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Proteome Comparison of Hypopharyngeal Gland Development between Italian and Royal Jelly Producing Worker Honeybees (*Apis mellifera* L.)

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The hypopharyngeal gland (HG) of the honeybee (*Apis mellifera* L.) produces royal jelly (RJ) that is essential to feed and raise broods and queens. A strain of bees (high royal jelly producing bee, RJb) has been selected for its high RJ production, but the mechanisms of its higher yield are not understood. In this study, we compared HG acini size, RJ production, and protein differential expressions between the RJb and nonselected honeybee (Italian bee, ITb) using proteomics in combination with an electron microscopy, Western blot, and quantitative real-time PCR (qRT-PCR). Generally, the HG of both bees showed age-dependent changes in acini sizes and protein expression as worker behaviors changed from brood nursing to nectar ripening, foraging, and storage activities. The electron microscopic analysis revealed that the HG acini diameter of the RJb strain was large and produced 5 times more RJ than the ITb, demonstrating a positive correlation between the yield and HG acini size. In addition, the proteomic analysis showed that RJb significantly upregulated a large group of proteins involved in carbohydrate metabolism and energy production, those involved in protein biosynthesis, development, amino acid metabolism, nucleotide and fatty acid, transporter, protein folding, cytoskeleton, and antioxidation, which coincides with the fact that the HGs of the RJb strain produce more RJ than the ITb strain that is owing to selection pressure. We also observed age-dependent major royal jelly proteins (MRJPs) changing both in form and expressional intensity concurrent with task-switching. In addition to MRJPs, the RJb overexpressed proteins such as enolase and transitional endoplasmic reticulum ATPase, protein biosynthesis, and development proteins compared to the ITb strain to support its large HG growth and RJ secretion. Because of selection pressure, RJb pursued a different strategy of increased RJ production by involving additional proteins compared to its original counterpart ITb. To our knowledge, this morphological and proteomic comparison study on the HG of the two strains of worker honeybees associated with their age-dependent division of labor is the first of its kind. The study provided not only the quantity and quality differences in the HG from the RJb and the ITb, but also addressed the cellular and behavioral biology development question of how the RJb strain can produce RJ more efficiently than its wild type strain (ITb).

Keywords: honeybee • hypopharyngeal gland • royal jelly • acini • proteomics

1. Introduction

Royal jelly (RJ), secreted from the hypopharyngeal gland (HG) in the heads of worker bees, is used for the rearing of larvae of all three castes, the adult queens, and adult workers.[1−3] The queen and worker caste differentiation is controlled epigenetically by differential feeding with RJ.[4,5] The HG of the worker honeybee was studied extensively both from the standpoint of honeybee biology and RJ production perspectives. Normally, the HG develops with two distinctly differential phases in worker bees. Initially, it produces RJ for brood food and then when worker bees start foraging, its function shifts to the production of enzymes involved in converting sucrose into its simple sugars.[2,6] Hence, the HG of the nurse bees expresses proteins associated with major royal jelly proteins (MRJPs) and proteins such as carbohydrate metabolism and energy production, while the HG of foragers expresses enzymes such as α-glucosidase, glucose oxidase, galactosidase, esterase, lipase, leucine arilamidase, and invertase.[7−10] The HG of the bees also goes through morphological and physiological changes.[11] Morphologically, the acini of the HG are well developed around 6 days in a worker bee to nurse brood with RJ[12] and begin to shrink in size after day 15 when a bee becomes a forager.[13,14] Physiologically, there is higher activity in protein synthesis during the nursing period and lower in foraging bees, suggesting a positive correlation between its morphology and physiology.[11,15]

RJ is now becoming a globally recognized health product with various claimed health benefits.[16] Medical properties of

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RJ and its health benefits have been well documented; these include effects in antiaging, antitumor, antibacterial, antifatigue, anti-inflammation, vasodilative and hypotensive activities, disinfectant action, antioxidant activity, and antihypercholesterolemic activity.[17−20] It is also important from a cellular and behavioral biology point of view as it raises interesting questions such as how one bee strain can produce more RJ than the other as well as what makes the contents different.

To increase RJ production, China has selected a strain of honeybee, called the RJ bee (RJb) from a strain of Italian bee (*Apis mellifera* L.) (ITb). The selected RJb produces significantly more RJ than its "wild type" ITb, reaching a production of 8 kg of RJ per colony per year.[21] This has made China the largest RJ producing country, accounting for more than 90% of the 3000 tons of the world annual production.[21] Previous studies indicated that RJ productivity is influenced by quantitative genetic components of the honeybee,[22] and molecular and morphological markers associated with higher RJ production were identified in RJb.[23,24] Despite of all these studies, comparative studies between the RJb and the ITb in terms of HG acini size, protein profiles, and RJ production have not been conducted. Furthermore, the involvement of several important key node proteins in the formation of biochemical networks of the developing HG of the ITb in recent investigations by our group[25] encouraged us to conduct this comparative proteomic study of the two bee strains.

2. Materials and Methods

2.1. Honeybee Samples. Mated hybrid ITb were purchased from Bologna, Italy, and queens of the RJb were from the Pinghu honeybee breeding station, Zhejiang Province, China. The RJb stain was intentionally selected from the normal ITb strain for its high RJ production traits. The queens from both bee strains were introduced into honeybee colonies at the Institute of Apicultural Research, Chinese Academy of Agricultural Science in May 2009. Newly emerged (<12 h, $N = 800$) worker bees were marked on their thoraxes and placed back into colonies. Then, the marked worker bees were collected on days 1, 3, 6, 12, 15 and 20, anesthetized on ice, and their HG dissected using a binocular microscope (at 25× magnification, XTL-2400). A total of 800 HG samples were pooled at each time point from the two bee strains, and HG morphometric measurements were taken immediately and frozen at −80 °C before protein extraction.

2.2. RJ Collection. Ten honeybee colonies from each bee strain received similar management practices to attain comparable colony size prior to RJ collections. Each colony was provided 150 queen cell cups with larvae grafted in, and the RJ collections were performed after 72 h of larval grafting. RJ collections of 12 batches from each strain were done and weighed with a digital scale (Mettler Toledo, America, accurate to 0.1 g).

2.3. HG Morphometric Measurements. The photographing HGs of the ultrastructure of HG from the two bee strains were done using a scanning electron microscope (SEM). The HGs were fixed with 2.5% glutaraldehyde in 0.1 M, pH 7.2 PBS (phosphate buffered saline) for several hours. Samples were rinsed three times with PBS for 15 min and then treated with 1% osmium tetroxide for 2 h. They were then dehydrated completely to a critical point, placed on stubs, sputter coated with palladium−platinum, and then measured (at 350× magnification) with a Hitachi S-3400N (Japan).

2.4. Identification of Altered Proteins and Statistics. The HG proteins from the two bee strains at six specific time points were extracted and separated by two-dimensional electrophoresis (2-DE) in accordance with our previously reported method.[25] Briefly, isoelectric focusing (IEF) was performed at 18 °C (Protean IEF Cell, Bio-Rad) according to the following program: 14 h at 50 V; 250 V for 30 min × 4 times; 1000 V for 60 min; 9000 V for 5 h; 9000 V, for 60 000 V·h. Prior to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), the immobilized pH gradient (IPH) 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 later in equilibration buffer 2 (6 M urea, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% SDS, 2.5% iodoacetoamide) for 15 min. After equilibration, the strip was transferred to an 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.

The 2-DE gels were stained with Coomassie Brilliant Blue (CBB) G-250, scanned (Pharos Fx Plus, Bio-Rad) and analyzed using PDQuest software (Version 8.0, Bio-Rad). The expression levels of protein spots were determined by the relative volume of each spot to the total volume of spots in gel and expressed as %Vol [%Vol = (spot volume/total volumes of all spots resolved in the gel)]. The values from the three independently obtained gel spots were analyzed using ANOVA and means compared using Duncan's parametric test (SAS, Version 6.12, SAS Institute). Means and standard deviations are presented throughout the text and $p < 0.05$ probability was considered statistically significant.

Hence, protein spots with significant differences between the two bee strains were excised and gel pieces were denatured, alkylated, trypsin digested as described previously.[25] The digested peptides were analyzed using Ultraflex II matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonics) under the control of FlexControl 2.2 software (Bruker Daltonics). MALDI-TOF spectra were recorded in the positive ion reflector mode in a mass range from 700−4000 Da, and the ion acceleration voltage was 25 kV. Acquired mass spectra were processed using the software Flex Analysis 2.2 (Bruker Daltonics): peak detection algorithm: Sort Neaten Assign and Place (SNAP); S/N threshold: 6; quality factor threshold: 1500. Spectra were calibrated by a protonated mass signal from a standard peptide calibration mixture consisting of eight peptides covering mass range from 700 to 3100 Da with Bruker, Billerica, MA Peptide Calibration Standard 206196. Spectra originating from parallel protein digestions were compared pairwise to discard common peaks derived from trypsin autodigestion or from contamination with keratins. The resulting peptide mass lists were used to search against the nonredundant NCBI (NCBInr, release date, June 26, 2008) using MASCOT 2.3 (Matrix Science). Search parameters were Taxonomy: all entries; trypsin cleavage; allow up to one missed cleavage; peptide mass tolerance 0.2 Da; fixed modification: carbamidomethyl (C); variable modification: oxidation (M). A total of 6 649 798 sequences and 2 279 950 795 residues in the database were searched.

Additionally, proteins with lower abundance that were not identified by MALDI-TOF/MS were identified by liquid chromatography-chip/electrospray ionization-quadropole time-of-flight mass spectrometry (LC-Chip/ESI-QTOF-MS) (QTOF G6530, Agilent Technologies), equipped with a capillary pump G1382A, a nano pump G2225A, an autosampler G1377D and the Chip

Table 1. Primer Sequences Used for Quantitative Real-Time PCR of Genes Differentially Expressed during the Development of Hypopharyngeal Gland of Royal Jelly Producing Bee (RJb) and Italian Bee (ITb) (*Apis mellifera* L.)

accession number	protein name	primer sense 5′-3′ antisense 5′-3′	product size (bp)	annealing temperature (°C)
NM_001185146	actin	ATCCTGGAATCGCAGATAGAATG TGTTGGAAGGTGGACAAAGAAG	145	56.2
gil58585098	major royal jelly protein 1	GAGATACAATGGCGTACCTTC TGAGTATTATTGACAAGACCTGAG	198	53.5
gil58585108	major royal jelly protein 2	ATTACAGTCCTCTCGCTTCTCAC TCGTTCCAGCAGCCAACAG	199	57.6
gil58585142	major royal jelly protein 3	GTGGACAGATGGCGTGATAAG TCAGGATATGGTCGTAGAAGAGG	122	56.1

Cube G4240A. The LC-Chip (Agilent Technologies) used was constituted of a Zorbax 300SB-C18 enrichment column (40 nL, 5 μm) and a Zorbax 300SB-C18 analytical column (75 μm $\times$ 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: 3% to 8% B for 1 min, 8% to 40% B for 5 min, 40% to 85% B for 1 min and 85% B for 1 min. The Chip flow rate was 300 nL/min. The MS condition was as follows: positive ion mode; Vcap: 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 water with 0.1% formic acid; centrifuged for 5 min at 10000g and about 15 μL of upper solution was injected for the analysis. Tandem mass spectra were retrieved using the Mass Hunter software (Version B.02.01, Agilent Technologies). Before MS/MS data searching, peak-lists were generated by Mascot Distiller software (version 3.2.1.0, Matrix Science) by the default setting. MS/MS data were searched against Mascot 2.3 (Matrix Science) applied to NCBInr (release date, June 26, 2008). Search parameters were: 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: $\pm$ 1.2 Da, MS/MS tolerance: $\pm$ 0.6 Da. The database searched was same as the above.

When the peptides were matched to multiple members of a protein family, or a protein appeared under the same name and accession number, the match was considered in terms of higher Mascot score, the putative function, and differential patterns on 2-DE gels. Protein identifications were accepted if they had greater than 95% probability and contained at least two identified peptides, maximum peptides coverage.

2.5. Validation of Differentially Expressed Proteins. Differentially expressed proteins MRJP1, MRJP2, and MRJP3 were further validated both at mRNA level through qRT-PCR and at protein level by Western blot. qRT-PCR was run to compare gene expression of MRJP1, MRJP2, and MRJP3 at day 1, 3, 6, 12, 15, and 20 from the two bee strains. On the basis of the results of the gel-based comparisons, specific primers suited to simultaneously amplify various target genes were designed according to the corresponding gene sequences of the identified proteins and available gene information deposited in the Gene Bank library using primer (Table 1) design software (Beacon Designer 7.51, PREMIER Biosoft International Palo Alto, CA). Total RNAs were prepared from the HG at day 1, 3, 6, 12, 15, and 20 using TRIzol regent (Takara bio), and cDNA synthesis was performed using a SYBR Green Real-time PCR Core Kit (Bio-Rad) according to the manufacturer's protocol. Real-time PCR amplification was conducted on iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad). Each sample was analyzed independently and processed in triplicate. Each reaction volume was performed in a total of 25 μL containing 1 μL of cDNA template and 10 pmol of primer, 12.5 μL of SYBR Green Supermix (dNTPs, iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green, fluorescein and stabilizers, RNase-free water) (Bio-Rad), and 9.5 μL of double distilled water. The real-time PCR was performed as 1 cycle of denaturation at 95 °C/10 min, followed by 40 cycles of amplification (95 °C/30 s, 60 °C/30 s, 72 °C/45 s) where the fluorescence was automatically measured during PCR. The specificity of the amplified product was monitored using its melting curve and β-actin was chosen as a reference to normalize the data. After verifying that the amplification efficiencies of the selected genes and β-actin were approximately equal, the difference in gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.[26]

For Western blot validation, each of MRJP1, MRJP2, and MRJP3 were subjected to three replication runs and 4 μg of protein samples were loaded onto each lane separated by stacking (4%) and separating (12%) SDS-PAGE gels. To ensure that the specific anti-MRJP1, MRJP2, MRJP3 bands could be detected, protein marker was loaded when running the gels. Gels were run at 120 V for approximately 1.5 h using Mini-Protein II Gel electrophoresis system (Bio-Rad Laboratories Ltd.). Resolved proteins were transferred to a nitrocellulose (NC) transfer membrane (0.2 μm pore size) (Invitrogen) using the iBlot apparatus (Invitrogen, Carlsbad, CA). Nonspecific binding was blocked with 5% (w/v) nonfat milk powder in the Tris buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.1% (v/v) Tween-20 (TBS-T) at room temperature for 1 h. The membranes were then incubated with primary rabbit polyclonal anti-MRJP1, MRJP2, MRJP3 antibodies (developed by our laboratory by synthesizing MRJP 1-3 polypeptides from 17, 19, and 22 amino acids by checking the immunogenicity of the corresponding protein sequences. The synthetic polypeptides were conjugated to keyhole limpet hemocyanin (KLH) as antigens and then injected into rabbits. Antibody titer was checked after each injection. Finally, whole blood was harvested to separate serum as anti-MRJPs polyclonal antibody after five injections) at a dilution of 1:5000 in 2% milk powder in TBS-T at 4 °C overnight. Following three washes, the membranes were further incubated with goat antirabbit IgG conjugated with horseradish peroxidase (Pierce, Rochford, IL) (1:10 000 in 2% milk powder in TBS-T), and were rolled for 1.5 h at room temperature. At the end of this process, the NC membranes were washed for 2 h, rolled at room temperature. Immunoreactive protein bands were then visual-

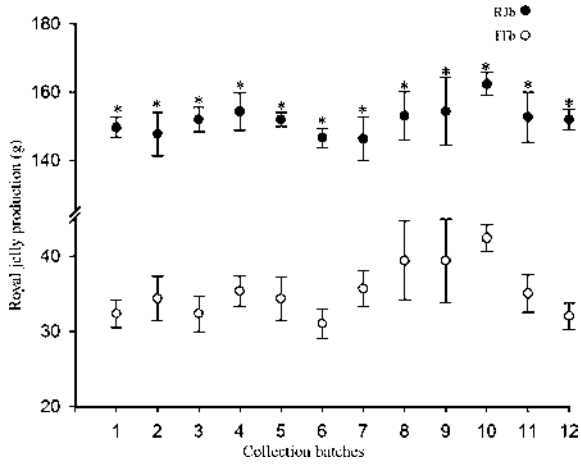


Figure 1. Royal jelly output represents as mean ± SE ($n = 12$). The "closed" and "open" circles represent the value for the RJb and ITb, respectively. Asterisks indicate the statistically significant differences between RJ yield of the two bee strains at different batches ($p < 0.05$).

ized by enhanced chemiluminescence detection (ECL, Pierce, Rochford, IL) regents and quantified by densitometry using the Quantity-one image analysis system (Bio-Rad Laboratories Ltd.). The human anti-β-actin antibody (1:5000, Sigma) was detected simultaneously as a loading control.

3. Results

3.1. Comparison of RJ Production and HG Acini Size. RJb produced significantly higher RJ (151.92 ± 1.49 g) as compared with its counterpart the ITb (35.28 ± 0.91 g) (Figure 1). Similarly, RJb possessed significantly larger acini size than the ITb and the difference is significant ($p < 0.05$), except for day 15 (Figure 2A,B). The HG acini diameters increased in both bee strains in a similar fashion until day 12 after which they decreased (Figure 2 B).

3.2. Identification of Differentially Expressed Proteins. About 400 protein spots were resolved on 2-DE images for HG samples collected day 1, 3, 5, 15, and 20 after the eclosion, and the corresponding spots were compared between the RJb and ITb strains of the same age. From these, 150 protein spots that exhibited a fold change of >1.5 with a significance level of $p < 0.05$ at each time point were chosen for identification via MS. Subsequently, out of 150 differentially expressed protein spots, 116 were successfully identified by MALDI-TOF and ESI-QTOF MS (Table 2 and Figure 3). The remaining proteins were not unidentified probably because of their low abundance to produce enough spectra, or the databases search scores were not higher than 95% to yield unambiguous results.

3.3. Qualitative and Quantitative Comparisons of Differentially Expressed Proteins. All bee strains upregulated proteins were classified into 10 functional groups showing different proportions of expressions in both bees. From the total proteins expressed in both bee strains, more than 74% were upregulated in RJb, and more interestingly, proteins as nucleotide and fatty acid metabolism, transporter and antioxidant were exclusively upregulated by the RJb (Figure 4). As further comparison of the upregulated protein number, the RJb overexpressed a considerably higher number of the upregulated proteins as MRJPs, carbohydrate metabolism and energy production, protein biosynthesis, protein folding, development, amino acid metabolism, and cytoskeleton compared to the ITb (Figure 5).

Furthermore, the two bees were compared to understand the protein expression profile differences. Consequently, from the total 56 upregulated protein spots on day 1, 44 of them were expressed by the RJb and 12 by the ITb. Among the 44

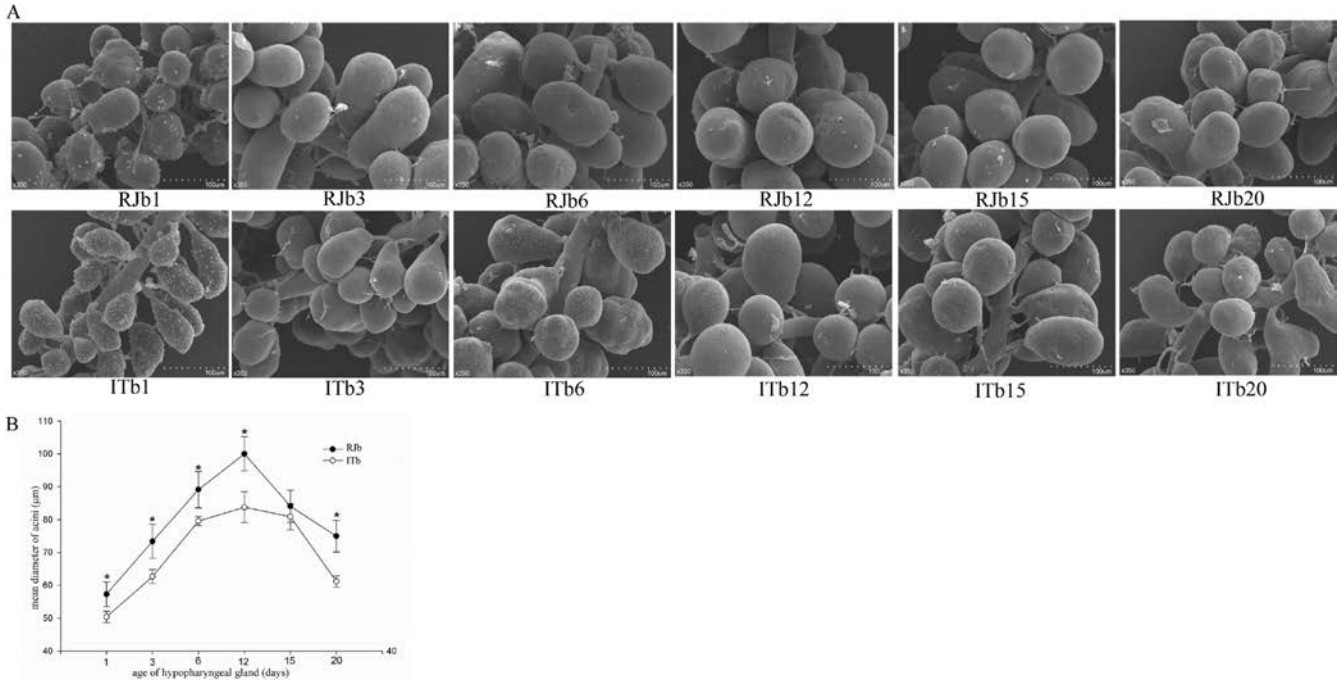


Figure 2. Panel A represents SEM profiles of HG of RJb and ITb workers on day 1, 3, 6, 12, 15, and 20 at 350-fold magnification, respectively. The number followed by RJb (RJb1−20) indicates the RJb HG 1−20 day after the eclosion. Similarly, the number followed by ITb (ITb1−20) indicates the ITb HG 1−20 day after the eclosion. Panel B is the HG acini mean diameter of the two bee strains. Asterisks indicate the statistically significant differences between the mean diameter of acini at each development stage ($p < 0.05$).

Table 2. The Differentially Expressed Proteins during the Development of Hypopharyngeal Gland of Royal Jelly Producing Bee (RJb) (*Apis mellifera* L.) and Italian Bee (ITb) (*Apis mellifera* L.) on Day 1, 3, 6, 12, 15, and 20[a]

spot no.	theoretical pI/M_r (kDa)	experimental pI/M_r (kDa)	sequence coverage (%)	matched/ searched	score	protein name	access number	Day 1		Day 3		Day 6		Day 12		Day 15		Day 20	
								$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value
Carbohydrate and Energy Metabolism																			
1	8.01/11.40	8.40/16.20	70	10/28	143	ATP phosphohydrolase (EC 3.6.1.3)	gil66547447	−1.25	0.04	−1.42	0.029								
2	6.45/15.21	5.17/16.22	15	14/26	81	lethal (1) G0196	gil28571287			1.27	0.046			1.96	0.027				
10	7.23/56.56	8.20/59.40	35	23/34	98	α-amylase (EC 3.2.1.1)	gil20377081	−1.06	0.008	1.12	0.034			−1.09	0.043				
24	9.36/35.40	7.41/31.44	19	6/8	83	phosphoglyceromutase (pglym78) (EC. 5.4.2.1)	gil66550890			−1.96	0.01							1.36	0.024
37	6.26/34.46	7.13/40.55	49	14/60	119	aldehyde reductase (EC 1.1.1.21)	gil66525576	−1.09	0.041			1.23	0.017						
42	6.69/55.94	6.81/52.38	34	17/56	112	aldehyde dehydrogenase (aldh) (EC 1.2.1.3)	gil66530423	1.07	0.025										
47	5.25/55.10	5.10/57.61	49	22/23	255	ATP synthase β subunit (atpsyn-beta) (EC 3.6.3.14)	gil110762902	1.19	0.04										
48	6.26/34.46	5.18/59.02	44	13/77	112	aldehyde reductase (EC 1.1.1.21)	gil66525576	1.73	0.025										
51	5.51/40.18	5.94/58.49	48	15/65	91	enolase (eno) (EC 4.2.1.11)	gil110761968	1.15	0.02	1.09	0.002	2.09	0.009						
52	9.00/59.76	7.58/58.51	27	13/29	105	ATP synthase α subunit (blw)	gil48100966	1.21	0.036										
54	8.15/45.14	9.01/65.98	19	9/13	86	phosphoglycerate kinase (pgk) (EC 2.7.2.3)	gil110763826	1.08	0.005	−1.22	0.028								
57	5.18/89.47	5.52/81.88	38	30/38	298	transitional endoplasmic reticulum ATPase (ter94)	gil66534286	1.34	0.04										
62	6.22/58.32	6.94/58.49	69	40/87	240	ATP phosphohydrolase (EC 3.6.1.3)	gil66533395	1.06	0.012										
64	5.06/65.69	5.20/69.42	39	31/100	147	α-glucosidase (EC 3.2.1.20)	gil58585164	1.78	0.03	1.17	0.023	2.08	0.032	1.04	0.048	1.11	0.031	−1.94	0.011
69	5.18/89.47	5.52/81.88	38	30/38	298	transitional endoplasmic reticulum ATPase (ter94)	gil66534286	1.22	0.032	−1.18	0.034	1.19	0.027	1.67	0.006			>10	<0.01
108	6.48/68.35	6.40/74.70	23	13/37	83	glucose oxidase (GOD) (EC 1.1.3.4)	gil58585090			−1.31	0.017							<−10	<0.01
109	6.48/68.35	6.50/75.10	32	16/34	120	glucose oxidase (GOD) (EC 1.1.3.4)	gil58585090			−1.36	0.022	1.14	0.044	<−10	<0.01			<−10	<0.01
110	6.48/68.35	6.60/74.40	26	18/85	82	glucose oxidase (GOD) (EC 1.1.3.4)	gil58585090			−1.09	0.030	1.98	0.006	<−10	<0.01	−1.29	0.018	−1.96	0.006
111	6.48/68.35	6.80/74.20	27	9/31	87	glucose oxidase (GOD) (EC 1.1.3.4)	gil58585090			−1.22	0.026	1.63	0.009	−1.28	0.026	−1.36	0.027	−2.16	0.008
112	6.48/68.35	6.90/74.50	32	20/63	106	glucose oxidase (GOD) (EC 1.1.3.4)	gil58585090			1.84	0.003	1.82	0.013	−1.32	0.037	1.46	0.017	−1.86	0.009
113	6.48/68.35	7.10/74.44	27	16/56	81	glucose oxidase (GOD) (EC 1.1.3.4)	gil58585090			1.97	0.001	1.32	0.034			1.62	0.011	−2.27	0.003
Amino Acid Metabolism																			
18	4.83/26.70	4.70/28.90	57%	19/275	668	proteasome subunit alpha type 5 (prosma5) (EC 3.4.25.1)	gil66541426	−1.03	0.044	−1.29	0.018			1.19	0.045			>10	<0.01
21	7.03/30.69	6.98/26.71	51	16/53	137	proteasome catalytic subunit 2 (prosbeta2) (EC 3.4.25.1)	gil66519842			1.04	0.042	1.07	0.0042						
36	5.78/31.25	6.51/30.65	59	20/65	165	proteasome subunit α type 1 (pros35) (EC 3.4.25.1)	gil66530404	1.78	0.04										
Nucleotide and Fatty Acid Metabolism																			
40	8.89/42.71	9.17/51.79	50	20/52	181	Yippee interacting protein 2 (yip2) (EC 2.3.1.16)	gil48097100	1.72	0.016										
71	8.69/59.61	9.20/61.00	39	19/87	99	sterol carrier protein X-related thiolase (ScpX) (EC 1.-.-.-)	gil66512039	1.85	0.022										

Table 2. Continued

spot no.	theoretical pI/M_r (kDa)	experimental pI/M_r (kDa)	sequence coverage (%)	matched/ searched	score	protein name	access number	Day 1		Day 3		Day 6		Day 12		Day 15		Day 20	
								$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value
Development																			
7	6.75/17.63	7.06/16.49	38	10/273	218	nucleoside diphosphate kinase (NDK) (EC 2.7.4.6)	gi|66520497			1.16	0.035	1.43	0.029	1.57	0.025				
19	4.79/20.06	5.38/30.60	56	27/306	903	14-3-3-like protein (14-3-3zeta)	gi|48097086	1.64	0.05	1.33	0.019			1.72	0.008			>10	<0.01
22	5.47/48.11	6.90/27.40	22	15/634	298	COP9 signalosome complex subunit 4 (CSN4)	gi|66521456	>10	<0.01	>10	<0.01								
23	6.54/29.99	7.12/30.32	81	16/50	174	lethal (2) 37Cc (l(2)37Cc)	gi|48097857			1.37	0.045	1.24	0.016					1.48	0.007
31	7.62/36.25	7.91/32.08	63	21/65	186	guanine nucleotide-binding protein subunit β-like protein (rack1)	gi|48104663	1.20	0.006	−1.75	0.032	−1.33	0.021	1.74	0.04	1.28	0.036	−1.39	0.023
32	7.62/36.25	8.22/31.76	50	16/67	126	guanine nucleotide-binding protein subunit β-like protein (rack1)	gi|48104663			−1.52	0.037	−1.58	0.028	−1.05	0.046			−1.12	0.036
33	4.60/35.86	4.61/39.68	73	44/286	1446	annexin IX (annIX)	gi|66530527	−1.20	0.048									>10	<0.01
Protein Biosynthesis																			
3	4.43/11.62	4.24/17.43	38	6/309	299	60S acidic ribosomal protein P1(rplp1)	gi|66546799					1.56	0.041	>10	<0.01	1.67	0.039		
4	5.19/17.92	5.63/18.52	49	12/52	88	eukaryotic translation initiation factor 5A (eIF-5A)	gi|110767655	1.70	0.04	1.44	0.021	1.36	0.039	1.43	0.017			1.76	0.013
8	9.84/12.65	9.80/16.65	59	8/41	82	ribosomal protein L30 (rpl30)	gi|66543659	1.66	0.003			1.04	0.041	>10	<0.01	>10	<0.01		
9	10.31/17.72	9.82/18.09	84	24/96	136	ribosomal protein S18 (rps18)	gi|66548968	2.73	0.002			1.45	0.027	>10	<0.01	>10	<0.01		
11	4.57/19.83	4.88/21.05	74	21/311	643	translationally controlled tumor protein (TCTP)	gi|66515987			1.84	0.007	1.69	0.005	1.77	0.007	−1.67	0.019	1.62	0.031
17	10.74/22.51	9.76/20.70	56	15/88	81	ribosomal protein S9 (rps9)	gi|48101950	2.01	0.011										
25	9.68/26.99	9.73/32.03	60	15/91	97	40S ribosomal protein S3 (rps3)	gi|66504343	1.69	0.013			1.17	0.042	>10	<0.01	>10	<0.01		
26	10.13/30.60	9.74/28.71	32	13/41	97	40S ribosomal protein S2 (sop)	gi|48103127	2.24	0.005			1.21	0.026	>10	<0.01	>10	<0.01		
27	9.67/30.14	9.74/29.78	59	18/59	142	40S ribosomal protein S3a (rps3a)	gi|66547340	1.72	0.008			1.16	0.035	>10	<0.01	>10	<0.01		
28	4.70/29.74	5.02/28.23	62	19/91	122	proliferating cell nuclear antigen (PCNA)	gi|110764909	−1.11	0.043			−1.09	0.044	1.63	0.013	1.38	0.041		
34	4.90/27.39	4.90/37.90	17	5/264	115	eukaryotic translation initiation factor 3 (eIF-3)	gi|66532125	1.08	0.037										
39	8.16/53.01	7.50/48.15	37	15/20	162	elongation factor Tu (eftum)	gi|66518848	1.51	0.035										
43	6.02/49.24	6.27/54.66	38	14/21	180	eukaryotic translation elongation factor 1-γ (ef1gamma)	gi|110761214	−1.81	0.026	1.83	0.007								
53	9.13/50.68	9.72/59.16	35	15/47	101	elongation factor 1-α (ef1alpha100e)	gi|62526112	2.44	0.001			1.77	0.013	2.13	0.004	1.31	0.016		
Transporter																			
16	6.96/24.86	7.23/23.57	54	15/59	134	GTP-binding nuclear protein Ran (ran)	gi|48097366			1.14	0.038	1.23	0.037						
30	6.20/31.76	7.06/35.06	46	17/73	96	phosphatidylinositol transfer protein (PITP)	gi|66557640	1.04	0.007										

Table 2. Continued

spot no.	theoretical pI/M_r (kDa)	experimental pI/M_r (kDa)	sequence coverage (%)	matched/ searched	score	protein name	access number	Day 1		Day 3		Day 6		Day 12		Day 15		Day 20	
								$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value
Protein Folding																			
14	6.01/25.61	5.83/27.07	38	11/28	118	small heat shock protein hsp20 family (small hsp20)	gil110757651	−1.14	0.039										
29	6.01/25.55	6.30/32.80	31	12/313	389	small heat shock protein hsp20 family (small hsp20)	gil110757651	1.05	0.045										
44	5.47/51.84	6.84/54.68	28	13/44	100	peptidylprolyl cis−trans isomerase (PPI) (EC 5.2.1.8)	gil66499186	1.27	0.035										
45	4.45/47.49	4.63/41.88	60	33/70	342	calreticulin (Crc)	gil66545506	−1.35	0.014	−1.61	0.005	1.27	0.023	2.23	0.016	1.53	0.026	>10	<0.01
46	5.64/60.55	5.13/60.09	15	8/9	97	60 kDa heat shock protein (Hsp60)	gil66547450	<−10	<0.01										
50	5.57/56.22	6.28/59.19	60	22/81	260	protein disulfide isomerase (PDI) (EC 5.3.4.1)	gil66546657	1.53	0.032	1.47	0.025	1.38	0.01	1.20	0.033			−1.18	0.049
56	4.74/24.44	5.52/81.88	61	16/31	152	protein disulfide isomerase precursor (PDI) (EC 5.3.4.1)	gil110768510	1.43	0.020										
58	5.64/60.55	5.13/60.09	30	16/43	116	60 kDa heat shock protein	gil66547450	1.91	0.03	−1.66	0.034	1.16	0.031	1.82	0.009	1.39	0.027	1.41	0.022
59	5.57/56.22	5.96/60.25	51	29/57	279	protein disulfide isomerase (PDI) (EC 5.3.4.1)	gil66546657	1.27	0.016										
60	5.57/56.22	6.00/60.24	45	22/41	182	protein disulfide isomerase (PDI) (EC 5.3.4.1)	gil66546657	1.78	0.009	−1.27	0.031	1.25	0.024	2.04	0.006			−1.72	0.011
61	5.70/59.89	6.13/60.35	34	19/41	168	T-complex chaperonin 5 (Tcp) (EC 3.6.1.3)	gil66522349	<−10	<0.01										
63	6.58/44.21	6.87/42.69	43	15/27	144	lethal (1) G0022	gil66513205	1.49	0.04										
65	5.43/71.39	5.87/66.61	31	17/33	132	heat shock protein cognate 4 (Hsc70-4)	gil229892210	2.02	0.038										
66	5.43/71.39	5.90/66.58	42	32/44	269	heat shock protein cognate 4 (Hsc70-4)	gil229892210	2.02	0.038	1.39	0.007								
67	6.38/75.64	6.12/66.41	30	19/47	105	heat shock protein cognate 5 (Hsc70-5)	gil229892203	1.64	0.02										
68	4.96/92.09	5.04/84.50	40	54/280	1894	glycoprotein 93 (gp93)	gil110758921			−1.81	0.003	−1.23	0.020			−1.19	0.039	>10	<0.01
70	5.21/72.50	5.42/74.76	45	28/47	233	heat shock protein cognate 3 (Hsc70-3)	gil110754998			−1.96	0.002	2.01	0.007	−1.21	0.022			>10	<0.01
72	6.24/55.78	7.13/61.38	30	17/33	130	Hsp70/Hsp90 organizing protein homologue (hop)	gil110756123	1.08	0.44										
73	5.57/56.22	5.96/60.25	60	32/81	260	protein disulfide isomerase (PDI) (EC 5.3.4.1)	gil66546657	<−10	<0.01										
Skeleton																			
5	6.17/17.05	6.20/17.60	70	11/32	123	cofilin/actin-depolymerizing factor (tsr)	gil110751158	1.44	0.39			1.37	0.013	1.29	0.032				
12	6.17/17.05	5.39/20.17	70	13/41	125	cofilin/actin-depolymerizing factor (tsr)	gil110751158	1.18	0.01			1.34	0.03						
35	5.37/32.95	5.84/36.80	70	15/69	136	F-actin capping protein α subunit (cpa)	gil66508517			−1.56	0.002	−1.03	0.030						
41	5.30/42.20	5.76/36.52	32	12/21	124	actin 87E (ptx1)	gil66509769					1.15	0.046	2.31	0.004			1.19	0.028
Antioxidant System																			
6	6.21/15.80	7.12/16.83	73	10/57	125	superoxide dismutase (sod) (EC 1.15.1.1)	gil66513527			1.44	0.039	1.27	0.026	1.68	0.006				
15	5.40/17.69	5.99/22.97	52	9/24	128	glutathione S transferase S1 (gsts1) (EC 2.5.1.18)	gil66534655	1.12	0.006			1.26	0.031	1.59	0.032			1.14	0.044
20	5.88/25.23	6.36/28.02	63	15/96	130	peroxiredoxin-like protein (prx2540) (EC 1.11.1.15)	gil283436152	1.36	0.024	1.51	0.009	1.62	0.005	1.53	0.002	1.42	0.009		

Table 2. Continued

spot no.	theoretical pI/M_r (kDa)	experimental pI/M_r (kDa)	sequence coverage (%)	matched/ searched	score	protein name	access number	Day 1		Day 3		Day 6		Day 12		Day 15		Day 20	
								$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value
Major Royal Jelly Proteins																			
55	5.10/48.86	4.97/59.90	22	11/26	102	major royal jelly protein 1 (MRJP1)	gil58585098	1.10	0.047	1.26	0.013	1.86	0.005	1.47	0.012	1.54	0.039		
74	6.83/51.45	6.73/58.57	32	15/42	125	major royal jelly protein 2 (MRJP2)	gil58585108			>10	<0.01	1.34	0.032	−1.26	0.004				
75	6.83/51.45	6.94/58.49	23	11/24	109	major royal jelly protein 2 (MRJP2)	gil58585108			1.15	0.005	1.65	0.027	−1.04	0.038	2.49	0.008	−1.16	0.044
76	6.83/51.45	7.13/58.01	23	10/19	93	major royal jelly protein 2 (MRJP2)	gil58585108			1.58	0.0047	3.56	0.0078	−1.29	0.008	1.57	0.026	−1.65	0.029
77	6.83/51.45	7.22/57.66	32	15/42	140	major royal jelly protein 2 (MRJP2)	gil58585108			1.71	0.0039	1.18	0.037	−1.33	0.003	1.73	0.004	−1.96	0.007
78	6.83/51.45	7.31/57.49	43	20/94	117	major royal jelly protein 2 (MRJP2)	gil58585108					1.39	0.004	1.08	0.046	1.03	0.031	−1.36	0.048
79	6.47/61.97	6.64/64.71	25	14/24	125	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01								
80	6.47/61.97	6.74/64.60	22	12/22	112	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01			−1.17	0.039				
81	6.47/61.97	6.97/62.92	20	10/13	128	major royal jelly protein 3 (MRJP3)	gil58585142			2.86	<0.01	1.50	0.046	1.96	0.008	>10	<0.01		
82	6.47/61.97	7.13/62.79	19	12/25	103	major royal jelly protein 3 (MRJP3)	gil58585142			1.39	0.0074	1.69	0.006	1.63	0.014	>10	<0.01	>10	<0.01
83	6.47/61.97	7.27/62.76	34	15/29	147	major royal jelly protein 3 (MRJP3)	gil58585142			2.46	0.0046	1.04	0.048	1.52	0.023	>10	<0.01	>10	<0.01
84	6.47/61.97	7.45/63.16	19	12/22	101	major royal jelly protein 3 (MRJP3)	gil58585142			1.39	0.031	1.52	0.004	−1.37	0.018	>10	<0.01	>10	<0.01
85	6.47/61.97	7.13/63.09	16	11/25	88	major royal jelly protein 3 (MRJP3)	gil58585142			<−10	<0.01	2.01	0.026			1.04	0.042	>10	<0.01
86	6.47/61.97	7.58/63.54	24	13/33	103	major royal jelly protein 3 (MRJP3)	gil58585142			<−10	<0.01	1.12	0.048					>10	<0.01
87	6.47/61.97	8.04/64.86	20	12/21	119	major royal jelly protein 3 (MRJP3)	gil58585142			<−10	<0.01	1.08	0.033					>10	<0.01
88	6.47/61.97	6.49/65.86	18	12/25	84	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01	1.77	0.014						
89	6.47/61.97	6.60/65.89	22	13/25	106	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01	1.56	0.008						
90	6.47/61.97	6.75/65.65	22	12/36	87	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01			1.36	0.022	>10	<0.01		
91	6.47/61.97	7.11/65.94	29	15/38	123	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01			−1.71	0.012	>10	<0.01		
92	6.47/61.97	7.25/65.18	16	9/13	92	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01			−1.86	0.008	2.75	0.01		
93	6.47/61.97	7.31/65.08	19	11/14	134	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01					1.78	0.018		
94	6.50/61.66	7.45/65.41	24	13/33	103	major royal jelly protein 3 (MRJP3)	gil58585142			>10	<0.01			−1.79	0.013	1.05	0.03		
97	6.47/61.97	7.21/66.81	18	10/23	92	major royal jelly protein 3 (MRJP3)	gil58585142			1.36	0.018	1.44	0.021	1.12	0.047			>10	<0.01
98	6.47/61.97	7.29/66.45	24	12/24	108	major royal jelly protein 3 (MRJP3)	gil58585142			−1.27	0.032	1.48	0.019	1.22	0.024	<−10	<0.01	>10	<0.01
99	6.47/61.97	7.38/66.69	21	10/13	118	major royal jelly protein 3 (MRJP3)	gil58585142			−1.29	0.042	1.17	0.046	1.02	0.044	−2.01	0.007	2.76	0.005
100	6.47/61.97	7.50/66.50	29	15/38	126	major royal jelly protein 3 (MRJP3)	gil58585142			−1.48	0.008	1.03	0.049	1.56	0.028	−2.59	0.017	3.08	0.008
101	6.47/61.97	7.60/66.10	30	17/44	143	major royal jelly protein 3 (MRJP3)	gil58585142			1.39	0.027	−1.05	0.030	1.43	0.033			1.88	0.016

Table 2. Continued

spot no.	theoretical pI/M_r (kDa)	experimental pI/M_r (kDa)	sequence coverage (%)	matched/ searched	score	protein name	access number	Day 1		Day 3		Day 6		Day 12		Day 15		Day 20	
								$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value	$\log_{1.5} R$	*P*-value
102	6.47/61.97	8.10/66.30	23	10/19	110	major royal jelly protein 3 (MRJP3)	gi|58585142			>10	<0.01	1.45	0.031	1.52	0.029	−1.04	0.044		
114	6.87/65.66	7.10/61.40	32	35/261	1055	major royal jelly protein 3 (MRJP3)	gi|56422035											<−10	<0.01
115	6.47/61.97	7.50/59.80	29	15/45	111	major royal jelly protein 3 (MRJP3)	gi|58585142											<−10	<0.01
116	6.47/61.97	7.60/60.00	31	17/49	128	major royal jelly protein 3 (MRJP3)	gi|58585142											<−10	<0.01
104	5.90/52.88	6.10/60.70	22	10/36	97	major royal jelly protein 4 (MRJP4)	gi|58585170					1.70	0.072	1.73	0.009			>10	<0.01
105	5.90/52.88	6.20/60.80	22	10/41	92	major royal jelly protein 4 (MRJP4)	gi|58585170			−2.00	0.008	1.51	0.039	−1.41	0.015			>10	<0.01
106	5.90/52.88	6.30/60.70	18	6/28	100	major royal jelly protein 4 (MRJP4)	gi|58585170			2.19	0.005			1.36	0.041				
107	5.90/52.88	6.40/60.80	25	8/29	90	major royal jelly protein 4 (MRJP4)	gi|58585170			2.17	0.013	1.68	0.011	1.15	0.044				
								Unknown											
13	5.20/23.51	4.70/21.02	41	7/265	294	RhoGDI	gi|48121613	1.76	0.03										

[a] Notes: Spot number corresponds to the number of protein spots in Figure 3. Theoretical molecular weight (M_r) and isoelectric point (p*I*) of the identified proteins were retrieved from the protein database of NCBInr. Experimental M_r and p*I* 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 MALDI-TOF/MS and LC-Chip/ESI-QTOF-MS. The taxonomy is Apis mellifera. 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 (RJb/ITb) were transformed and proteins with $|\log_{1.5} R| \geq 1$ and a *p*-value ≤ 0.05 was considered as differentially expressed proteins. The log ratios of uniquely expressed proteins in each bee strain were limited to 10.

proteins upregulated by the RJb, the most represented forms were protein folding consisting 12, followed by 10 proteins as carbohydrate metabolism and energy production, 10 proteins as biosynthesis, 3 proteins as development, 2 proteins as fatty acid metabolism, 2 proteins as cytoskeleton, 2 proteins as antioxidants, 1 protein as metabolism of amino acid, 1 protein as a transporter, 1 protein as MRJP1. Likewise, among the 12 upregulated proteins in the ITb, 5 as protein folding, 3 as carbohydrate metabolism and energy production, 2 proteins as development, 1 protein as protein biosynthesis and 1 as amino acid metabolism (Figure 6, day 1). The upregulated protein species were listed under each functional class as described in Figure 6.

On day 3, 66 proteins were upregulated and 42 were in the RJb and 24 in the ITb. Among the 42 upregulated proteins in the RJb, 23 proteins were as MRJPs, 6 as carbohydrate metabolism and energy production proteins, 5 as developmental proteins, 2 as protein biosynthesis, 2 as antioxidant, 2 as protein folding, 1 protein as transporter, and 1 in amino acid metabolism. Likewise, among the 24 upregulated proteins in the ITb on day 3, 7 as MRJPs, 8 as carbohydrate metabolism and energy production, 7 as protein folding, 2 as developmental proteins. An interesting point of this time was that proteins as biosynthesis, antioxidant, and transporter did not altered in the ITb as well as cytoskeleton in the RJb. Moreover, proteins such as nucleotide and fatty acid metabolism proteins were not upregulated in both bees (Figure 6, day 3). Furthermore, CSN4 was uniquely expressed only in the RJb on day 1 and day 3 (Figure 6).

On day 6, 63 proteins were upregulated in both bee strains in which 56 of them were in the RJb and only 7 in the ITb. Among the 56 proteins altered their expressions in the RJb, 22 as MRJPs, 9 as carbohydrate metabolism and energy production, 8 as protein biosynthesis, 5 as protein folding, 3 as development, 3 as antioxidant, 3 as cytoskeleton, 1 as amino acid metabolism and 1 as transporter. Similarly, among the 7 proteins altered their expressions in the ITb, 3 as development, 2 as MRJPs, 1 as protein folding and 1 as cytoskeleton (Figure 6, day 6). Interesting notice of this developmental stage was that changes in protein profile between the two bees became more apparent; proteins as carbohydrate metabolism and energy production were significantly overexpressed in the RJb but not in the ITb. The RJb moved on upregulating amino acid metabolizing protein such as prosbeta2 (spot 21), while the ITb completely stopped upregulating of this class even from day 3 onward. More interestingly, the RJb maintained constant expressions of MRJPs as previous days and the ITb significantly reduced and rather expressed protein as cytoskeleton that it did not on the day 3.

On day 12, 58 protein spots were upregulated in both bee strains of which 41 were in the RJb and 17 in the ITb. Among the 41 upregulated proteins in the RJb, 15 as MRJPs, 8 as biosynthesis, 5 as development, 4 as protein folding, 3 as carbohydrate metabolism and energy production, 3 as antioxidant, 2 as cytoskeleton and 1 as amino acid metabolism. As well, among the 17 proteins that altered their expression in the ITb, 10 of them were as MRJPs, 5 as carbohydrate metabolism and energy production, 1 as development, and 1 as protein folding. In addition, subsequent situations were regarded as this developmental junction variation between the two bee strains. The ITb upregulated more numbers (5) of proteins as carbohydrate metabolism and energy production, the differences in proteins as development became more significant, the

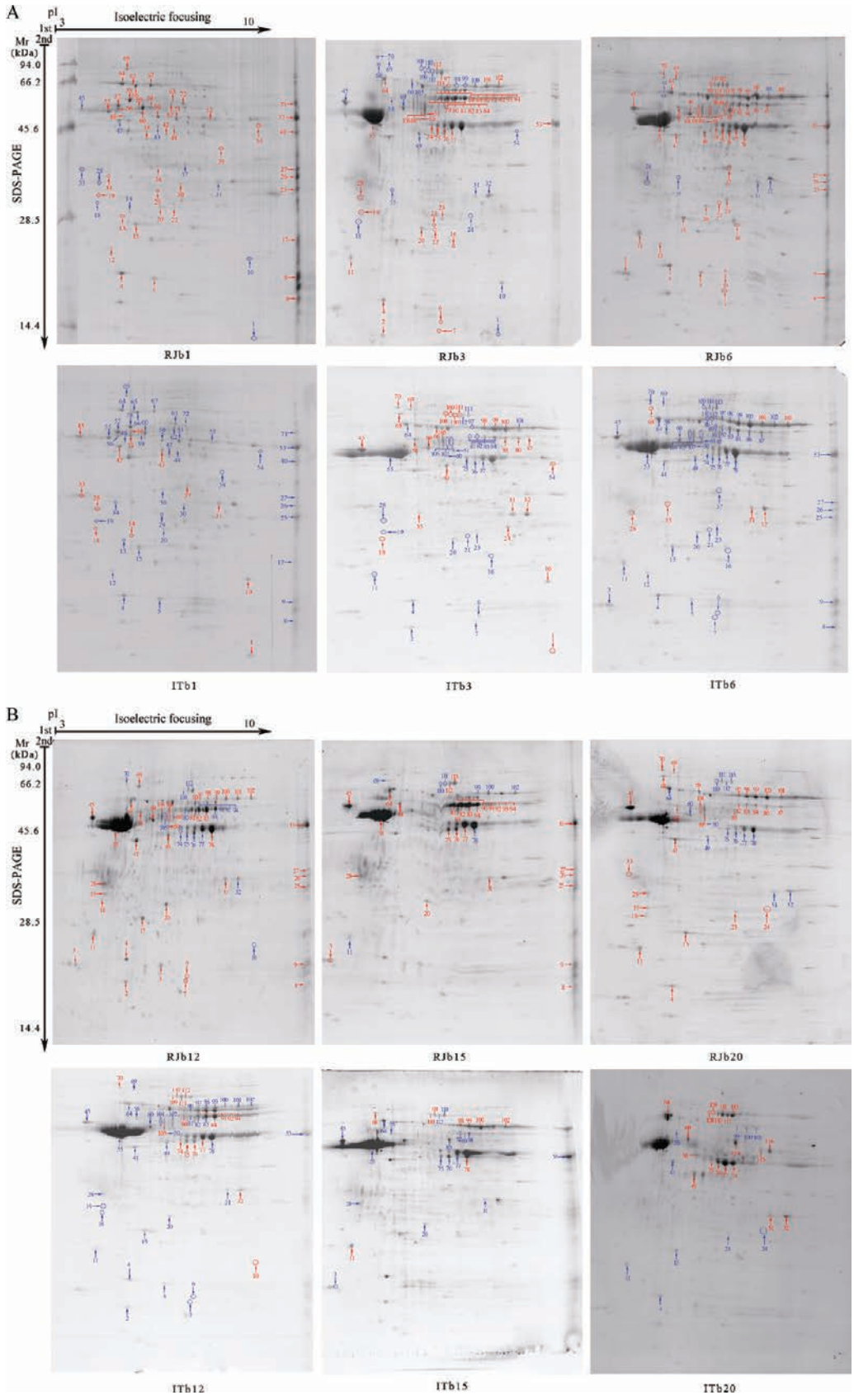


Figure 3. 2-DE profiles of HG of RJb and ITb on different development stages. 200 μg of each sample was subjected to 2-DE and stained by CBB G-250. Panel A represents protein profiles on day 1, 3 and 6, whereas panel B represents day 12, 15, and 20. The red and blue labels indicate the proteins upregulated and downregulated.

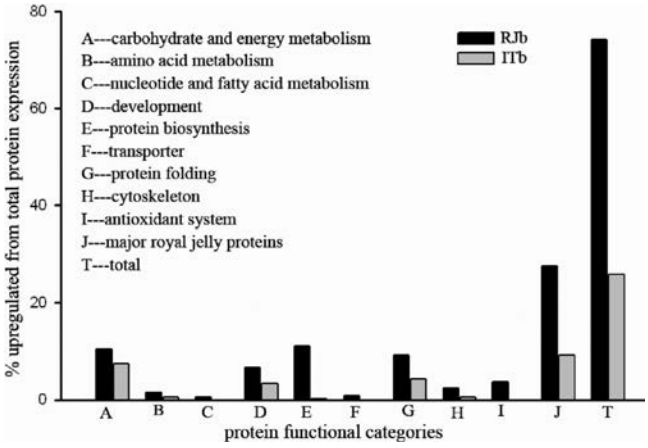


Figure 4. Proportion of upregulated proteins from the total expression in both bee strains by each functional category. The letters A−J and T represents short descriptions of each functional class and total, respectively.

differences in proteins as biosynthesis, protein folding, and amino acid metabolism look similar with the difference on day 6, the merely representations of cytoskeleton on day 3 and 6 in the ITb stopped, the differences in MRJPs expressions were significant, and protein as transporter was completely absent in both bees (Figure 6, day 12).

On day 15, 38 proteins were upregulated in both bees of which 30 were in the RJb and 8 were in the ITb. Among the 30 proteins overexpressed in the RJb, 15 were as MRJPs, 7 as biosynthesis, 3 as carbohydrate metabolism and energy production, 2 as protein folding, and 1 as antioxidant. Also, among the 8 upregulated proteins in ITb, 4 as MRJPs, 2 as carbohydrate metabolism and energy production, 1 as development and 1 as protein folding. Unlike the previously days, an interesting point of this period was that both bees downregulated most of the protein categories (except for protein biosynthesis and MRJPs) and did not altered any of the proteins as metabolism of amino acid, nucleotide and fatty acid, transporter and cytoskeleton. Protein expressional differences of the two bees were significant for protein biosynthesis, antioxidant, and MRJPs (Figure 6, day 15).

On day 20, 45 proteins were upregulated in both and the RJb upregulated 27 as well the ITb 18. From the 27 upregulated in the RJb, 13 as MRJPs, 4 proteins as development, 4 as protein folding, 2 as carbohydrate metabolism and energy production, 1 as amino acid metabolism, 1 as biosynthesis, and 1 as antioxidant. Likewise, among the 18 proteins upregulated in the ITb, 7 as MRJPs, 7 as carbohydrate metabolism and energy production, 2 as development, and 2 as protein folding. Summing-up the difference of the two bees on the day, besides the MRJPs and carbohydrate metabolism and energy production, proteins involved in "development", "protein biosynthesis", "protein folding", and "antioxidant" contributed more to the differential expression of proteins between the RJb and ITb strains (Figure 5). As in the earlier days, proteins as development were considerably upregulated in the RJb than in the ITb, while the representation of protein biosynthesis was drastically reduced even in the RJb. The proportion of folding protein was reincreased in both bee strains and proteins as cytoskeleton and antioxidant were upregulated only in the RJb (Figure 6, day 20).

In addition to the qualitative HG protein expressional comparisons, protein expression intensity comparisons were also done between the two bee strains using ANOVA log ratio ($|\log_{1.5}$ Ratio$| \geq 1$). The p-values for the differentially expressed proteins were calculated as the ratio of the protein abundance (RJb/ITb) and protein spots with $p \leq 0.05$ were considered (Figure 6). As the two bees share similar complements of proteins, this method of protein quantification was particularly effective at comparing protein expression intensity between them. In other words, even if the two bees have similar upregulated protein types and numbers, the expressional intensity might be different, which could help to estimate the extent of its biological function. Hence, vertical log ratios were computed for all the upregulated proteins in both bee strains. Generally, the computed results established that all the upregulated proteins in either of the bee strains were with high expressional intensity (Figure 6). In this view, the number of proteins with high expressional volume on day 1 was 44 in the RJb as compared with 12 in the ITb. However, the expressional volume of a development protein (CSN4, spot 22) in particular was >10-fold in the RJb as compared with the ITb. On the other hand, the expressional volumes of protein folding Hsp60, tcp, and PDI were >10-fold in the ITb (Figure 6, day 1). Similarly, the number of proteins with high expressional volume on day 3 in the RJb were 42 as compared with 14 in the ITb and in particular the overexpressions of proteins as CSN4 (spot 22), MRJP2 (spot 74) and MRJP3 (spots 83, 84, 89−94) were >10-fold. The ITb also uniquely expressed some proteins as MRJP3 (spots 83, 85, 87) 10-fold than the RJb on day 3 (Figure 6, day 3). On day 6, almost all the proteins of the day (56 out of 63) showed ≥1.5-fold higher expressional intensities in the RJb than in the ITb. However, MRJPs proteins (MRJP3, spot 102) overexpressed ≥2.5-fold more in the ITb and MRJP3 (spots 83, 85, 87) that was used to be >10-fold more in the ITb on day 3 overexpressed ≥2 fold in the RJb (Figure 6, day 6). Similar to previous days, the high expressional intensity proteins number was higher in the RJb (41 out of 58) on day 12 as compared with the ITb (17 out of 58). In particular, the RJb expressed >10-fold more proteins as biosynthesis, rplp1 (spot 3), rpl30 (spot 8), rps18 (spot 9), rps3 (spot 25) and sop (spot 26) over the ITb. And the ITb expressed 10-fold proteins as carbohydrate metabolism and energy production GOD (spots 109, 110) than the RJb (Figure 6, day 12). On day 15, the RJb showed >10-fold expressions in most of the proteins like as biosynthesis, rpl30 (spot 8), rps18 (spot 9), rps3 (spot 25), sop (spot 26), and rsp3a (spot 27) over the ITb in a similar trend with day 12 (Figure 6, day 15). Also on day 20, the RJb expressed >10-fold proteins as carbohydrate metabolism and energy production, ter94 (spot 108), as amino acid metabolism, prosma5 (spot 18), as development 14−3−3zeta (spot 19) and annIX (spot 32), as protein folding Crc (spot 45), gp93 (spot 68) and Hsc70−3 (spot 70) and MRJP3 (spots 83−99) and as MRJPs MRJP4 (spots 104, 105) than the ITb, whereas ITb uniquely overexpressed proteins as carbohydrate metabolism and energy production GOD (spots 108, 109) and proteins as MRJPs (MRJP3, spots 114−116) over the RJb on same day (Figure 6, day 20).

3.4. Validation of Differentially Expressed Proteins. Significantly higher expressions of MRJP1, MRJP2, and MRJP3 in the RJb than in the ITb was confirmed at the corresponding mRNA level by qRT-PCR and at the protein level by Western blot (Figure 7). Hence, there were considerable expressional volume differences at protein level and the achieved differences were in line with the 2-DE images. Likewise, the difference in overall expression profile at the mRNA level was analogous to the difference at the protein level (Figure 7). But, some

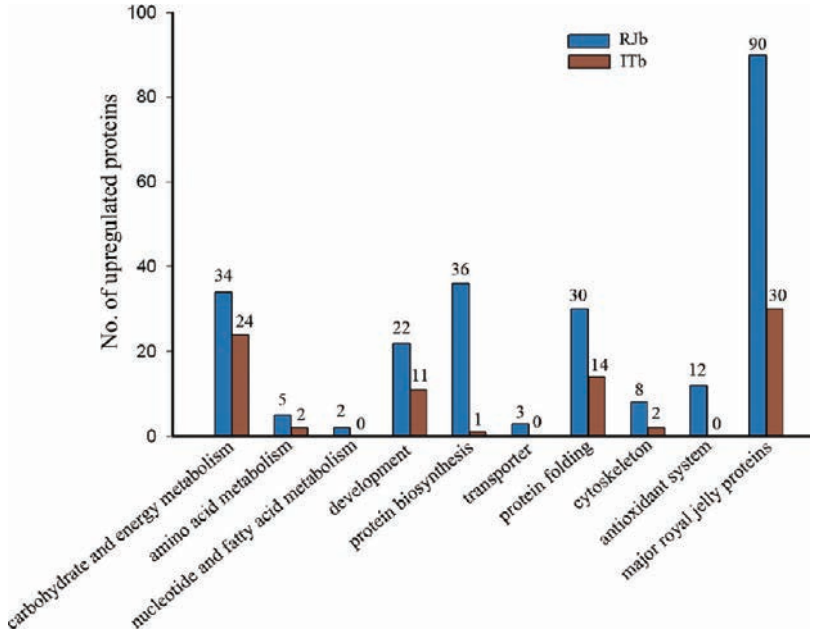


Figure 5. Comparisons of the upregulated number of proteins in the HG of RJb and ITb honeybee workers across the functional groups. Blue and red represent the number in the RJb and the ITb, respectively.

expression profile differences at the mRNA level were not statistically significant, which might be attributed to differences in regulation mechanisms (such as synthesis and degradation rates) that act on both the synthesized mRNA and the synthesized protein and ultimately affect molecular amounts from both sides.[27]

4. Discussion

We compared the HG of the two bee strains based on RJ production, morphology (acini size), and major proteins differentially expressed. This study identified molecular elements involved in the development of the HG size and the RJ productivities in the two bee strains. The identified proteins showed variations in relative molecular mass (M_r) and isoelectric point (p*I*) as well as in the experimental and theoretical values (Figure 3, Table 2). In particular, the variations in proteins as MRJPs were more noticeable, which might be due to naturally occurring protein isoforms that result from in vivo post-translational modifications such as phosphorylation, glycosylation, or acetylation.[28]

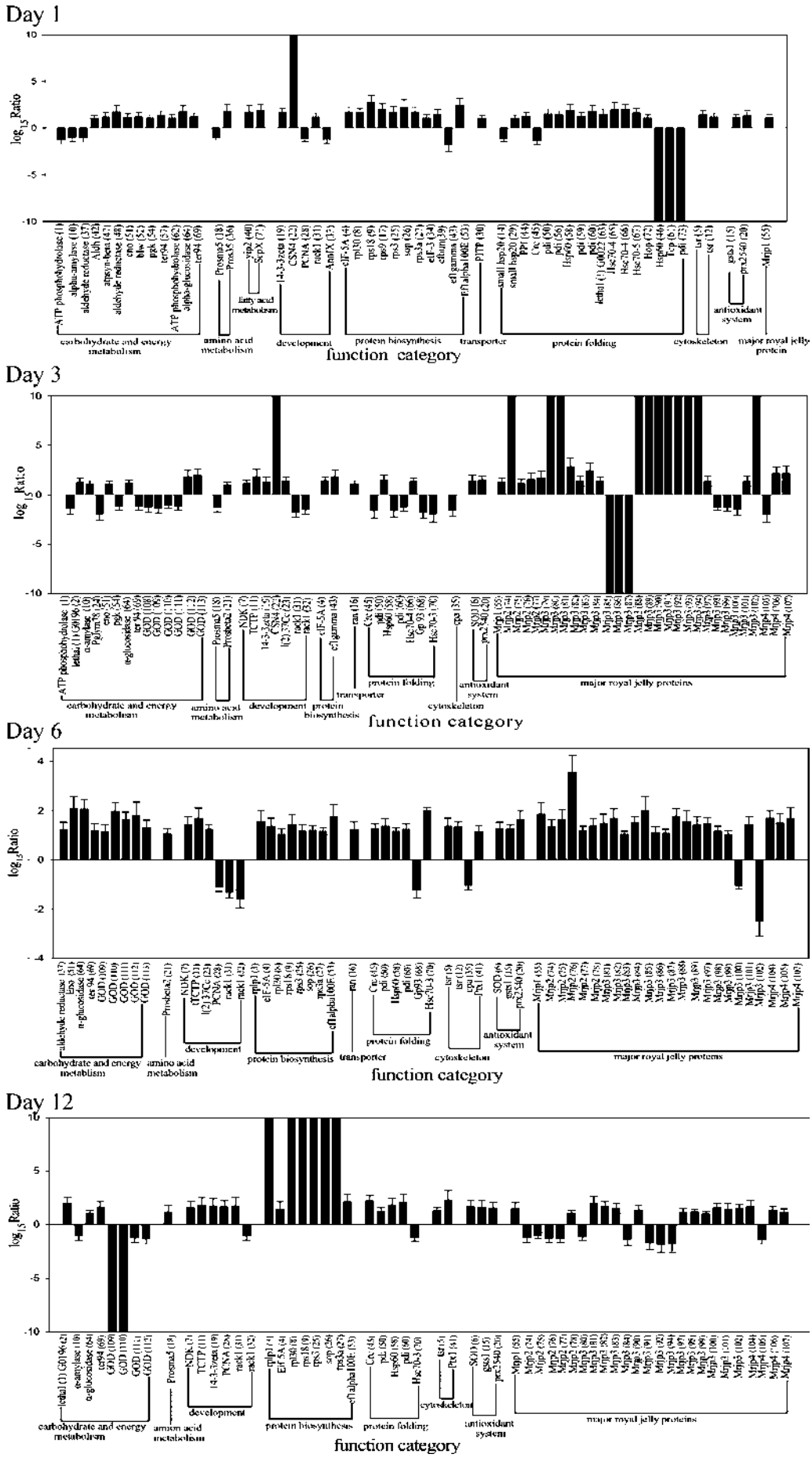
The significant (5 times) overproduction of RJ by the RJb compared to the ITb suggests that selections affected RJ yield through a higher rate of queen cell acceptance,[23] large acini size, and extended time for RJ deposit by the individual bee. Similarly, the larger HG acini diameter in the RJb suggests HG morphological adjustment to cope with high activities.[8] Equally, the HG acini size increased up to day 12 and declined thereafter in both bees' suggesting HG morphological readjustments in line with age-dependent labor division of the worker bees.[6,15,25]

Different protein types were involved during the process of RJb HG development as compared to the ITb, suggesting the fact that fast developing and high yielding HG demands large and diverse amounts of proteins to ensure its organogenesis and high performance. The representation of MRJPs in both bees was expected as the HG is specialized to secrete RJ proteins.[15,22] However, the significant difference in MRJPs amount and content between the two bees suggests polymorphism owing to repeated selection of RJb for the desired trait, that is, high RJ yield. Even though MRJPs in RJ are mainly used as a nutrition source for the developing larvae and queen,[29] different MRJPs vary in amino acid content that play an individual role in honeybee physiology.[30] For instance, MRJP1 and MRJP2 contain high amounts of essential amino acids for larvae and MRJP3 and MRJP4 contain lower amounts.[30] The RJb constantly overexpressed MRJP1 and MRJP2, and the ITb overexpressed MRJP3 and MRJP4, suggesting the effects of selection pressure on RJb to follow a different strategy toward increased RJ production through engaging additional proteins than its original counterpart ITb. In addition, the age-dependent shifting of MRJPs from high essential amino acid MRJP types to lower amino acid content types in RJb suggests the HG task shifts in line with age and acini size. The expressional differences of MRJP1−3 between the HG of the two bees have been confirmed by Western blot and qRT-PCR.

Protein biosynthesis was significantly upregulated in the RJb than in the ITb, indicating special relevancies and functions in translation of cellular process[31] in the gland growing fast through RNA-binding. The none overexpressions of nucleotide and fatty acid metabolism in the HG of the ITb was in line with our previous finding[25] and its termination after day 1 in the RJb further substantiate its minimal participations in either formations or secretions processes of the HG. However, the substantial overexpressions of proteins as transporter in the RJb only up to 6 days, suggests their early stage to assist in fast growing of the RJb HG by facilitating cell divisions, shaping and transporting cell contents as reported in *Drosophila*.[32]

Antioxidant proteins terminate oxidation reactions that damage cells through removing free radicals, stop and restrain other oxidation reactions by being oxidizing themselves.[33] The simultaneous overexpressions of cellular detoxificant gsts1and metabolizing peroxide,[34−36] and prx2540 by the RJb coincides with big HG acini size and high RJ yield suggests their partaking in restraining oxidation reactions due to high metabolic activities.[33]

Carbohydrate metabolism and energy production proteins involve glycolysis, citric acid circle, ATP generation, converting nectar to honey and serving as preservatives.[25] In this study,


Day 1
log2 Ratio
carbohydrate and energy metabolism
amino acid metabolism
development
protein biosynthesis
transporter
protein folding
cytoskeleton
major royal jelly protein
antioxidant system
fatty acid metabolism
function category
Day 3
log2 Ratio
carbohydrate and energy metabolism
amino acid metabolism
development
protein biosynthesis
transporter
protein folding
cytoskeleton
antioxidant system
major royal jelly proteins
function category
Day 6
log2 Ratio
carbohydrate and energy metabolism
amino acid metabolism
development
protein biosynthesis
transporter
protein folding
cytoskeleton
antioxidant system
major royal jelly proteins
function category
Day 12
log2 Ratio
carbohydrate and energy metabolism
amino acid metabolism
development
protein biosynthesis
protein folding
cytoskeleton
antioxidant system
major royal jelly proteins
function category

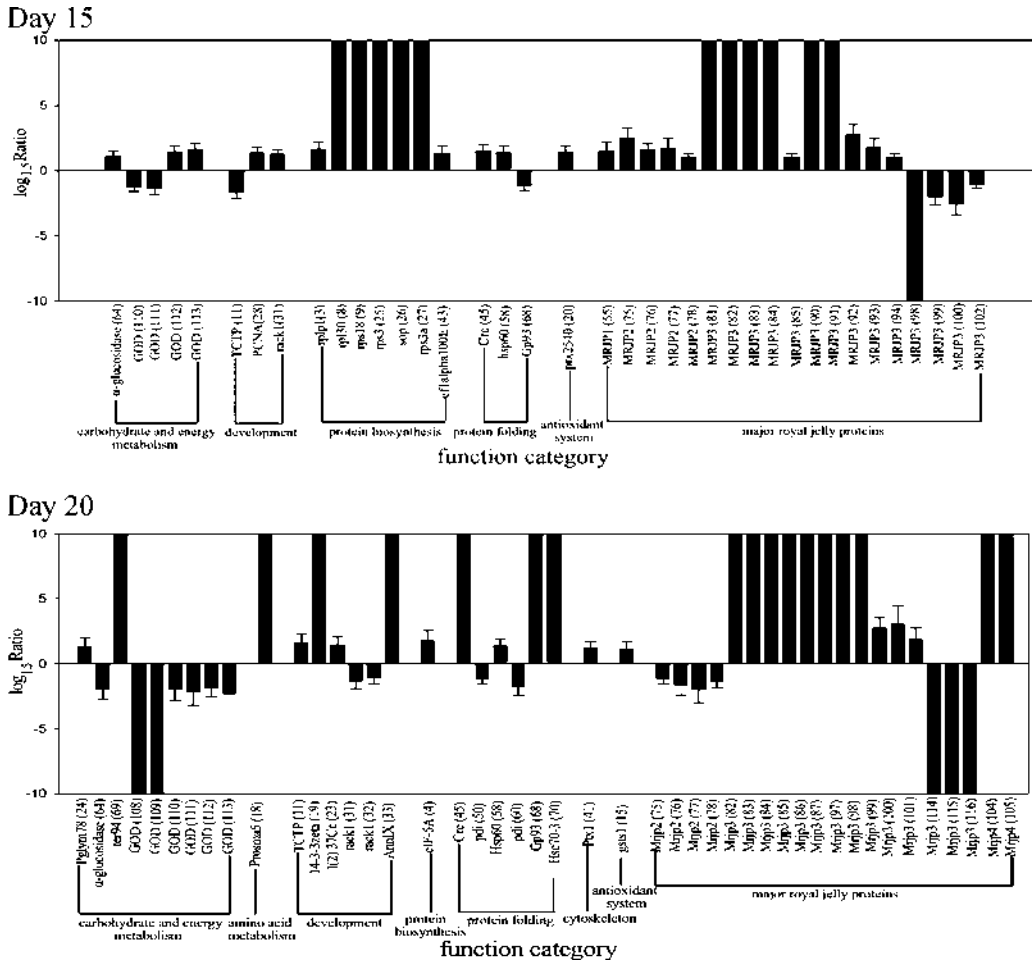


Figure 6. Quantitative analysis of differentially expressed proteins at each development stage during the HG development of RJb and ITb. The ratios of the protein abundance (RJb/ITb) were transformed and the protein spots with $|\log_{1.5} \text{Ratio}| \geq 1$ and $p \leq 0.05$ were selected as the differentially expressed proteins. A, B, C, D, E, and F represent the proteins differentially expressed on day 1, 3, 6, 12, 15, and 20, respectively, and protein name (in abbreviation) and protein numbers are listed, where + values indicate RJb expression over ITb and − values are ITb over RJb. The $|\log_{1.5} \text{Ratio}|$ of the uniquely expressed proteins is limited to 10.

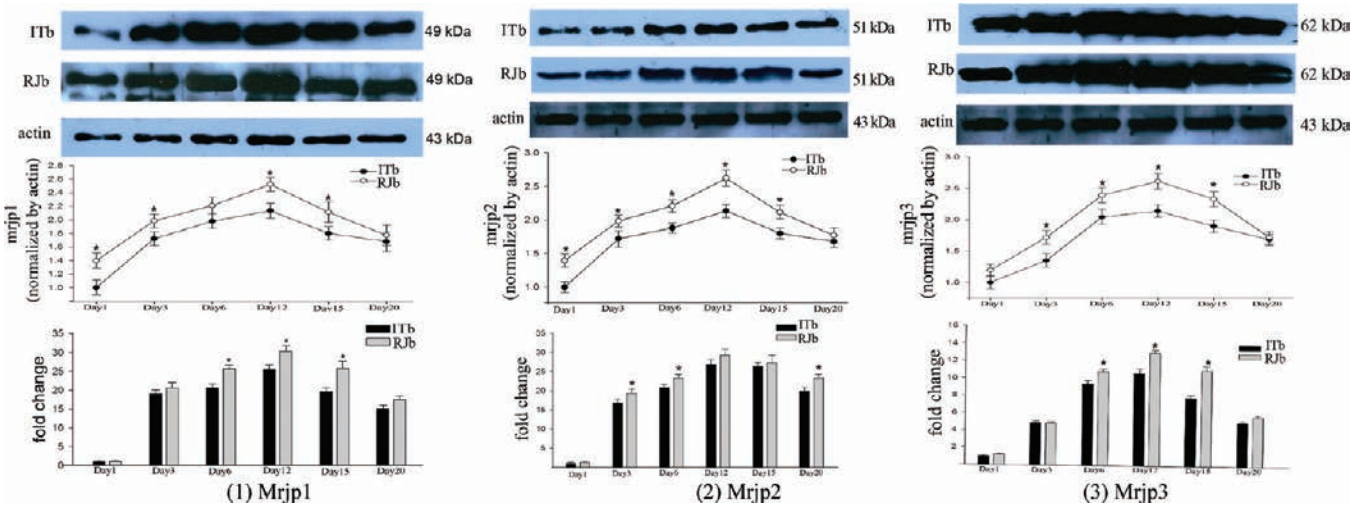


Figure 7. Western blot and real-time PCR analysis of major royal jelly protein 1 (MRJP1) (A), MRJP2 (B), and MRJP3 (C) of RJb and ITb. Whole cell lysates from HG on day 1, 3, 6, 12, 15, and 20 of honeybee worker were subjected to SDS-PAGE followed by Western blot analysis. MRJP1, 2, and 3 were detected using polyclonal antibody anti-MRJP1, 2, and 3, respectively. Equal loading of lanes was maintained by performing a total protein assay and confirmed by Western blot analysis using an antiactin antibody. Upper panel, Western blot analysis of ITb and RJb; middle panel, relative densitometry analysis (normalized by actin); lower panel, transcript alteration of MRJP1, 2, and 3, total RNA of HG were extracted and samples were normalized with *actin* gene as the control housekeeping gene. Asterisks indicate the statistically significant differences of the transcription level between the honeybee strains at each development stage ($p < 0.05$).

the HG of the two bee strains differed in amounts and varieties of proteins such as carbohydrate metabolism and energy production across all the developmental stages and overexpressed in the RJb as compared to the ITb. RJb overexpressed this class of proteins during early (1−6 days) developmental periods, suggesting high energy demand to facilitate proper cell divisions toward proper morphological and physiological development of the fast growing as well as high yielding HG.[6] Likewise, age-based shifting of protein species from important energy generating enzymes to preservative enzymes (that maintain the state of honey) suggests an evolutionary strategy in line with an age-based task shift or labor division of the worker bee from brood nursing to nectar ripening and storage activities.[15,37] On day 3, the ITb upregulated large amounts of proteins as carbohydrate metabolism and energy production and this was despite the small growth of the HG on the day, which might be attributed to the expressional time differences. This expressional time variation (push of day 3 protein expression to day 6) was reasonable through day 6 HG normal growth in the nonexistence of these proteins. In particular, the continuous overexpressions of eno (spot 51) and ter94 (spots 57, 69) in the HG of RJb during the early stages (day 1−6) suggest their key roles in enhancing metabolic activities through catalyzing phosphoenolpyruvate reaction toward glycolytic and gluconeogenesis pathway and ATP-generation in the fast growing and high secretion HG.[25] The HG growth of the ITb on day 12 was small as compared to the RJb, and this was despite the amounts of proteins, suggesting proteins of the day were not metabolic enzymes; rather, they were enzymes with activities of converting nectar to honey (α-amylase, spot 10) and honey preservative (GOD, spots 109−112).[38] On the contrary, the growth attained by the HG of the RJb was big and this was due to the contributions of (lethal(1)G0196, spot 2 and ter94, spot 69) that involve in energy production and cell division processes. Similarly, the HG acini diameter of the two bees decreased on day 15 in a similar fashion, suggesting the missing of protein types that have growth stimulating functions. The enlargements of the RJb HG on day 20 was in line with the overexpression of proteins as pglym78 (spot 24) and ter94 (spot 69), enzymes involved in catalyzing high energy transfer and cell division, respectively. On the other hand, the HG of the ITb continuously shrank as the overexpressed proteins of the day were honey preservative enzymes GOD (spots 108−113).

The RJb overexpressed higher numbers of proteasome protein family across all the developmental stages (except on day 15), suggesting higher protein synthesis and degradation activities in the HG of the RJb that correspond to its growth and RJ secretions.

Growth factors are important to guarantee the development of the HG. Specifically, 14-3-3 protein is known to act as an adaptor molecule stimulating protein−protein interactions, regulating the subcellular localization of proteins and activating or inhibiting enzymes.[39] Its overexpression in the RJb on day 1, 3, and 12 are most likely to assist the HG by localizing important proteins among the subcellular regions of the gland and activating protein−protein interactions toward fast growing and achieving high secretions of the HG. Protein as rack1 is known to play essential roles at multiple steps in *Drosophila* development and acts as a growth factor in the HG development of the honeybee workers.[25,40] However, it was interchangeably represented at all the places in the HG of both bee strains and this made difficult to refer its specific purpose in the HG, which calls further study. Protein as a development family, CSN4 is required for multicellular differentiation and regulates multiple pleiotropic pathways in compound eye photoreceptor cell differentiation and cephalopharyngeal skeleton in *Drosophila*.[41,42] Even if the specific function of CSN4 in the honeybee HG is not exactly known, the special and continuous expressions in the RJb on day 1 and 3 was most probably to take part in early stage multicellular differentiations toward HG growth, acini ducts emergences and elongations.

Protein synthesis is a multistep process; many regulators related to transcription and translations are needed to ensure it proceeds correctly. Accordingly, seven ribosomal proteins known to repair damaged DNA and affect the fidelity of mRNA translation, translational initiation, and polypeptide chain elongation[43,44] were identified in this study. Among them, eIF-5A is involved in peptide bond formation in translation,[45] and PCNA plays an essential role in DNA replication and DNA repair.[46] Hence, the overexpression of large numbers of ribosomal proteins in the HG of the RJb in this study were essential at least for two main reasons, that is, to participate in the HG acini differentiation and cellular growth control as well as to enable the production of large amounts of RJ in a short period.[47]

In this study, proteins with folding functions were expressed in the early and middle stages of the HG development, suggesting more molecular chaperone requirement aligned with fast HG growth to facilitate the nascent protein folding and targeting.[25] Specifically, the early stage expressions of PDI (spots 44, 50, 56, 60, 59, 73), hop (spot 72), and small hsp20 (spots 14, 29) in the HG of the RJb suggest their vitality during the fast growing period to ease nascent protein folding, whereas the mid stage expressions of Crc (spot 45), Hsp60 (spot 58), gp93 (spot 68), Hsc70-3 (spot 70) signifies their taking part in assisting the correct folding of nascent proteins at the peak RJ secreting stage.[25]

The roles of cytoskeleton proteins in intracellular transport and cellular division were already established.[11,15,25,48−50] The upregulation of tsr (spots 5, 12), cpa (spot 35), and ptx1 (spot 41) in both bees, but with 4 times larger amounts in the RJb, suggests their important role in assisting proper cellular divisions as well as regulating cell shapes in the fast growing HG of the RJb.

The HG acini size, RJ production, and protein differential expressions were compared between the RJb and nonselected honeybee (ITb) using proteomics in combination with an electron microscopy, Western blot, and qRT-PCR. In general, the HG of both bees showed age-dependent changes in acini sizes and protein expression as worker tasks shifted from brood nursing to nectar ripening and foraging and storage activities. The HG acini diameter of the RJb was large and produced 5 times more RJ than the ITb, demonstrating a positive correlation between the yield and HG acini size. In addition, the HG of the RJb considerably upregulated a large group of proteins which are involved in carbohydrate metabolism and energy production, protein biosynthesis, development, amino acid metabolism, nucleotide and fatty acid, transporter, protein folding, cytoskeleton and antioxidation to assist its high HG development and bulky RJ secretions. There were also MRJPs age-dependent variations both in forms and expressional intensities simultaneous with task-switching. Owing to selection pressure, RJb pursued different strategy of increased RJ production by involving addition proteins than its original counterpart ITb. To our knowledge, this morphological and pro-

teomic analyses of the HG of the worker honeybees associated with their age-dependent division of labor in both the RJb and the nonselected ITb strains study is the first of its kind. Supported by morphological, phonotypical, and molecular analysis, the study provided not only quantity and quality differences between the RJb and the ITb HG, but also contributed to cellular and behavioral biology by addressing an interesting question of how the RJb strain can produce RJ more efficiently compared to its wild type strain (ITb).

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