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Differential Expressions of Nuclear Proteomes between Honeybee (*Apis mellifera* L.) Queen and Worker Larvae: A Deep Insight into Caste Pathway Decisions

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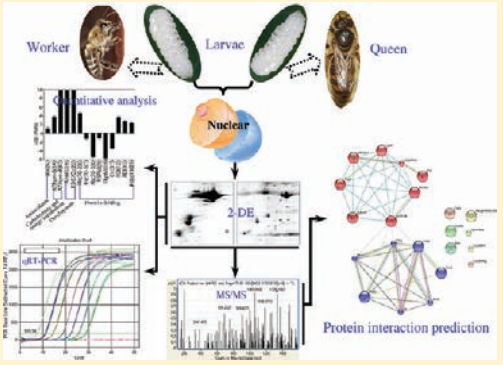
Supporting Information

ABSTRACT: Honeybees (*Apis mellifera* L.) possess individuals (castes) in their colonies, to which specific tasks are allocated. Owing to a difference in nutrition, the young female larvae develop into either a fertile queen or a sterile worker. Despite a series of investigations on the underlying mechanisms of honeybee caste polyphenism, information on proteins and enzymes involved in DNA and RNA regulation in the nucleus is still missing. The techniques of nuclear protein enrichment, two-dimensional electrophoresis, mass spectrometry and bioinformatics were applied to understand the nuclear proteome changes in response to changes in environmental settings (nutrition and time) during the early developmental stages at the third (72 h), fourth (96 h), and fifth (120 h) instars of the two caste intended larvae. A total of 120 differentially expressed nuclear proteins were identified in both caste intended larvae during these developmental stages. The third, fourth and fifth instars of queen prospective larvae expressed 69%, 84%, and 68% of the proteins that had altered expression, respectively. Particularly, the prospective queen larvae up-regulated most of the proteins with nuclear functions. In general, this changing nuclear proteome of the two caste intended larvae over the three developmental stages suggests variations in DNA and RNA regulating proteins and enzymes. These variations of proteins and enzymes involved in DNA and RNA regulation in response to differential nutrition between the two caste intended larvae lead the two caste larvae to pursue different developmental trajectories. Hence, this first data set of the nuclear proteome helps us to explore the innermost biological makings of queen and worker bee castes as early as before the 72 h (3rd instar). Also, it provides new insights into the honeybee's polymorphism at nuclear proteome level and paves new ways to understand mechanisms of caste decision in other eusocial insects.



KEYWORDS: *nucleus, proteome, honeybee, caste, pathway*

1. INTRODUCTION

The honeybee (*Apis mellifera* L.) colony possesses three types of individuals (castes); the male drone, the workers, and the queen. While the male bee (drone) develops from unfertilized egg, the two female castes (queen and worker bees) inherit exactly the same set of genetic materials from a fertilized egg. This fantastic phenomenon of caste polymorphism in the female honeybee has been a focus of scientific research for centuries. As a result, many researches have addressed the differences between the two castes in morphology, reproduction, and complex social interactions or traits.[1,2] The influence of nutrition in shifting the caste developmental trajectories during the early larval growth stage has been a main focus of researchers past and present.[2−9] It has reported that the quantity and quality of the nutrition influence on the early honeybee larva developmental directions. A larva fed on royal jelly throughout the whole larval stage develops to be an adult queen, which is large in size, with a long lifespan and an active reproductive system. On the contrary, a larva fed on worker jelly develops to be an adult worker, which is small in size, with a short lifespan and is sterile.[10,11] As dictated by their evolutionary successes, the two castes develop possessing distinctive energy and metabolism requirements.[2] Recently, the influence of cell size on the larva caste development has been reported,[12] which is also linked to the quantity of the feed provided to the larvae. Therefore, in response to environmental stimuli (quality and quantity of feed); one genome gives rise to two or more phenotypes that lead to the creations of polyphenisms in the honeybee.[13] On the other hands, these changes in metabolic pathways in response to environmental stimuli are organized by enzymes.[14] Proteins act as enzymes that play important roles in catalyzing chemical reactions in metabolism[15] and also serve in cell signals, immune response, cell adhesion, active transport across membranes and cell cycle.[16]

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Nowadays, several approaches have been developed to assist detail investigations of honeybee caste determination.[17] Consequently, several research outputs such as mRNA profiling,[18] RNA differential display,[2] subtractive hybridization,[19] differential protein expressions,[20,21] differential genes expression,[14,22] and DNA methylation[23,24] significantly contribute to the understanding on the fundamental causes of honeybee polyphenisms. More recently, our group employed subcellular proteomic strategy to address this issue of honeybee caste polyphenisms through analyzing differential expressions of mitochondrial proteins at early larval developmental stages of the queen and worker intended larvae.[25] However, information localized in the other subcellular organelles still require further investigation. In particular, from the point of its biological significance of nuclear localized proteins in caste determinations are not yet addressed.

The nucleus contains most of the genetic material in cell's DNA with large varieties of proteins and serves as a control center of the cell through maintaining the integrity of the genes and regulating gene expression. Hence, it is the place where a large proportion of the transcriptional and RNA processing activity of most cells are undertaken.[26] Therefore, investigating the mechanisms of the caste differentiation in honeybee queen and worker intended larvae through their nuclear proteome differential expression could provide deeper insight into possible candidate proteins that regulate caste pathways and further extend our understanding of honeybee biology.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

Urea, Tris-base, sodium dodecyl sulfate (SDS), sodium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), iodoacetamide, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, U.S.A.). Biolyte was from Bio-Rad (Hercules, CA, U.S.A.). Acrylamide, *N*,*N*′-methylenebisacrylamide, ammonium persulfate (AP), *N*,*N*,*N*′,*N*′-tetramethylethylene diamine (TEMED), 3-[(3-cholamidopropyl)-dimethylammonio]-propane sulfonate (CHAPS), glycerol, bromophenol blue, Coomassie brilliant blue (CBB) G-250, and α-cyano-4-hydroxycinnamic acid (CHCA) were from Bruker Daltonics (Billerica, MA, U.S.A.). Trypsin was from Roche (Modified, Sequencing grade, Roche, Mannheim, Germany), and trifluoroacetic acid (TFA) and acetonitrile were from J. T. Baker (Phillipsburg, NJ, U.S.A.). Other chemicals used but not specified here were noted with their sources in the text.

2.2. Biological Samples

The collections of worker and queen larvae from five honeybee (*Apis mellifera* L.) colonies were done at the third larval instar (72 h posthatching), fourth larval instar (96 h posthatching), and fifth larval instar (120 h posthatching) from the apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Science according to our previously used method.[21] Briefly, to ensure the exact age of the larvae to be sampled, the egg laying queen bee was confined to a single wax comb frame containing worker cells for five hours with a cage made of a queen excluder, through which workers but not the queen could pass. Subsequently, the queen was removed, and the fertilized eggs contained in the frame were maintained in the honeybee colony for further development. After the eggs hatched, the larvae intended to be in the queen caste were transferred from the worker cells to the queen cell cups in a queen rearing frame and put into the queenless colony for further development from which each experimental time point of queen larvae were sampled. However, the larvae intended for the worker's caste were collected directly from the worker cells. From each caste and each developmental stage 300 larvae (three biological replicates each) were collected and frozen at −80 °C until use.

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

Nuclear fractions of the collected larvae were prepared according to the method as previously described[27] with minor modifications. Briefly, the collected larvae were washed with ice-cold PBS buffer and laid on ice-cold tissue paper to absorb the PBS buffer. The total protein extraction was done by finely mincing and homogenizing in ice-cold lysis buffer 1(LB1) (250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF using a tight fitting Teflon pestle with a ratio of 1 g (larvae)/5 mL (LB1). (All subsequent steps were performed at 4 °C or on ice). The total protein extract was centrifuged in a benchtop centrifuge at 1000× *g* for 15 min, and the pellets were recovered serving as the source of crude nuclear protein material. Once again, the pellets were rehomogenized in LB1 (2 mL/1 g of sample), recentrifuged at 1000× *g* for 15 min and the pellets were recovered. Subsequently, the recovered nuclei pellets were suspended in a cushin buffer (2 M sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 m M DTT, 1 mM PMSF and pelleted by ultracentrifugation at 80 000× *g* for 1 h (Beckman, JA-30.50 Rotor). The nuclei proteins were extracted by resuspending the recovered nuclei pellets in LB2 (1 mg/1 mL) (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT, 2% Biolyte pH 3−10) followed by homogenization for 5 min on ice and sonicated for 2 min. The debris was removed by centrifugation at 13 000 × *g* for 30 min. With these preparations, nuclear fraction samples from the third, fourth and fifth instars of the queen and worker larvae were obtained and stored at −80 °C until use. Protein concentration was determined according to Bradford method[28] using BSA as the standard and the absorption measured at 595 nm (Beckman, spectrophotometer DU800).

The protein sample (1000 μg) from each larva described above was suspended in 336 μL of rehydration buffer [8 M urea, 2% CHAPS, 0.001% bromophenol blue, 45 mM DTT, and 0.2% Biolyte (pH 3−10)]. The mixture was loaded onto a 17 cm IPG strip (pH 3−10, linear, Bio-Rad). Isoelectric focusing (IEF) was performed (Protean IEF Cell, Bio-Rad) at 18 °C according to the following program: 14 h at 50 V, 250 V for 30 min (four times), 1000 V for 60 min, 9000 V for 5 h, 9000 V for 60000 V h. Prior to 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, and 2% DTT] and later in equilibration buffer 2 [6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, and 2.5% iodoacetamide] for 15 min. After equilibration, the strip was transferred to an SDS-PAGE, 12% T separating gel (1.00 mm). 2-DE, SDS-PAGE, was performed in a Protean II Xi Cell (Bio-Rad) at 25 mA/gel for 6 h.

2.4. Image Acquisition and Statistical 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 MS analysis. Three independent 2-DE gel images from triplicate samples of each

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honeybee caste 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 the pixels making up the spot) analyses were performed using PDQuest (version 8.0.1, Bio-Rad). All gels were matched with one of the selected reference gels. The match analysis was performed in an automatic mode, and further manual editing was performed to correct the mismatched and unmatched spots. The expression level of a given protein spot was expressed in terms of the volume of the spot. To compare spot quantities between gels accurately, we normalized the spot volumes as percentages of the total volume of all of the spots in the gel. The means and standard deviations from the triplicate experiments were calculated, and the statistical significance of the level of expression of the protein and mRNA between the larvae of queen and worker 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 the means of the expression level of the two honeybee castes. A $p < 0.05$ error probability was considered to be statistically significant with respect to at least 1.5-fold changes.

2.5. Trypsin Digestion for MS

Trypsin digestion for MS was performed according to a method previously established.[29] Briefly, the CBB-stained spots were manually excised from each gel of queen and worker larvae samples and destained for 30 min using 100 mL of acetonitrile (50%) and 25 mM NH_4HCO_3 (pH 8, 50%) three to four times until the gels were transparent. The gels were dehydrated for 10 min with acetonitrile (100%) and dried for 30 min using a Speed-Vac system. To prepare the trypsin solution, 2.5 mL of 25 mM NH_4HCO_3 was added to 25 μg of trypsin (final concentration of 10 ng/μL, Roche). The trypsin solution (10 μL) was pipetted onto each dried protein spot and incubated for 60 min at 4 °C. To avoid trypsin autodigestion, the excess trypsin was removed. Then the sample was incubated for 14 h at 37 °C.

To extract the peptide fragments from the tryptic digests, 20 μL of 5% (v/v) TFA was added and incubated for 60 min at 37 °C. Thereafter, 20 μL of 50% (v/v) acetonitrile [containing 2.5% (v/v) TFA] was added to gel pieces and incubated for 60 min at 37 °C. After each step, the supernatants were pooled and dried using a Speed Vac system.

2.6. Identification of Proteins by MS

The digested protein spots were identified by liquid chromatography-chip/electrospray ionization-quadruple time-of-flight/MS (LC-Chip-ESI-QTOF-MS) (QTOF 6520, Agilent Technologies), equipped with a capillary pump G1382A, a nano pump G2225A, an autosampler G1377D, and the Chip Cube G4240A. The LC-Chip used (Agilent Technologies) was constituted of a Zorbax 300SB-C18 enrichment column (40 nL, 5 μm) and a Zorbax 300SB-C18 analytical column (75 μm × 43 mm, 5 μm). The loading flow rate was 4 μL/min and the 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. The Chip flow rate was 300 nL/min. The MS conditions were as follows: positive ion mode; V_{cap}, 1900 V; drying gas flow rate, 5 L/min; drying gas temperature, 350 °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 μL of water with 0.1% formic acid; centrifuged for 5 min at 10 000× g and about 15 μL of upper solution was analyzed and used to inject 8 μL of sample for analysis. Spectra were calibrated by mass reference standard purine and HP-0921 (121. 050873, 922.009798, Agilent Technologies). Tandem mass spectra were retrieved using the Mass Hunter software with default parameter setting (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 NCBInr (release date, Aprial 12, 2011). Search parameters: Oxidations (M) were selected as variable modifications and fixed modification was selected as carbamidomethyl (C). The other parameters used were as follows: taxonomy, apis mellifera; enzyme, trypsin; missed cleavages, 1; peptide tolerance, ±1.2 Da; MS/MS tolerance, ± 0.6 Da. A total of 13 473 798 sequences and 4 621 495 809 residues in the database were actually searched. When the identified peptides matched to multiple members of a protein family or a protein appeared under the same names 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.

2.7. Protein Subcellular Prediction and Biological Network Analysis

The identified proteins were searched against the Uniprot database (http://www.uniprot.org/) and combined Flybase (http://flybase.org/) search results and grouped on the basis of their biochemical functions. Subsequently, the FASTA of the identified proteins were submitted to different subcellular predictors to know their subcellular locations. Protein subcellular location predictors, SubLoc v1.0, (http://www.bioinfo.tsinghua.edu.cn/SubLoc/eu_predict.htm), WoLF PSORT (http://wolfpsort.org/), PSORT-II (http://psort.hgc.jp/form2.html), and NucPred http://www.sbc.su.se/~maccallr/nucpred/ were used. Also, as organelles' composition is always changing based on the molecules being delivered to and transported from,[30] the previous history of the protein from different literatures were also used to establish its multiple organelles localization. In general, in this study, a given protein to be designated as a nuclear localized protein, it should be qualified at least by one of these predictors or historically supported by literature evidence. Protein interaction networks of the nuclear enriched and differentially regulated proteins were analyzed using the online database resource Search Tool for the Retrieval of Interacting Genes (STRING).[31]

2.8. Test of Protein Expression by Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted from both queen and worker larvae using TRIzol reagent (Bio Rad). Reverse transcription was performed using an RNA PCR kit (Bio Rad), according to the manufacturer's instructions. The analysis of qRT-PCR was conducted on 24 differentially expressed proteins. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference. All the premiers were listed in Table S1 as Supporting Information.

Real-time PCR was performed using an iQ5Multicolor Real-Time PCR Detection System (Bio-Rad) in a 25 μL reaction system containing 1 μL of cDNA, 5 pmol of forward and reverse primers, 12.5 μL of SYBR Green Supermix (Bio-Rad),

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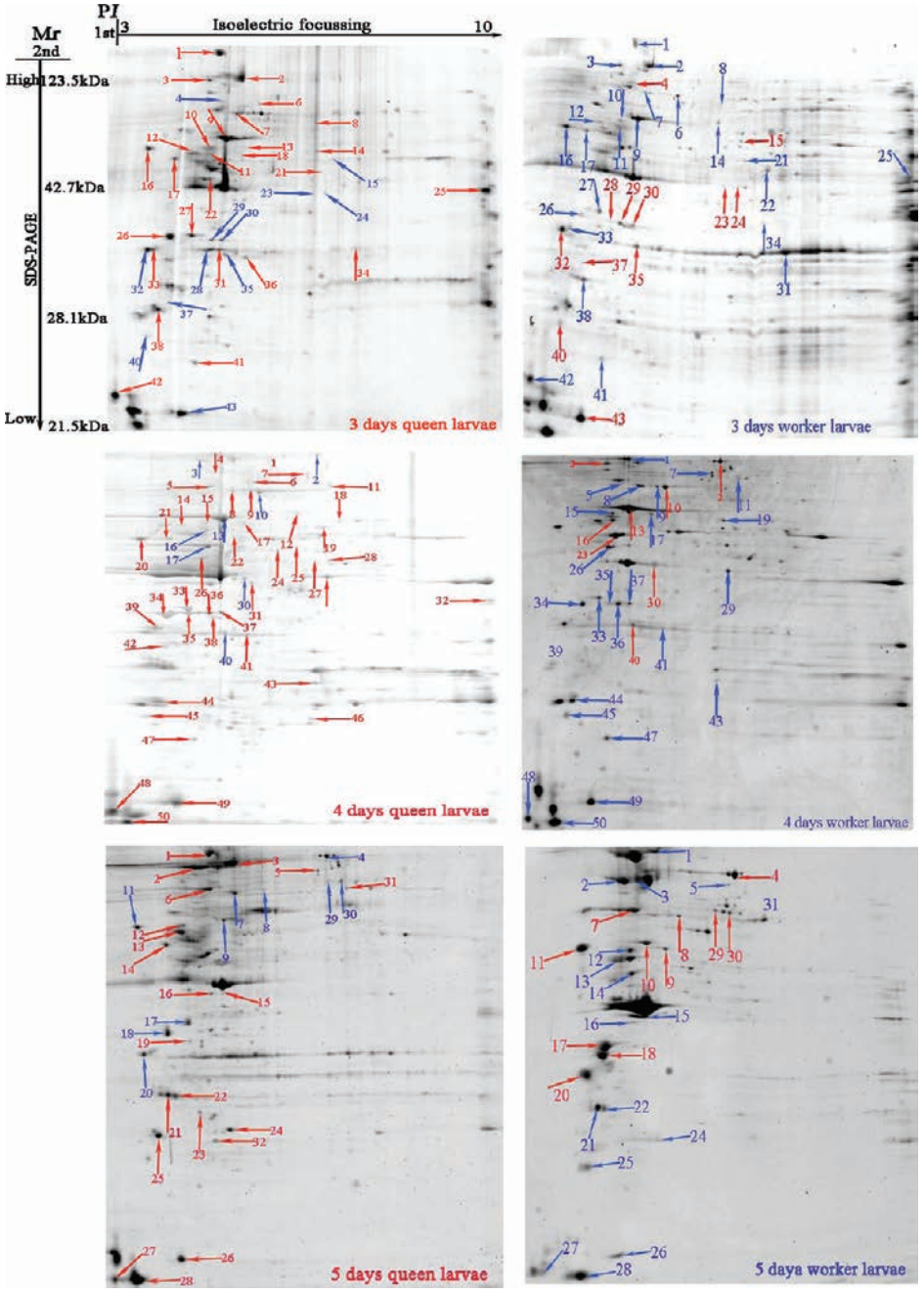

Figure 1. Representative 2-DE nuclear proteome images of honeybee queen and worker larvae at 72 (3 days), 96 (4 days), and 120 (5 days) hours, respectively. Proteins are separated on IPG gel strips (17 cm, 3−10 L) with 1000 μg sample loaded, followed by SDS-PAGE on a vertical slab gel (12.5%) and detected 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.

and water. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.[32] Each sample was analyzed independently and processed in triplicate. The values from the three independently obtained results were compared using students t test. An error probability $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Identification of Differentially Expressed Proteins

To better understand the mechanism of caste determination in the honeybee, we analyzed the nuclear proteome of queen and worker intended larvae at three developmental time points. On the basis of the numbers of protein spots visualized on 2-DE gel images, 250, 180, and 130 spots were detected on both larvae at the third, fourth, and fifth instars, respectively (Figure 1). From these 80, 100, and 58 protein spots showed differential expressions (>1.5 fold, $p \leq 0.05$) of which 58, 72, and 46 proteins identified as *A. mellifera* origin at the third, fourth and fifth instars, respectively. Subsequent analysis of the *A.mellifera* origin proteins reproducibly yielded 39, 50, and 31 spots as nuclear proteins at the third, fourth and fifth instars, correspondingly (Table 1−3). However, the unidentified differentially expressed protein spots might be attributed to their low abundance to produce enough spectra to identify them. Also, all the proteins were not identified as nuclear localized proteins and this could be attributed to a common

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Table 1. Identification of Differentially Expressed Nuclear Proteins during Honeybee (*Apis mellifera* L) Caste Determination of Queen and Worker Larvae at 72 h (day 3)

spot no.	experimental pI/M_r (kDa)	theoretical pI/M_r (kDa)	sequence coverage (%)	matched	score	protein name	accession number	log ratio (1.5) (+ or −)	*p*-value	subcellular predictor(s)
						cytoskeleton				
1	5.78/224.60	5.78/224.60	36	92	1518	myosin heavy chain CG17927-PB, isoform B isoform 1 (Mhc)	gil110759191	2.8	0.009	1, 2
2	6.03/126.64	5.33/102.05	66	77	1266	paramyosin CG5939-PA, isoform A (Prm)	gil66510482	1.8	0.010	1, 2, 3
3	5.71/133.29	5.33/102.05	38	40	368	paramyosin CG5939-PA, isoform A (Prm)	gil66510482	1.2	0.007	1, 2, 3
4	5.79/223.23	5.78/224.60	11	22	288	myosin heavy chain CG17927-PB, isoform B isoform 1 (Mhc)	gil110759191	−4.5	0.000	1, 2
12	5.58/56.13	4.73/48.18	44	17	220	TPA: TPA_exp: troponin T isoform 2 (up), CG7107	gil78101798	4.0	0.001	1, 2, 3,
15	7.11/22.74	6.82/21.56	12	2	47	cuticular protein 64Aa, CG15006-PA (Cpr64Aa	gil110764439	−1.6	0.001	4
23	6.93/38.55	6.82/21.56	24	4	59	cuticular protein 64Aa, CG15006-PA (Cpr64Aa)	gil110764439	−1.2	0.000	4
24	7.11/32.74	6.82/21.56	24	4	97	cuticular protein 64Aa, CG15006-PA (Cpr64Aa)	gil110764439	−1.0	0.000	4
26	5.29/33.91	4.74/32.99	44	17	235	tropomyosin 1 CG4898-PD, isoform D isoform 1(Tm1)	gil48094441	5.7	0.003	1, 2, 3
27	5.48/35.06	4.73/32.25	32	9	240	tropomyosin 2 CG4843-PB, isoform B (Tm2)	gil66522386	3.4	0.000	1, 2, 3
28	5.53/33.02	5.54/34.33	21	6	16	Ccp84Ag CG2342-PA (Ccp84Ag)	gil110764443	−1.1	0.000	2, 4
29	5.71/34.04	5.54/34.33	18	4	38	Ccp84Ag CG2342-PA (Ccp84Ag)	gil110764443	−1.1	0.023	2, 4
30	5.86/34.00	5.54/34.33	18	4	38	Ccp84Ag CG2342-PA (Ccp84Ag)	gil110764443	−1.1	0.002	2, 4
32	5.09/33.42	4.73/48.17	14	02	55	TPA: TPA_exp: troponin T isoform 2 (up), CG7107	gil78101798	−1.4	0.002	1, 2, 3,
33	5.17/33.60	4.73/48.17	13	01	43	TPA: TPA_exp: troponin T isoform 2 (up), CG7107	gil78101798	1.9	0.009	1, 2, 3,
37	5.37/31.54	4.70/29.24	27	7	86	tropomyosin 1 CG4898-PA, isoform A(Tm1)	gil66500390	−1.0	0.000	1, 2, 3
42	4.78/22.50	4.78/23.50	26	13	216	myosin regulatory light chain 2 (Mlc2), CG2184	gil66555437	1.5	0.000	1, 2
						development/growth factors				
17	5.31/59.88	4.72/58.49	25	12	108	chromatin assembly factor 1 subunit CG4236-PA, (Caf1)	gil66534191	2.3	0.005	2, 3
21	7.17/50.52	6.05/67.33	32	17	121	lamin CG6944-PA, partial (Lam),	gil66530257	1.1	0.000	6 and (26)
25	9.04/37.16	9.04/37.17	24	10	31	toutatis CG10897-PA, isoform A (tou)	gil110750089	2.4	0.042	6
						carbohydrate and energy metabolism				
41	5.58/25.53	5.00/23.23	28	07	33	ATP synthase D chain (ATPsyn-d), CG6030	gil48098315	1.5	0.004	4 and (26)
18	6.04/62.05	5.41/55.39	12	5	49	vacuolar H^+-ATPase 55kD B subunit CG17369-PB, isoform B, (Vha55) EC 3.6.3.14	gil66531434	10		2, 3
						nucleic-acid-binding proteins				
6	6.48/126.06	8.26/114.45	21	14	123	DNA topoisomerase I (CG6146), Top1 EC = 5.99.1.2	gil110760942	3.3	0.002	6
34	7.56/32.60	10.02/32.34	16	04	21	fibrillarin CG9888-PA (81)	gil66504777	1.2	0.000	3
35	5.85/32.41	10.02/32.34	17	05	60	fibrillarin CG9888-PA (81)	gil66504777	−1.4	0.001	3
						protein biosynthesis				
38	5.39/30.35	4.65/26.60	18	03	51	eIF6 CG17611-PA isoform 1 (eIF6)	gil48094369	1.5	0.002	
7	6.00/73.65	7.68/64.27	18	5	41	no on or off transient A CG4211-PB, isoform B (nonA)	gil110750119	3.5	0.000	6
8	6.91/78.55	7.68/64.27	33	22	93	no on or off transient A CG4211-PB, isoform B (nonA)	gil110750119	4.7	0.000	6
9	5.92/45.43	11.45/39.50	16	14	438	B52 CG10851-PB, isoform B (B52)	gil110761019	2.2	0.010	6
14	6.84/76.47	7.68/64.27	18	5	41	no on or off transient A CG4211-PB, isoform B (nonA)	gil110750119	1.1	0.045	6
36	6.17/31.94	9.88/28.47	35	10	103	SF2 CG6987-PA (SF2)	gil66548276	2.1	0.010	6
						protein folding				
43	5.43/22.75	5.52/14.72	22	04	54	CG3884-PB, isoform B	gil110755727	−1.6	0.000	
16	5.03/56.42	4.45/47.49	60	32	359	calreticulin CG9429-PA isoform 1 (Crc)	gil66545506	1.1	0.016	4

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Table 1. continued

spot no.	experimental pI/M_r (kDa)	theoretical pI/M_r (kDa)	sequence coverage (%)	matched	score	protein name	accession number	log ratio (1.5) (+ or −)	*p*-value	subcellular predictor (s)
						protein folding				
22	5.67/45.63	5.12/47.68	15	10	92	CaBP1 CG5809-PA isoform 1, EC = 5.3.4.1 (CaBP1)	gil66551889	10		4
10	5.68/36.73	4.74/24.44	22	4	57	protein disulfide-isomerase precursor (PDI), partial, CG6988	gil110768510	4.1	0.003	(26, 56)
11	5.64/27.68	4.74/24.44	52	21	170	protein disulfide-isomerase precursor (PDI), partial, CG6989	gil110768510	1.0	0.000	(26)
13	6.09/68.05	5.57/56.22	26	14	108	ERp60 CG8983-PA, isoform A isoform 2, EC 5.3.4.1	gil66546657	10	0.000	(26)
40	5.12/27.18	4.55/17.00	55	10	147	Fkbp13 CG9847-PA, isoform A, (Fkbp13)	gil66510528	−1.7	0.000	3,4
						unknown				
31	5.70/32.77	7.6/29.32	10	02	40	CG1240-PA	gil66525165	10		6

limitation of differential centrifugation enrichment processes.[33] To show the changing nuclear proteome profiles that were identified in both caste intended larvae, we included all the nuclear enriched proteins in the data analysis. However we gave due emphases only to those proteins that have nuclear functions and provided their functional interpretations.

3.2. Qualitative Comparisons of Differentially Expressed Proteins

The qualitative comparisons mainly based on the number of differentially expressed nuclear proteins indicated significant differences between the two caste intended larvae. The queen intended larvae expressed 27 (69%), 42 (84%), and 22 (69%) of the total differentially regulated proteins in both larvae at the third, fourth and fifth instars, respectively. Furthermore, there were considerable variations between the two caste larvae in terms of the amounts of up-regulated nuclear proteins across each functional category. To this fact, the third instar queen larvae exclusively up-regulated proteins with nuclear functions like development/growth factors and large proportions (almost all) of nucleic-acid-binding (Figure 2). Also, the fourth instar queen larvae up-regulated almost all (16 out of 19) of the cytoskeletal proteins. Likewise, the fifth instar queen larvae overexpressed all the proteins in which the cellular function indicates a nuclear function (Figure 2).

3.3. Quantitative Comparisons of Differentially Expressed Proteins

Also, to deeply understand the biological significance of each protein, the expression level of each protein in each of the two caste intended larvae was compared using the ANOVA log ratio ($|\log_{1.5}$ ratio$| \geq 1$). Only proteins with ≥1.5 fold changes and *p*-values ≤0.05, which was calculated as protein abundance ratio (queen/worker larvae), were considered (Tables 1−3). According to the results, all proteins that were up-regulated in both caste larvae showed stronger expressions (fold change >2) (Figure 3), which was more than the minimum threshold (1.5 fold change). Once again, this quantitative comparison established existences of great variations among the two caste intended larvae in the levels of protein expressed in each of them. As shown in the Figure 3A, the third instar queen larvae expressed most of the proteins with high expression levels (>2 fold change). In particular, cytoskeletal proteins as "up" (spot 12), Tm1 (spot 26) and Tm2 (spot 27), development related proteins as Caf1 (spot 17) and two (spot 25), nucleic-acid-binding proteins as Top1 (spot 6), were represented with stronger expression levels in the queen intended larvae. Whereas, only Mhc (spot 4) showed a stronger expression level (>4 fold changes) in the worker larvae of a same age (Figure 3A).

Similarly, the fourth instar queen larvae expressed large majorities of the proteins uniquely and with strong expression levels (Figure 3B). More specifically, proteins with strong expression levels included cytoskeletal, Mhc (spot 4), "up" (spots 14, 39), Cpr64Aa (spots 19, 27, 28), Act88F (spots 30, 31), Tm1 (spots 10, 33), Tm2 (spot 34), and Ccp84Ag (spots 35, 37); nucleic-acid-binding proteins, Top1 (spot 6) and SF2 (spot 41) and all the development related proteins expressed exclusively (Figure 3B). Whereas its counterpart fourth instar worker larvae strongly upregulated (>2 fold changes), only cytoskeletal proteins such as futsch (spot 2) and prm (spot 3) (Figure 3B).

Also the fifth instar queen larvae expressed large proportions of cytoskeletal proteins with strong expression levels (Figure 3C). Interestingly, the fifth instar queen larvae overexpressed all the development/growth factor proteins as annIX (spot 19) and l(2)37Cc (spot 23). Whereas the fifth instar worker larvae overexpressed only one cytoskeletal protein (Tm1, spot 17) (Figure 3C).

3.4. Network Analysis of Enriched Nuclear Proteins

Proteins function jointly in a living cell through networks by forming protein−protein interactions (PPI), modifications and regulation of expression relationships.[34] Therefore, only 23 proteins which have established nuclear functions been selected and their PPI mapping was constructed using STRING. The result showed two distinct PPI clusters with seven others operating without connectivity (Figure 4). The first cluster primarily consisted of cytoskeletal proteins that included Tm1, Tm2, Mlc2, Prm, Act87E, Mlc1, Up, Act88F, and Mhc. While, the second cluster mainly consisted proteins involved in regulation of the development and this included (l(2)37Cc, Lam, Top1, 1alpha48D (FBpp0087143), futsch, and Fib (Figure 4). Proteins connected in the network served as hub proteins and probably played central roles in the process of caste decision.

3.5. Verification of Differentially Expressed Proteins

Because of the apparent protein differential expressions between the two caste larvae at the third and fourth instars,

Table 2. Identification of Differentially Expressed Nuclear Proteins during Honeybee (*Apis mellifera* L) Caste Determination of Queen and Worker Larvae at 96 h (day 4)

spot no.	experimental pI/M_r (kDa)	theoretical pI/M_r (kDa)	sequence coverage (%)	matched	score	protein name	accession no.	log ratio (1.5) (+ or −)	*p*-value	subcellular predictor
						cytoskeleton				
1	5.59/119.76	5.33/102.051	66	77	1266	paramyosin CG5939-PA, isoform A (Prm)	gil66510482	1.8	0.003	1, 2, 3
2	7.14/179.55	4.93/170.54	25	29	317	futsch CG3064-PB (futsch)	gil110758074	−5.56	0.003	6
3	5.40/173.39	5.33/102.05	38	40	368	paramyosin CG5939-PA, isoform A	gil66510482	−1.98	0.007	1, 2, 3
4	5.82/232.63	5.78/225.64	11	22	288	myosin heavy chain CG17927-PB, isoform B isoform 1 (Mhc)	gil110759191	10	0.000	1, 2
14	5.00/56.71	4.73/48.18	44	17	220	TPA: TPA_exp: troponin T isoform 2, CG7107 (TnT)	gil78101798	10	0.000	4
19	7.18/24.12	6.82/21.56	12	2	47	cuticular protein 64Aa, CG15006-PA (Cpr64Aa)	gil110764439	1.1	0.001	4
27	7.10/31.56	6.82/21.56	24	14	59	cuticular protein 64Aa, CG15006-PA (Cpr64Aa)	gil110764439	10	0.000	4
28	7.29/34.40	6.82/21.56	24	14	97	cuticular protein 64Aa, CG15006-PA (Cpr64Aa)	gil110764439	10	0.000	4
30	6.19/39.01	5.56/41.74	11	13	25	actin 88F CG5178-PA, Act88F	gil66509789	−1.58	0.011	2
31	6.28/38.09	5.56/41.74	39	15	157	actin 88F CG5178-PA, Act88F	gil66509789	10	0.000	2
33	5.19/33.32	4.74/33.00	44	17	235	tropomyosin 1 CG4898-PD, isoform D isoform 1(Tm1)	gil48094441	4.23	0.043	1, 2, 3
34	4.73/32.25	4.73/32.30	32	9	240	tropomyosin 2 CG4843-PB, isoform B (Tm2)	gil66522386	3.4	0.018	1, 2, 3
35	5.46/32.67	5.54/34.30	21	16	16	Ccp84Ag CG2342-PA (Ccp84Ag)	gil110764443	3.99	0.000	2, 4
36	5.71/32.29	5.54/34.30	18	11	38	Ccp84Ag CG2342-PA (Ccp84Ag)	gil110764443	1.5	0.032	2, 4
37	5.9/32.35	5.54/34.30	18	14	38	Ccp84Ag CG2342-PA (Ccp84Ag)	gil110764443	2.93	0.000	2, 4
39	4.61/39.94	4.73/48.18	4	12	55	TPA: TPA_exp: troponin T isoform 2, CG7107 (up)	gil78101798	6.35	0.010	4
42	4.69/31.49	4.7/29.24	27	7	86	tropomyosin 1 CG4898-PA, isoform A (Tm1)	gil66500390	10	0.000	1, 2, 3
48	4.43/28.10	4.78/23.50	26	13	216	myosin regulatory light chain 2 (Mlc2), CG2184	gil66555437	1.2	0.001	1, 2, 3
50	4.45/27.43	4.33/19.57	29	05	106	myosin alkali light chain 1 CG5596-PA, isoform A isoform 4 (Mlc1)	gil110768236	1.75	0.022	2, 3
						carbohydrate and energy metabolism				
11	7.32/88.15	6.58/70.80	12	18	49	CG9518-PA	gil110749126	3.9	0.009	2
22	6.05/47.60	5.41/55.394	12	15	49	vacuolar H^+-ATPase 55kD B subunit CG17369-PB, isoform B, (Vha55) EC 3.6.3.14	gil66531434	10	0.000	3
23	5.64/45.19	5.25/55.10	71	57	1828	ATP synthase- CG11154-PA, isoform A (ATPsyn-β)	gil110762902	−2	0.028	4
29	7.24/38.08	8.51/46.83	53	35	344	mushroom bodies tiny (mbt), CG18582	gil66499429	1.7	0.041	6
43	7.08/30.76	6.46/27.74	42	10	39	CG7834-PA, isoform A	gil66520256	2.65	0.036	2
47	5.41/29.59	5.00/20.30	28	07	33	ATP synthase D chain (ATPsyn-d), CG6030	gil48098315	2.26	0.004	4 and (26)
						protein folding				
5	5.73/122.87	5.29/72.90	38	33	396	heat shock protein cognate 3 (Hsc70−3), CG4147	gil229892214	1.2	0.020	2
9	6.19/110.89	6.38/75.64	17	11	43	heat shock protein cognate 5 (Hsc70−5), CG8542	gil229892203	2.1	0.008	2
10	6.28/89.62	6.38/75.64	46	51	871	heat shock protein cognate 5 (Hsc70−5), CG8542	gil229892203	−2.52	0.001	2
15	5.56/62.94	4.74/24.44	22	4	57	protein disulfide-isomerase precursor (PDI), partial, CG6988	gil110768510	1.26	0.020	(26), (56)
16	5.57/34.54	4.74/24.44	52	21	170	protein disulfide-isomerase precursor (PDI), partial, CG6989	gil110768510	−1.21	0.014	(26), (56)
17	6.20/61.80	5.57/56.22	26	14	108	ERp60 CG8983-PA, isoform A isoform 2, EC 5.3.4.1	gil66546657	1.39	0.014	(26)
20	4.45/47.48	4.45/47.49	60	32	359	calreticulin CG9429-PA isoform 1 (Crc)	gil66545506	1.5	0.003	4
45	4.59/29.86	4.55/17.00	55	10	147	Fkbp13 CG9847-PA, isoform A, (Fkbp13)	gil66510528	1.55	0.031	2,3,4
26	5.58/43.00	5.12/47.68	15	10	92	CaBP1 CG5809-PA isoform 1 (CaBP1)	gil66551889	4.33	0.071	4
						development				
18	7.43/40.07	4.85/43.48	11	13	35	cadherin-N CG7100-PL, isoform L (CadN)	gil110764123	10	0.000	1, 2, 4
21	4.72/47.33	4.72/48.49	25	12	108	chromatin assembly factor 1 subunit CG4236-PA	gil66534191	10	0.000	2, 3

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Table 2. continued

spot no.	experimental pI/M_r (kDa)	theoretical pI/M_r (kDa)	sequence coverage (%)	matched	score	protein name	accession no.	log ratio (1.5) (+ or −)	*p*-value	subcellular predictor
						development				
24	6.62/43.87	8.87/50.54	6	3	60	EF-1-alpha (EF-1-alpha), CG8280	gil7915	10	0.000	2
25	6.87/54.61	6.05/67.33	32	17	121	lamin CG6944-PA, partial (Lam)	gil66530257	10	0.000	6
32	9.59/32.15	9.04/37.17	24	10	31	toutatis CG10897-PA, isoform A (tou)	gil110750089	10	0.000	6
						protein biosynthesis				
8	6.04/69.62	7.68/64.27	18	5	41	no on or off transient A CG4211-PB, isoform B (nonA)	gil110750119	1.81	0.001	6
12	6.88/70.50	7.68/64.27	33	22	93	no on or off transient A CG4211-PB, isoform B (nonA)	gil110750119	10	0.000	6
13	5.92/45.46	11.45/39.50	16	14	438	B52 CG10851-PB, isoform B (B52)	gil110761019	−2.48	0.005	6
44	4.50/30.00	4.65/26.60	18	13	51	eIF6 CG17611-PA isoform 1(eIF6)	gil48094369	2.56	0.003	2, 3,
41	6.22/31.76	9.88/28.47	35	10	103	SF2 CG6987-PA (SF2)	gil66548276	2.76	0.009	6
						nucleic-acid-binding proteins				
6	6.33/121.69	8.26/114.45	21	14	123	DNA topoisomerase I (CG6146), (Top1), EC 5.99.1.2	gil110760942	10	0.000	all
40	5.88/31.81	10.02/32.30	17	15	60	fibrillarin CG9888-PA (81)	gil66504777	−1.2	0.043	4
						antioxidant				
46	6.08/29.93	5.65/21.94	31	05	78	thioredoxin peroxidase 1 CG1633-PA, isoform A isoform 1 (Jafrac1)	gil66548188	4.46	0.001	2, 3
						transporter				
7	7.02/144.56	6.43/112.18	12	11	63	hexamerin 110 (hex110)	gil155369750	1.12	0.034	1, 4
						unknown				
38	5.87/32.14	7.6/29.32	7	12	40	CG1240-PA	gil66525165	10	0.000	
49	5.02/28.23	5.52/14.72	22	04	54	CG3884-PB, isoform B	gil110755727	2.3	0.017	2

we paid much attention to proteins from these two developmental stages to further ascertain the information obtained from mass spectrometry with the biological function. Accordingly, from the 24 qRT-PCR tested proteins, 23 of them showed consistent mRNA expressions with proteome expressions (Figure 5).

DISCUSSION

To get deep insight into the mechanisms of the honeybee caste determination, we analyzed the nuclear proteome of the queen and worker intended larvae at three early developmental time points. The two larvae showed considerable variations in nuclear proteomes expressions, suggesting differences in DNA and RNA regulation functions in the nucleus. To this fact, from the total 120 differentially regulated proteins, the queen destined larvae up-regulated 91 proteins further emphasizing the marking of honeybee caste development through differential expressions of the nuclear proteins. The queen destined larvae massively up-regulated most of the proteins to enhance its high metabolic rate that could lead to rapid weight gain.[22] In particular, the third instar queen larvae overwhelmingly up-regulated (27 out of the total 39 proteins) and this clearly indicates the marking of caste trajectory before 72 h. Also, the wide variations in the nuclear proteome of both caste larvae during the subsequent analysis at the 4th and 5th instars further affirmed the fact that the two caste intended larvae are growing through different ways.

It is well-known that nuclear enriched proteins perform "RNA functions" and strongly associate with cytoskeletal protein binding, cell adhesion, and "extracellular matrix structural constituent".[35] Also, gene regulation involves the entire tissue skeleton through interconnected proteins from the skeleton in tissue to the nucleus.[36−38] To this reality, the PPI network indicated the interconnections of different proteins working jointly to effect the development of both larvae. In this study, many cytoskeletal proteins carrying out different nuclear functions have been enriched and differentially regulated in both castes' intended larvae. The third instar queen larvae involved myosin (Mhc, Mlc2, and Prm) for its nuclear phenotypic alterations to assist in the myofibril assembly; muscle cell differentiation and muscle system process, and troponin (Tm1 and Tm2) for cellular calcium ion homeostasis and muscle organ morphogenesis.[39] Moreover, the queen larvae expressed Tm1 in alignment with Tm2 is most likely as a natural strategy to regulate motor systems required to maintain nuclear integrity and apico-basal polarity as in *Drosophila*.[40] On the other hand, the worker intended larvae involve more of chitin-based larval cuticle building proteins (Cpr64Aa and Ccp84Ag) to support the development of anatomical structures.[39] Similarly, strong directional divergence of the two castes' intended larvae were manifested through considerable cytoskeletal protein differential expressions at the fourth and fifth instars. This suggests regulations in the nuclear functions is in response to nutrition and time programmed.[41−44] In particular, the fast growing queen larvae massively engage cytoskeletal proteins to involve in several nuclear activities like gene regulation, protein binding and cellular division[35] for its muscle cell differentiation and muscle system process development.

Nucleic acids are important to store and transmit genetic information and use the information to direct the synthesis of new proteins.[45] DNA is the permanent storage place for genetic information in the nucleus and controls the synthesis of RNA that transmits genetic information from DNA to the protein synthesizers in the cell.[46] It has been reported that DNA topoisomerase I (topo I) mutant ovary and embryo exhibit abnormal proliferation and irregular nuclear morphology with chromosome condensation and segregation defects in *Drsophila*.[46] Therefore, the third and fourth instar queen larvae up-regulation of an essential enzyme, topo I is most likely to

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Table 3. Identification of Differentially Expressed Nuclear Proteins during Honeybee (*Apis mellifera* L) Caste Determination of Queen and Worker Larvae at 120 h (day 5)

spot no.	experimental pI/M_r (kDa)	theoretical pI/M_r (kDa)	sequence coverage (%)	matched	score	protein name	accession no.	log ratio (1.5) (+ or −)	*p*-value	subcellular predictor
						cytoskeleton				
1	5.78/224.70	5.78/224.70	29	93	2767	myosin heavy chain CG17927-PB, isoform B isoform 1, Mhc	gil110759191	2.45	0.000	1, 2
2	5.56/112.94	5.33/102.00	17	13	368	paramyosin CG5939-PA, isoform A, Prm	gil66510482	4.15	0.000	1, 2
3	5.82/109.36	5.33/102.00	55	99	2326	paramyosin CG5939-PA, isoform A, Prm	gil66510482	1.115	0.000	1, 2
5	6.58/98.77	5.33/102.00	24	18	437	paramyosin CG5939-PA, isoform A, Prm	gil66510482	2	0.004	1, 2
15	5.94/42.17	5.3/42.00	66	71	1057	actin-87E isoform 1,1,CG18290 (Act87E)	gil66509780	4.06	0.000	(57), (58)
16	5.81/40.63	5.3/42.00	60	46	927	actin-87E isoform 1,CG18290 (Act87E)	gil66509780	1.58	0.005	(57), (58)
21	4.94/33.73	4.78/36.09	40	12	183	myosin regulatory light chain 2 (MLC-2), CG2184	gil66555437	2.4	0.040	3
22	5.08/33.55	4.78/23.50	36	18	270	myosin regulatory light chain 2 (MLC-2), CG2184	gil66555437	1.91	0.004	3
27	4.30/19.34	4.78/23.50	26	13	216	myosin regulatory light chain 2 (MLC-2), CG 2184	gil66555437	1.2	0.000	3
28	4.33/19.30	4.33/19.30	28	7	243	myosin alkali light chain 1 CG5596-PA, isoform A isoform 4	gil110768236	5.07	0.000	4
17	5.37/38.47	4.74/33.00	41	35	769	tropomyosin 1 CG4898-PD, isoform D isoform 1 (Tm1)	gil48094441	−2.01	0.000	3
18	4.97/37.70	4.73/32.00	48	28	762	tropomyosin 2 CG4843-PB, isoform B (Tm2)	gil66522386	−1.45	0.009	3, uniprot
20	4.65/35.98	4.73/48.00	13	12	658	troponin T isoform 2, CG7107 (up)	gil78101798	−1.45	0.043	uniprot
						protein folding				
6	5.80/84.53	5.29/72.80	34	34	992	heat shock protein cognate 3 (Hsc70−3), CG4147	gil229892214	4.62	0.002	(58), (59)
7	5.99/88.21	5.29/72.80	44	66	1340	heat shock protein cognate 3(Hsc70−3), CG4147	gil229892214	−1.16	0.040	(58), (59)
8	6.11/86.06	5.29/72.80	23	13	367	heat shock protein cognate 3(Hsc70−3), CG4147	gil229892214	−5.33	0.020	(58), (59)
9	5.89/54.59	5.64/60.40	53	53	1178	60 kDa heat shock protein (HSP-60), CG12101	gil66547450	−1.05	0.006	(58), (59)
10	5.97/49.97	5.57/55.80	38	22	696	ERp60 CG8983-PA, isoform A isoform 2, (ERp60)	gil66546657	−10	0.000	(56)
11	4.45/47.00	4.45/47.00	42	25	555	calreticulin CG9429-PA isoform 1 (Crc)	gil66545506	−3.41	0.000	(56)
12	5.30/46.78	5.27/30.00	32	18	505	protein disulfide-isomerase precursor (PDI), partial, CG6988, EC 5.3.4.1	gil66531851	3.7	0.031	(56)
13	5.27/46.08	5.27/30.00	39	30	640	protein disulfide-isomerase precursor (PDI), partial, CG6988, EC 5.3.4.1	gil66531851	2.79	0.000	(56)
25	4.76/23.62	4.55/17.00	55	10	147	Fkbp13 CG9847-PA, isoform A (Fkbp13)	gil66510528	2.35	0.000	3,4
						carbohydrate and energy metabolism				
14	5.75/44.03	5.25/55.00	58	58	1399	ATP synthase- CG11154-PA, isoform A, (ATPsyn-β)EC 3.6.3.14	gil110762902	3.82	0.010	(56)
32	5.93/30.62	5.00/20.30	53		96	ATP synthase, subunit D CG6030, (ATPsyn-d)	gil48098315	10	0.000	4
						development				
19	4.65/36.54	4.60/36.00	31	32	95	annexin IX CG5730-PC, isoform C isoform 2 (AnnIX)	gil66530527	10	0.000	(60)
23	6.29/34.39	6.54/30.00	45	19	154	lethal (2) 37Cc CG10691-PA, isoform A (l(2)37Cc)	gil48097857	10	0.000	2, 3
						transporter				
4	6.51/126.14	6.43/112.00	34	68	152	hexamerin 110 (hex110)	gil156637469	−1.82	0.000	1, 2
29	6.45/102.70	6.43/112.00	22	32	647	hexamerin 110 (hex110)	gil155369750	10	0.000	1, 2
30	6.59/132.46	6.43/112.00	26	32	818	hexamerin 110 (hex110)	gil156637469	−2.19	0.001	1, 2
31	6.53/95.34	6.43/112.00	20	20	372	hexamerin 110 (hex110)	gil155369750	10	0.000	1, 2
						antioxidants				
24	5.81/31.74	5.24/27.00	23	6	146	short-chain dehydrogenase/reductase (sro), CG12068, EC 1.1.1.30	gil58585184	1.14	0.023	1, 2, 3
						unknown				
26	5.13/22.35	5.52/14.40	36	8	184	CG3884-PB, isoform B	gil110755727	5.258	0.000	2, 3

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Table 3. continued

Spot numbers corresponds to the number of the protein spots in Figure 1. The theoretical molecular weight (*Mr*) and isoelectric point (pI) of the identified proteins were retrieved from the protein database of NCBInr limited to *apis mellifera*. Experimental M_r and pI values were calculated with PDQuest 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 paring an experimental fragmentation spectrum to a theoretical segment of protein, and searched is the total searched peptide. The Mascot score was searched against the *A. mellifera* database. Protein name is given when proteins were identified by LC-Chip/ESI-QTOF-MS. The taxonomy is *A. mellifera*. Accession number is the unique number given to mark the entry of a protein in the NCBInr database. The ratios (*R*) of the protein abundance (queen/worker larvae) were transformed and a protein with $|\log_{1.5}$ ratio$| \geq 1$ and a *p*-value ≤ 0.05 was considered as differentially expressed proteins. Positive (+) log ratios indicate proteins upregulated in the queen and the negative (−) by the worker larvae. The log ratios of uniquely expressed proteins in each bee caste larvae were limited to 10. Subcellular Predictor(s) is to indicate the predictor(s) and/or the literature sources used in ascertaining the identified protein is fully or partly localized in the nuclear subcellular organelle with the following keys: 1 = NucPred, 2 = PSORT II, 3 = WoLF PSORT, 4 = subLoc, 5 = FlyBase, 6 = by all the predictors (1−5) and numbers in the bracket stands for the number of predictors listed in the reference part.

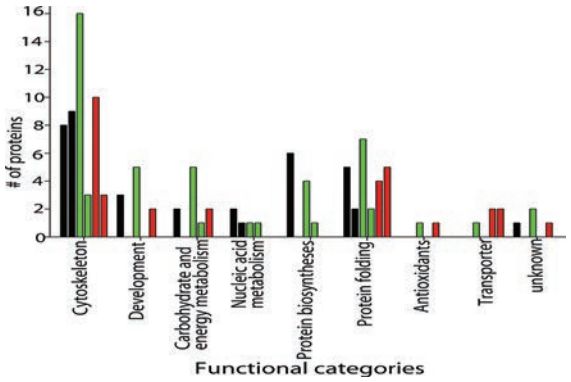

Figure 2. Comparison of up-regulated protein numbers of queen and worker intended larvae at 72 (3rd instar), 96 (4th instar), and 120 (5th instar) hours, where the black, green and red color codes stand for the 3rd, 4th, and 5th instar larvae, respectively. And the 1st bar of similar color code from each functional category represents value for the "queen" and the 2nd for the "worker" larvae.

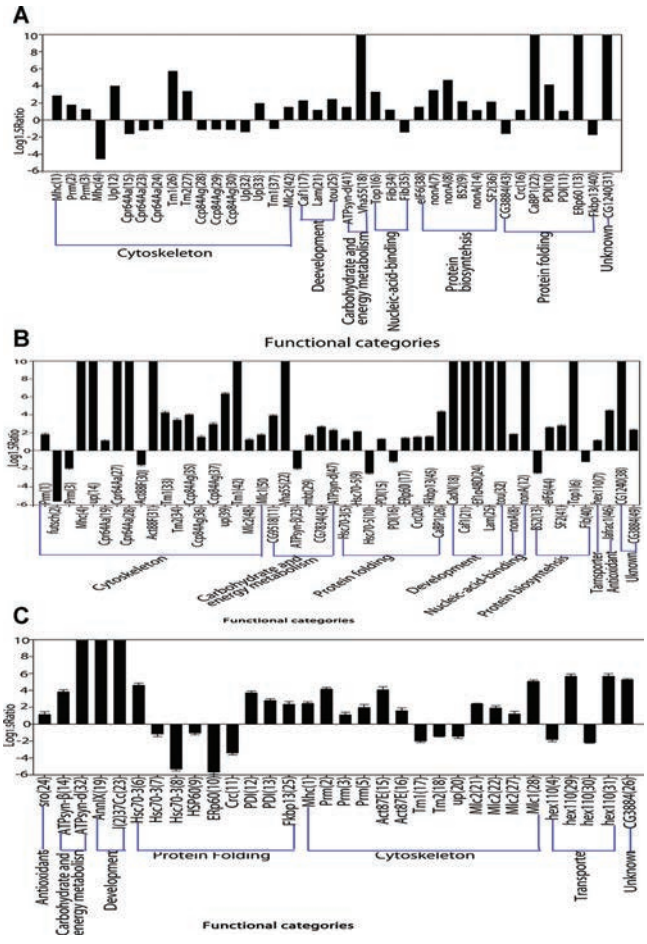

Figure 3. Quantitative comparisons of differentially expressed nuclear proteins in the honeybee queen and worker prospective larvae. The ratios of the protein abundance (queen to worker larvae) were transformed, and the protein spots with $|\log 1.5$ ratio$| \geq 1$ and $p \leq 0.05$ were selected as the differentially expressed proteins. A, B and C show the level of differentially expressed proteins at 72 (3rd instar), 96 (4th instar) and 120 (5th instar) hours, respectively. Protein names (in abbreviations) and protein numbers are listed as in the Table 1. Positive and negative values indicate the queen and the worker larva expressed proteins, respectively. The $|\log 1.5$ ratio$|$ of the uniquely expressed proteins is limited to 10.

involve in the processes of replication, transcription, recombination and cell proliferation in imaginal disk tissue development as well as to maintain its nuclear structure.[46] However, its down-regulations in the third and fourth instar worker larvae suggest its strong obedience to the fertile ovary than to the steps in the metamorphic degradation of ovarioles in worker-bee ovaries. On the other hand, fibrillarin (fib), an important nucleolar protein that maintains nuclear shape and cellular growth,[47] up-regulated in the third instar of both castes suggests task similarities in both growing larvae in supporting nuclear shape and cell growth. Nonetheless, the biological significance of its down-regulation in the fourth instar queen and its up-regulation in the fourth instar worker larvae require separate investigations.

Growth factors or development regulating proteins are important to guarantee the development of an organism through genetic differential expression. In this study, the queen destined larvae up-regulated entirely the identified development proteins at all the developmental stages. This is most likely to enhance the genetic differential expression[48] and to enhance its fast growth for the later phenotype distinction. This queen larvae exclusively expressions of all of the development related proteins is despite the existing record for the equal expression with worker larvae, which was analyzed at the third and fifth instars using the global protein differential expression.[21] On the other hand, this signifies the unambiguous approach of the subcellular method in providing precise information through localizing to specific organelles or subcells. Also, the third and fourth instars queen larvae up-regulated nuclear lamina (lam)

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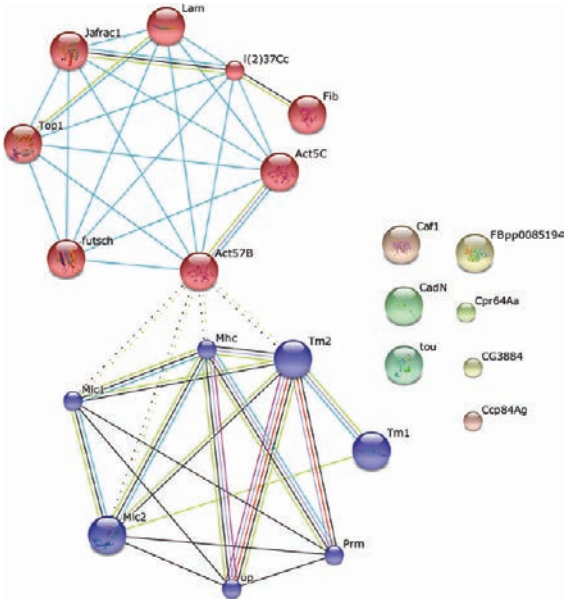

Figure 4. Protein−protein interaction networks of 23 protein species with known nuclear functions during the caste development in honeybee larvae that are constructed by the online database resource, Search Tool for the Retrieval of Interacting Genes (STRING). Lines represent the existence of the different types of evidence used in predicting the associations. A red line indicates the presence of fusion evidence; a green line, neighborhood evidence; a blue line, co-occurrence evidence; a purple line, experimental evidence; a yellow line, text mining evidence; a light blue line, database evidence; a black line, coexpression evidence.

that acts as a transcriptional repressor when targeted to promoters for its efficient genome organization and gene expression during cellular differentiation.[48] Moreover, the third and fourth instars queen larvae up-regulated caf1 in association with lam are to attain its desired phenotypic markings through their interactive effects in regulating gene expression and cellular differentiation.[49,50] Furthermore, to initiatiate the development of wing disk and nervous systems,[51] the third and fourth instar queen larvae up-regulated tou, a multiprotein complex in chromatin remodeling that activates gene expression.[51] On the other hand, for neuron differentiation and sensory organ development,[39] the fourth instar queen larvae up-regulated nuclear protein CadN that commands DNA toward synthesizing a protein of demand. Also, to support its high metabolic rate and rapid growth though increased nuclear activities, the fourth instar queen larvae up-regulated Ef1α48D that boosts the nuclear translation during new protein biosyntheses. However, in order to control cell apoptosis, one of the problems in a fast growing situation, the fifth instar queen larvae overexpressed annIX that manages programmed cell death timing.[52−54] Furthermore, to regulate DNA replication[55] during the process of metabolism or progressions into a pupae, the fifth instar queen larvae up-regulated l(2) 37Cc. Generally, the queen intended larvae overexpression of growth factor proteins is possibly due to the various parts of the protein networks associated with the formations and regulation of the honeybee queen phenotype like differential size and fertility.

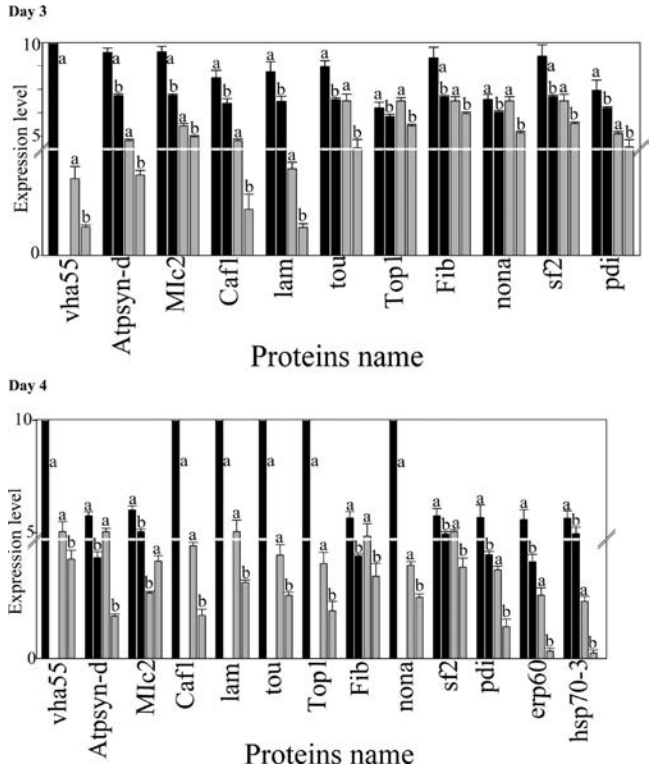

Figure 5. qRT-PCR analysis of the differentially expressed proteins of the queen and the worker intended larvae at 72 (3rd instar) and 96 (4th instar) hours . The mRNA expression is measured by qRT-PCR and normalized with reference gene (GAPDH). The black and the gray bars designate protein and mRNA expression, respectively. The 1st and the 2nd bars in each of the black and the gray bars represent the queen and the worker larvae, respectively, where "a" is significant higher than "b". Error bar is standard deviation. Abbreviated protein names indicate different proteins as in Table 1 and 2. The |log1.5 ratio| of the uniquely expressed proteins are limited to 10.

5. CONCLUSION

The caste decision regulations of the honeybee queen and worker intended larvae have been analyzed through their nuclear proteome differential expressions at three developmental stages (third, fourth and fifth instars). The identified proteins are known for enhancing genetic differential expression through which queen and worker features can be ensured. There are significant variations between the two caste larvae in their nuclear proteome at all the developmental stages, with the queen intended larvae up-regulating significantly large proportions. More specifically, the queen larvae considerably up-regulate nuclear proteins involved in DNA and RNA regulations, such as cytoskeleton, development, and nucleic-acid-binding. Interestingly, the third instar proteome data allows us to recognize the two caste intended larvae on different pathways as early as before 72 h. Therefore, this nuclear proteome data helps us to decipher the mechanism of caste decision during the development of the two caste larvae at the subcellular level and significantly advanced our system's level of understanding on the reactions of nuclear proteomes in responses to the process of caste determinations. The constructed PPI network and the tested results (qRT-PCR) between expression of proteins and genes provide potential target proteins or genes to be selected for further functional analysis, such as gene knock down and RNA interference.

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■ ASSOCIATED CONTENT

Supporting Information

Table S1 is primer sequences used for qRT-PCR analysis of the differentially expressed nuclear proteins during the honeybee (*Apis mellifera* L.) larva caste differentiation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Conceived and designed the experiments: LJ. Performed the experiments, analyzed the data: DB. Wrote the paper: DB and LJ. Contributed reagents/materials/analysis tools: YF and HB. Collected larvae sample: MF.

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