Comp. Biochem. Physiol. C* **1988**, *90* (2), 439–444.
- (44) Kucharski, R.; Maleszka, R. Arginine kinase is highly expressed in the compound eye of the honey bee, *Apis mellifera*. *Gene* **1998**, *211* (2), 343–349.
- (45) Thorpe, C.; Kim, J. J. Structure and mechanism of action of the acyl-CoA dehydrogenases. *FASEB J.* **1995**, *9* (9), 718–725.
- (46) Hasegawa, M.; Asanuma, S.; Fujiyuki, T.; Kiya, T.; Sasaki, T.; Endo, D.; Morioka, M.; Kubo, T. Differential gene expression in the mandibular glands of queen and worker honeybees, *Apis mellifera* L.: implications for caste-selective aldehyde and fatty acid metabolism. *Insect Biochem. Mol. Biol.* **2009**, *39* (10), 661–667.
- (47) Torroja, L.; Ortuno-Sahagun, D.; Ferrus, A.; Hammerle, B.; Barbas, J. A. scully, an essential gene of *Drosophila*, is homologous to mammalian mitochondrial type II L-3-hydroxyacyl-CoA dehydrogenase/amyloid-beta peptide-binding protein. *J. Cell Biol.* **1998**, *141* (4), 1009–1017.
- (48) Xun, Z.; Sowell, R. A.; Kaufman, T. C.; Clemmer, D. E. Protein expression in a *Drosophila* model of Parkinson's disease. *J. Proteome Res.* **2007**, *6* (1), 348–357.
- (49) Feng, M.; Fang, Y.; Li, J. Proteomic analysis of honeybee worker (*Apis mellifera*) hypopharyngeal gland development. *BMC Genomics* **2009**, *10*, 645.
- (50) Jianke, L.; Mao, F.; Begna, D.; Yu, F.; Aijuan, Z. Proteome comparison of hypopharyngeal gland development between Italian and royal jelly producing worker honeybees (*Apis mellifera* L.). *J. Proteome Res.* **2010**, *9* (12), 6578–6594.
- (51) Eveleth, D. D., Jr; Marsh, J. L. Sequence and expression of the Cc gene, a member of the dopa decarboxylase gene cluster of *Drosophila*: possible translational regulation. *Nucleic Acids Res.* **1986**, *14* (15), 6169–6183.
- (52) Li, J.; Fang, Y.; Zhang, L.; Begna, D. Honeybee (*Apis mellifera* ligustica) drone embryo proteomes. *J. Insect Physiol.* **2011**, *57* (3), 372–384.
- (53) Park, J.; Kim, Y.; Choi, S.; Koh, H.; Lee, S. H.; Kim, J. M.; Chung, J. *Drosophila* Porin/VDAC affects mitochondrial morphology. *PLoS One* **2010**, *5* (10), e13151.
- (54) Koc, E. C.; Burkhardt, W.; Blackburn, K.; Moseley, A.; Koc, H.; Spremulli, L. L. A proteomics approach to the identification of mammalian mitochondrial small subunit ribosomal proteins. *J. Biol. Chem.* **2000**, *275* (42), 32585–32591.
- (55) Goshima, G.; Wollman, R.; Goodwin, S. S.; Zhang, N.; Scholey, J. M.; Vale, R. D.; Stuurman, N. Genes required for mitotic spindle assembly in *Drosophila* S2 cells. *Science* **2007**, *316* (5823), 417–421.
- (56) Ni, J. Q.; Liu, L. P.; Hess, D.; Rietdorf, J.; Sun, F. L. *Drosophila* ribosomal proteins are associated with linker histone H1 and suppress gene transcription. *Genes Dev.* **2006**, *20* (14), 1959–1973.
- (57) Spradling, A. C.; Stern, D.; Beaton, A.; Rhem, E. J.; Laverty, T.; Mozden, N.; Misra, S.; Rubin, G. M. The Berkeley *Drosophila* Genome Project gene disruption project: Single P-element insertions mutating 25% of vital *Drosophila* genes. *Genetics* **1999**, *153* (1), 135–177.
- (58) Sies, H. Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* **1997**, *82* (2), 291–295.
- (59) Vertuani, S.; Angusti, A.; Manfredini, S. The antioxidants and pro-antioxidants network: an overview. *Curr. Pharm. Des.* **2004**, *10* (14), 1677–1694.
- (60) Li, J.; Zhang, L.; Feng, M.; Zhang, Z.; Pan, Y. Identification of the proteome composition occurring during the course of embryonic development of bees (*Apis mellifera*). *Insect Mol. Biol.* **2009**, *18* (1), 1–9.
- (61) Baer, B.; Eubel, H.; Taylor, N. L.; O'Toole, N.; Millar, A. H. Insights into female sperm storage from the spermathecal fluid proteome of the honeybee *Apis mellifera*. *Genome Biol.* **2009**, *10* (6), R67.
- (62) Dreger, M. Proteome analysis at the level of subcellular structures. *Eur. J. Biochem.* **2003**, *270* (4), 589–599.