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Identification of the proteome composition occurring during the course of embryonic development of bees (*Apis mellifera*)

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Abstract

To investigate the proteome during embryonic development of honeybees, *Apis mellifera*, proteins were identified by two-dimensional gel electrophoresis, mass spectrometry and protein engine identification tools that were applied to MASCOT and Xproteo search engines. 312, 320, 315 proteins were detected in 24, 48 and 72 h embryos. Thirty-eight highly abundant proteins were identified at the three time points by MS fingerprinting. All 21 proteins could be identified as products of annotated genes of the honeybee. Identified proteins included six proteins related to the metabolism of carbohydrates and energy production, six proteins belonging to the heat shock protein family, three cytoskeletal proteins, four proteins related to the antioxidant system of the embryo and two proteins related to growth regulation of the embryo. Quantitative proteomics was applied to analyze differences in amounts of these proteins during the three above mentioned developmental stages. Our data present an initial molecular picture of honeybee embryos, and will hopefully pave the way for future research on this animal.

Keywords: honeybees (*Apis mellifera* L.), honeybee embryo, peptide mass fingerprint, proteome, MALDI-TOF.

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Introduction

The honeybee, *Apis mellifera*, is one of relatively few species whose evolution has culminated in an advanced society (Wilson & Holldobler, 2005). The perspectives and interests for functional genomic research on the honeybee (*Apis mellifera* L.) are expected to expand since the release of the honeybee genome sequence and development of new molecular methods (Aase *et al*., 2005). However, rearing honeybee larvae after they hatch is an obstacle to the use of honeybees for molecular genetics research. In the hive, the rearing of a worker honeybee requires hundreds of feeding visits by nurse bees. Honeybee workers can be reared to adulthood *in vitro* (Rembold & Lackner, 1981; Shuel & Dixon, 1986; Czoppelt & Rembold, 1988; Peng *et al*., 1992), but these techniques are laborious and the resulting bees may vary greatly in their weight and morphology (Michael & Abramovitz, 1955; Rembold *et al*., 1974, 1980; Shuel & Dixon, 1986). Rearing *in vitro* is therefore inappropriate in cases where it is necessary to detect subtle phenotypic differences between mutants and wild type bees.

The honeybee embryo is approximately 1.6–1.8 mm long, with a maximal diameter of 0.35 mm (DuPraw, 1967). In the course of 72 h from oviposition to hatching, embryonic development proceeds through 10 developmental stages (Fleig & Sander, 1986). During this stage of ontogenesis, queen and worker castes are not discerned. Subsequent feeding of larvae with differing amounts of royal jelly determines the developmental pathway of the embryo (Drapeau *et al*., 2006). Optimal relative humidity for normal hatching of honeybee eggs is between 90 and 95% (Doull, 1976), Hatching is cold sensitive as reported for other insect embryos (Collins & Mazu, 2006). Given that the honeybee egg is very amenable to laboratory manipulations (DuPraw, 1967), researchers have developed methods allowing frequent collection of eggs of a defined age (Omholt *et al*., 1995) and the rearing of genetically manipulated honey bee workers from manipulated embryos (Aase *et al*., 2005). Introduction of genetic material into honeybee embryos requires in most cases considerable manipulations of

young eggs (Beye *et al.*, 1998, 2002; Amdam *et al.*, 2003; Dearden, 2006). A robust protocol for preparing honeybee embryos and ovaries for in-situ hybridization exists to study the function and expression of genes identified in the honeybee genome sequence (Osborne & Dearden, 2005).

The scarcity of experimental work on what kind of gene is expressed during the course of embryogenesis and how it varies is surprising.

Today the honeybee is becoming an increasingly important model organism for the investigation of developmental biology, reverse genetics, aging (Amdam & Omholt, 2002), social behavior (Robinson *et al.*, 2005) and neurobiology (Heisenberg, 2004). Its genome provides a new basis for honeybee genetics investigations (The Honeybee Genome Sequencing Consortium, 2006) and for application of a proteomic approach in characterization of proteomic profiles during embryogenesis. The aims of this study were to identify differentially regulated genes by proteomics during honeybee embryonic development and in addition to, expand knowledge about the small number of honeybee embryonic proteins presently known, to aid future biochemical studies of bee ontogenesis and biology.

Results

Protein complement of honeybee eggs

Figure 1 shows a representative 2-DE images showing the soluble proteins extracted from 24, 48, and 72 h-old honeybee eggs. A total of 312, 320, 315 protein spots were detected during the first three days of development, respectively. 38 highly abundant proteins present on all images were cut out and subjected to functional identification. Of 21 identified proteins, all were identified as representing annotated genes of the honey bee (Table 1). For the other 17 proteins we could not identify orthologs in other species by the present methods.

The first group of identified proteins was related to the metabolism of carbohydrates and energy production. Six proteins (spots 1, 10, 15, 16, 19, 20, Table 1), Enolase (EC4.2.1.11), F1 ATP synthase β subunit (EC3.6.1.34), arginine kinase (EC2.7.3.3), F1 ATP synthase α subunit (EC 3.6.3.14), phosphoglycerate kinase (EC2.7.2.3), transketolase dimmer (EC2.2.1.1), respectively, displayed MW values ranging from 17.7 to 67.4 kDa and pI values ranging from 5.25 to 9.00. Among them, four spots (1, 15, 19, 20) were identified as proteins involved in carbohydrate and energy metabolism, and two were alpha and beta subunits of F1 ATPase (Table 1).

The second group of identified proteins belonged to heat shock proteins. Six different heat shock proteins were identified in spots 2, 6, 8, 11, 13 and 21, in the MW range from 11.4 to 80.0 kDa and with pI values ranging from 5.21 to 8.00. Among them were *Hsc*70/*Hsp*90-organizing protein (HOP), chaperonin 10 (*Hsp*10, CG11267-PA), heat shock protein 8 (*Hsp*a8), heat shock protein cognate 5 (*Hsc*-5), heat shock protein cognate 3 (*Hsc*-3) and 60 kDa heat shock protein, mitochondrial precursor (*Hsp*60) (Table 1).

Three different cytoskeletal proteins were identified in spots 9, 14 and 17, ranging in pI from 4.75 to 5.60 and in MW from 13.7 to 50.1 kDa (Fig. 1 and Table 1). Spot 9 was tubulin (pI of 4.75, MW of 50.1 kDa), spot 14 was a profilin, with a pI of 5.6 and a MW of 13.7 kDa and spot 17 was an actin-5C isoform 1 (Table 1).

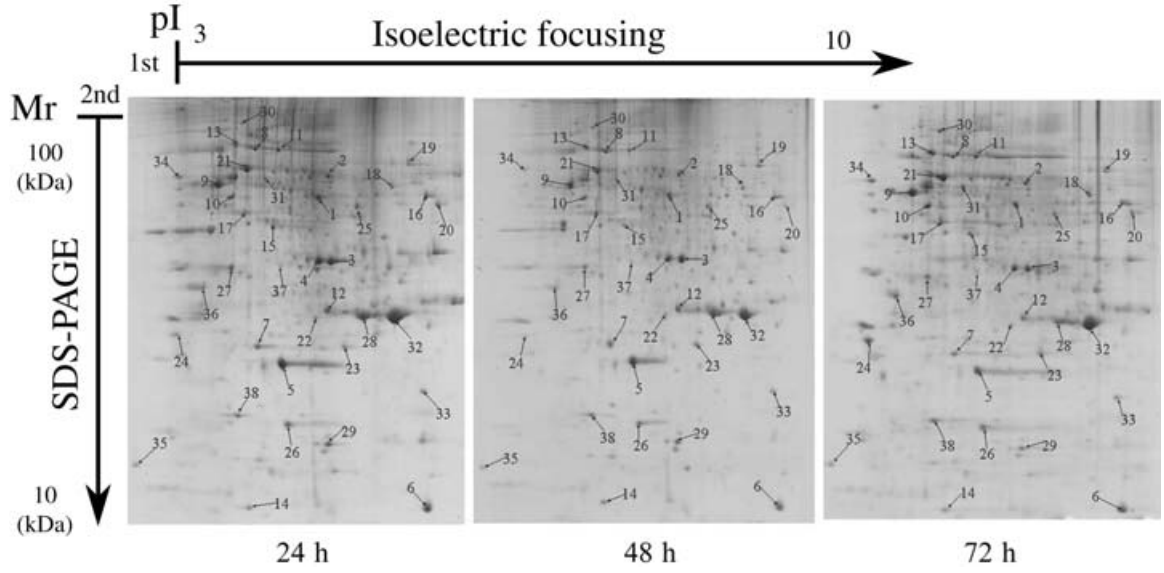

Figure 1. CBB G-250 stained 2D gels of proteins of the egg of honeybee (*Apis mellifera* L.). Number-labeled spots were cut out and subjected to tryptic digestion and subequent mass spectrum analysis.

Table 1. Proteins identified in the egg of honey bee (*Apis mellifera* L.) divided into functional groups

Spot Number	PMF pI/MW	Sequence Coverage	Matched/unmatched peptides	Score	Protein Name	Accession Number
Energy metabolism						
1	5.51/40.12	29.0%	9/8	103	Enolase CG17654-PA, isoform A, partial [Apis mellifera]	*gi|110761968*
10	5.25/55.1	44.0%	22/20	199	ATP synthase-CG11154-PA, isoform A [Apis mellifera]	*gi|110762902*
15	5.66/39.98	44.0%	15/14	152	Arginine kinase(EC 2.7.3.3) [Apis mellifera]	*gi|58585146*
16	9.00/59.51	33.6%	16/59	d′ = 5.7	Bellwether CG3612-PA isoform 1 [Apis mellifera]	*gi|48100966*
19	7.62/67.4	33.0%	17/33	135	CG8036-PB, isoform B isoform 1 [Apis mellifera]	*gi|110751363*
20	8.15/45	23.0%	8/9	84	Phosphoglycerate kinase isoform 1 [Apis mellifera]	*gi|110763826*
Heat shock protein						
2	6.24/55.44	34.9%	15/2	86	*Hsc*70/*Hsp*90-organizing protein homolog CG2720-PA isoform 1 [Apis mellifera]	*gi|110756123*
6	8.00/11.4	70.0%	8/12	128	CG11267-PA [Apis mellifera]	*gi|66547447*
8	5.40/71.7	41.0%	27/22	212	Heat shock protein 8 isoform 1 [Apis mellifera]	*gi|66537940*
11	6.38/75.4	28.0%	17/26	95	Heat shock protein cognate 5 CG8542-PA [Apis mellifera]	*gi|66501507*
13	5.21/72.4	42.0%	25/19	211	Heat shock protein cognate 3 CG4147-PA, isoform A [Apis mellifera]	*gi|110754998*
21	5.6/60.4	33.0%	17/36	112	60 kDa heat shock protein, mitochondrial precursor (*Hsp*60) (60 kDa chaperonin) (CPN60) [Apis mellifera]	*gi|66547450*
Cytoskeleton						
9	4.75/50.1	50.0%	33/53	204	Tubulin at 56D CG9277-PB, isoform B [Apis mellifera]	*gi|48095525*
14	5.64/13.79	60.0%	6/18	84	Profilin [Apis mellifera]	*gi|147902613*
17	5.30/41.78	32.0%	10/12	102	Actin-5C isoform 1 [Apis mellifera]	*gi|48137684*
Antioxidant protection						
3	6.26/36.1	46.0%	10/33	97	CG6084-PA, isoform A isoform 1 [Apis mellifera]	*gi|66525576*
4	6.26/36.46	28.0%	6/12	82	CG6084-PA, isoform A isoform 1 [Apis mellifera]	*gi|66525576*
5	5.65/21.8	54.0%	11/64	87	Thioredoxin peroxidase 1 CG1633-PA, isoform A isoform 1 [Apis mellifera]	*gi|66548188*
7	5.40/17.7	45.0%	10/29	120	Glutathione S transferase S1 CG8938-PA, isoform A, partial [Apis mellifera]	*gi|66534655*
Growth factors						
12	6.54/30.0	78.0%	15/24	179	Lethal (2) 37Cc CG10691-PA, isoform A [Apis mellifera]	*gi|48097857*
18	6.50/41.99	19.7%	7/11	d′ = 6.6	Imaginal disc growth factor 4 CG1780-PA, isoform A, partial [Apis mellifera]	*gi|110773851*

Note: Protein scores in Mascot greater than 81 are significant ($P < 0.05$) and when d′=4 the probability to identify a candidate is ~0.99 at false alarm rate 0.05 in Xproteo, respectively. Spot Number corresponds to the number of a protein spot in Fig. 1. PMF represents the peptide mass fingerprinting, a protein identification technique in which MS is used to measure the masses of proteolytic peptide fragments.
MW (molecular weight) and pI (isoelectric point) in PMF are the theoretical results identified in Genbank or MSDB. Sequence coverage is the ratio of the number of amino acid in every peptide that matches with the mass spectrum divided by the total number of amino acid in the protein sequence. Matched peptide is the score of paring an experimental fragmentation spectrum to a theoretical segment of protein. Accession Number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e. GenBank).

Four proteins related to the antioxidant system of the embryo were identified in spots 3, 4, 5 and 7, with a pI of 5.4–6.4 and a MW of 17.7–61.6 kDa (Table 1). Spots 3, 4 were Aldo/keto reductase (EC1.1.1.2), spot 5 was thioredoxin peroxidase 1 (EC1.11.1.15), spot 7 was glutathione S-transferase (GST) isoenzymes (EC2.5.1.18) (Table 1).

Two proteins that could regulate embryogenesis were identified in spots 12 and 18 (Fig. 1 and Table 1). Spot 12 is a lethal (2) 37Cc, (pI of 6.50 and a MW of 30.0 kDa Fig. 1 and Table 1). An imaginal disk growth factor protein 4, or Chitinase-like protein Idgf4 precursor, was identified in spot 18, with a pI of 8.1 and a MW of 48.7 kDa (Table 1).

Expression analysis of the identified proteins

The normalized spot volume in gels of the identified proteins is presented in Fig. 2. Among the energy metabolism proteins, five were expressed significantly at a higher level on day two than on day three and day one. Only phosphoglycerate kinase was significantly reduced within the three days of observation (ANOVA, $n = 5$, $P < 0.009$) (Fig. 2a). All the six heat shock proteins were expressed with no significant differences during the three days development (ANOVA, $n = 5$, $P > 0.0823$) (Fig. 2b). Out of three cytoskeletal proteins, two (profilin and actin-5C isoform 1) were expressed with no statistic differences (ANOVA, $n = 5$, $P > 0.1002$), whereas tubulin was significantly higher on day three than on day one, day two, respectively (ANOVA, $n = 5$, $P < 0.007$) (Fig. 2c). All the four antioxidant proteins increased with embryo age (ANOVA, $n = 5$, $P > 0.0009$) (Fig. 2d). Of the proteins related to cell metabolism and cell cycle, lethal (2) 37Cc and imaginal disc growth factor 4 increased on day three as compared to day

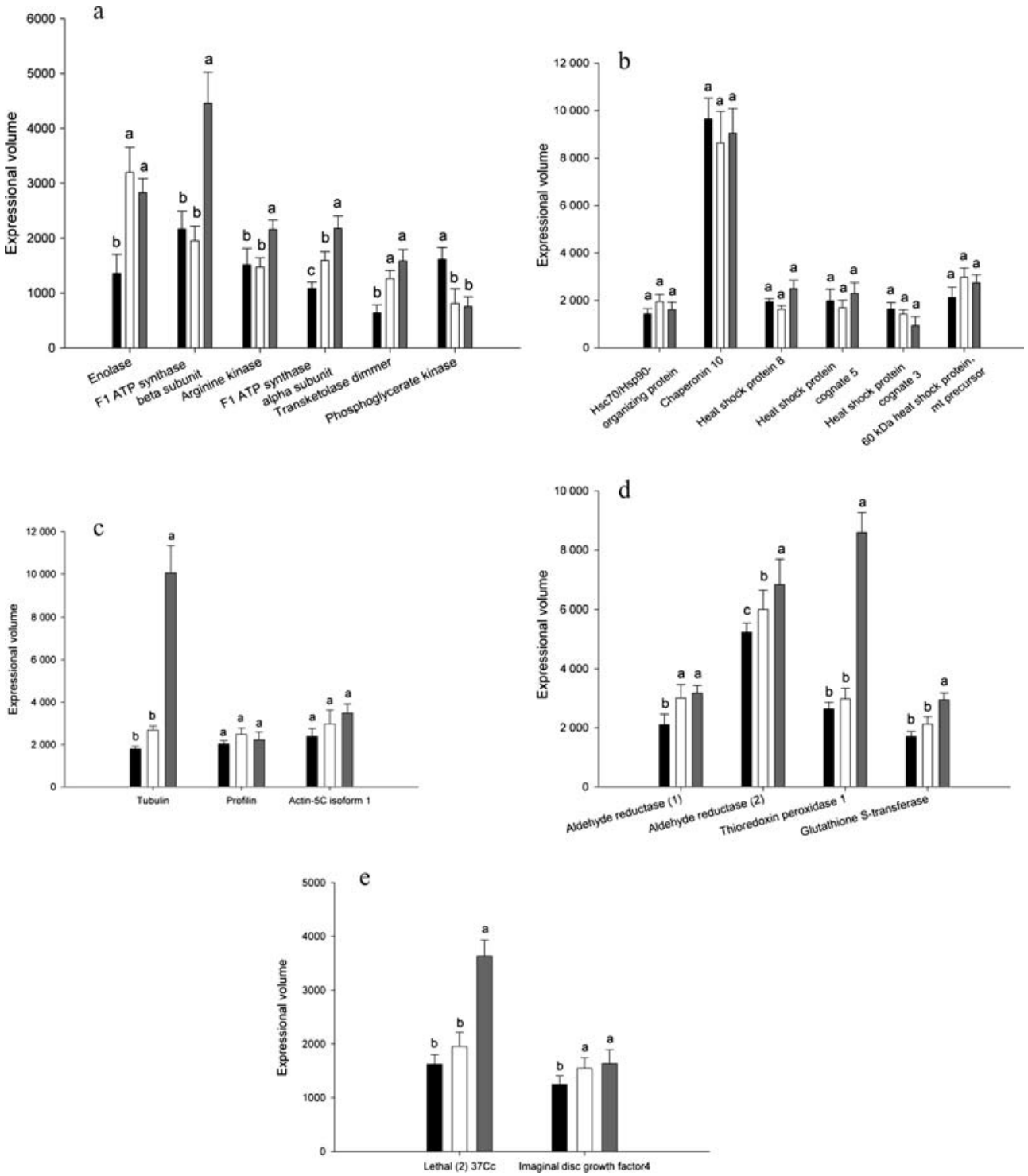

Figure 2. Expression levels of identified proteins. a, b, c, d and e represent six proteins related to the metabolism of carbohydrates and energy production, six heat shock proteins and four other proteins, three cytoskeletal proteins, four proteins related to the antioxidant system and two proteins related to growth regulation, respectively. Different lower case letters (a, b, c) above the bars indicate significant differences between day one, day two and day three eggs ($P < 0.05$). a is significant higher than b and c, b is significant higher than c. Black, white and gray bar represent the day one, day two and day three along with the embryonic development.

one and day two, respectively (ANOVA, $n = 5$, $P > 0.0016$) (Fig. 2e).

Discussion

The data presented here provide an experimental basis for identification of redundant proteins during the embryonic development of the honeybee. Based on detected protein spots, it can be concluded that the honeybee embryonic development is associated with a wide range of proteins. Among them, 38 highly abundant proteins were chosen for analysis because they were expressed across all three days of embryonic development. Of these, 21 gave mass fingerprinting spectra that allowed an unambiguous match to another honey bee protein. Although the most identified proteins increased, 14 percent of the unidentified proteins are decreased with embryo age, while 36 percent are over-expressed in older embryos.

The proteins identified as carbohydrate and energy metabolic enzymes are related to glycolysis (Enolase, Phosphoglycerate kinase, spots 1, 20), pentose phosphate pathway (transketolase, spot 19), ATP generation (F1 ATP synthase alpha and beta subunit, spots 16, 10, 15) (Table 1). Five of them were significantly increased with the age of embryos (Fig. 2a).This suggests that much metabolic energy is required to produce the honeybee embryo since some tissues must respond to the initiation of metamorphosis by increasing their metabolic activity for additional cell divisions and tissue metamorphosis. These results are in accordance with observations that the tracheal network becomes visible about 2 h before egg hatch, the upright embryos slowly flexes and abdominal peristalsis occurs (Collins, 2004). In comparison with *Drosophila*, the expression levels of genes involved in cellular differentiation change dynamically during metamorphosis, the genes encoding enzymes in the glycolytic pathway, citric acid cycle, glycogen synthesis and breakdown, and the pentose phosphate pathway are down-regulated during the late larval ecdysone pulse. This reduction may represent an early response in certain tissues that are destined to undergo programmed cell death (White *et al*., 1999).

Heat shock proteins (Hsps) and heat shock cognate proteins (Hscs) both belong to the heat shock protein superfamily. They are a group of proteins that are present in all cells in all life forms. They act like 'chaperones,' making sure that the cell's proteins are in the right shape and in the right place at the right time. They also shuttle proteins from one compartment to another inside the cell, and transport old proteins to 'garbage disposals' inside the cell (Binder, 2006; Hirsch *et al*., 2006). The enhanced expression of Hsps seen in cells from stressed bees suggests that this phenomenon could be a defense mechanism to prevent against stress tolerance. The only studies of Hsps and cell death in bee tissues have been with the midgut of *A. mellifera* larvae infected with *Paenibacillus larvae* or *Bacillus larvae* (Gregorc & Bowen, 1998; Gregorc & Bowen, 1999) and the larval salivary glands of *A. mellifera* treated with acaricides (Silva-Zacarin *et al*., 2006). The identified *Hsp*s, *Hsc*70/*Hsp*90-organizing protein (HOP, spot 2), *Hsp*10 (spot 6), *Hsp*a8 (spot 8), *Hsc*-5 (spot 11), *Hsc*-3 (spot13) and *Hsp*60 (spot 21), probably act as molecular chaperones, by taking part in functions such as transporting proteins into cellular compartments, folding of newly synthesized proteins in the cytosol, endoplasmic reticulum, and mitochondria, degradation of unstable or unfolded proteins, and prevention of their aggregation, thus contributing to cell maintenance (Cheng *et al*., 1990; Johnson *et al*., 2003; Goldfarb *et al*., 2006). Duo to our experiment was carried out at normal temperature conditions for the embryonic development, relative stable expression of these proteins (Fig. 2b) probably act as chaperones during protein folding and protein assembly processes (Lindquist & Craig, 1988; Binder, 2006; Hirsch *et al*., 2006) within the stage of the egg development. In this regard, this agrees well with the larval development of worker (Li *et al*., 2007).

Among the cytoskeletal proteins identified were β-Tubulin (spot 9), profilin (spot 14), actin-5C isoform 1 (spot 17). These proteins are components of a system of microtubules (MTs), cylindrical protein filaments found in all eukaryotes and are critically involved in a variety of cellular processes including cellular motility, cytoplasmic transport and mitosis (Tuszynski *et al*., 2006). MTs undergo cycles of rapid growth and disassembly in a process known as dynamic instability which has been extensively studied both *in vivo* and *in vitro* (Mitchison & Kirschner, 1984). This instability appears to be critical for proper MT function, especially during mitosis (Kirschner & Schulze, 1986). This could be indicative that deletion of gene encoding profilin of Drosophila results in a late embryonic lethal phenotype (Verheyen & Cooley, 1994). Queenright workers showed signs of a disorganized microtubule and microfilament system that could explain the histological evidence for progressive cell death observed in their germaria (Tanaka & Hartfelder, 2004). The increased β-tubulin (Fig. 2c) may agree with one of the two peaks arising in the longitudinal distribution pattern of the periplasm which coincides with the sites of the differentiation and mesodermal centers during cell division of honeybee eggs (Maul, 2005). Dynamic expression of these three structural proteins suggests that they play an important role in the egg mitosis and proliferation.

One class of proteins that in honeybees appears to deviate from general biochemical reasoning (Seehuus *et al*., 2006a) is involved in protection against reactive oxygen species (ROS). A major threat for organisms that depend on oxygen for metabolism is the production of ROS that cause oxidative damage of cellular components. It is assumed that due to a high demand for oxygen during embryonic development there is an increase in ROS. An

increase in oxidative damage has been documented (Corona & Robinson, 2006; Seehuus *et al*., 2006b). The present study identified thioredoxin peroxidase 1 (spot 5), a member of a thioredoxin system, which protects against oxidative stress by reducing peroxides such as H_2O_2 to harmless products (Chae *et al*., 1994; Netto *et al*., 1996); glutathione S-transferases (spot 7), another superfamily of detoxication proteins involved in the xenobiotic metabolic processes (Hayes & Pulford, 1995); and aldehyde reductase (spot 3, 4), one of several enzymes constituting the aldo-keto reductase superfamily that catalyzes the NADPH-dependent reduction of a wide variety of aldehydes (Barski *et al*., 1995), that are important components of this protective system. The increase of these proteins from days one to three (Fig. 2d) implies that embryos invest in higher levels of proteins involved in ROS protection during development.

As is the case with other animals, honeybee embryonic development requires the presence of growth factors in order to ensure its regulation. Within this group, lethal (2) 37Cc (spot 12) and imaginal disc growth factor 4 (IDGF) (spot 18) cooperate with insulin-like peptides to stimulate the proliferation, polarization and motility of imaginal disc cells (Kawamura *et al*., 1999). Lethal (2) 37Cc is required for larval metabolism from larvae to pupae, and is expressed in early embryos, late embryos, late third instar larvae and adults of the *Drosophila* (Black *et al*., 1987; McCrady & Tolin, 1994; Wright, 1996). The IDGFs function as cofactors of a *Drosophila* insulin-like molecule stimulating imaginal disc cell growth (Kawamura *et al*., 1999). The two proteins increased in this study (Fig. 2e) suggesting that three-day-older embryos need higher growth factors titers in general to regulate the embryonic development.

An initial goal of this study was to identify all the highly abundant proteins in embryogenesis. However, we identified just a part of them because our method could not effectively identify all the proteins. Therefore, an alternative strategy including LC-ESI-MS/MS or equivalent, capable of providing the sequence of the peptides should be taken into account for future work. Despite that many proteins acting in honeybee embryogenesis remain unidentified, our preliminary results provide solid experimental evidence for establishing an initial molecular picture of honeybee embryos. Hopefully these results will be useful for future genetic manipulation.

Experimental procedures

Chemicals

Immobilized pH gradients (IPG) strip (pH 3–10, Linear), two-dimensional gel electrophoresis (2-DE) marker, Bio-lyte (pH 3–10), mineral oil were purchased from Bio-Rad Laboratories Ltd (Hercules, CA, USA). Tris-base, ammonium persulfate (AP), sodium dodecyl sulfate (SDS), N,N,N′,N′-tetramethylethylene diamine (TEMED) and glycin were from Sigma (St. Louis, MO, USA). Acrylamide, N,N′-methylenebisacrylamide, Bromophenol Blue, Coomassie Brilliant Blue (CBB) G-250, thiourea, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), glycerol, bovine serum albumin (BSA) were purchased from Amresco (Solon, Ohio, USA). Agarose and urea were from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT) and iodoacetamide were from Merck. Trypsin was from Roche (Basel, Switzerland), trifluoroacetic acid (TFA), acetonitrile were from J. T. Baker (Phillipsburg, NJ, USA).

Biological samples

The bees (*Apis mellifera* L.) were from Pisa, Italy and were raised in an experimental apiary of the Bee Research Institute of the Chinese Academy of Agricultural Science, Beijing, China.

Worker bee eggs aged 24, 48 and 72 h were collected from frames containing eggs from March to June, 2007. In order to ensure that the eggs on day one, day two and day three would be sampled, the queen was confined to a chamber (the queen confinement frame) where only one empty frame could be placed. The queen was then left undisturbed to lay eggs in the cells on the frame for 5 h. Subsequently, the queen was removed from the chamber and the frame containing the eggs was placed in an area without queen access. The eggs of the worker bees of the first day were collected at 24 h with a plastic transfer tool. Employing the same method, the eggs of the second and the third day were collected at 48 and 72 h, respectively. A total of five batches of 280 eggs were collected per time point (i.e. at 24, 48 and 72 h respectively) from four colonies and analyzed by two-dimensional gel electrophoresis (2-DE).

Protein extraction

Protein extraction was according to Li *et al*. (2007). The eggs (1 mg eggs/10 μl buffer) were mixed in a phosphate buffer (PB) pH 7.6, containing 32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4, 400 mM NaCl. The mixture was homogenized for 20 min on ice and sonicated for 2 min, then centrifuged at 12 000 g and 4 °C for 10 min and centrifuged again at 15 000 g and 4 °C for 10 min. The supernatant was removed for subsequent use. The pellets (1 mg eggs/2 μl buffer) were mixed with PB pH 7.6, then centrifuged at 15 000 g and 4 °C for 10 min. The supernatant was removed and transferred into the tube containing the supernatant as a PB-soluble proteins extraction, while the pellets (1 mg eggs/10 μl buffer) and PB-insoluble proteins were mixed in a lysis buffer (LB) composed of 8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT, 2% Bio-lyte (pH 3–10). Next, the mixture was homogenized for 10 min on ice and sonicated for 2 min and centrifuged at 15 000 g and 4 °C for 10 min. The supernatant was removed and mixed with the PB-soluble proteins extraction and the debris was discarded. Trichloracetic (TCA) was added to the collected supernatants to final concentration of 10% and the mixture was kept on ice for 10 min to ensure the precipitating of proteins and desalting. Subsequently, the mixture was centrifuged at 15 000 g at 4 °C for 10 min. The supernatant was discarded and the pellets (1 mg eggs/4 μl buffer) were resolved in LB, then the mixture was homogenized for 5 min on ice, sonicated for 2 min and subsequently adjusted to pH 7.0 with 2 M NaOH. The final mixture containing the protein extracts of the worker bee eggs was stored at −70 °C for further use. Total protein concentration was determined according to Bradford (1976) using BSA as a standard.

Two-dimensional gel electrophoresis (2-DE)

The protein sample was suspended in LB and then mixed with rehydration buffer (8 M urea, 2% CHAPS, 0.001% bromophenol

blue, 45 mM DTT, 0.2% Bio-lyte pH 3–10). The mixture containing 470 μg proteins was loaded on a 17 cm IPG strip (pH 3–10, linear, Bio-Rad). 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 000Vh. 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, 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 on 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 6.5 h.

Image acquisition and analysis

Gels were fixed overnight in 50% (v/v) ethanol with 10% (v/v) acetic acid, washed with water, and stained with Colloidal Coomassie Blue G-250 (CCB). After washing with water, gels were scanned, annotated, and analysed for spot number using PDQuest V 7.3.0 (Bio-Rad). The best five runs with good reproducibility from five replicate samples were subjected to analysis.

Statistics

In order to accurately compare spot quantities between gels with variation in spot size and intensity not due to differential protein expression, the mean normalized volume of spots was performed with profilin as a protein standard compensating for the non-expression-related variations in spot intensity. ANOVA (Version 6.12, SAS Institute, Cary, NC, USA), was used to test the significance of the normalized volume in total density of identified proteins in all gels. An error probability of $P < 0.05$ was considered to be statistically significant.

Tryptic digestion

The CBB stained spots were excised and destained for 30 min using 100 ml acetonitrile (50%) and 25 mM $(NH_4)HCO_3$ pH 8 (50%) repeated two or three times until the gel was transparent, and dehydrated for 10 min with acetonitrile (100%). The gels were dried for 30 min using a Speed-Vac system. Then 2.5 ml of 25 mM $(NH_4)HCO_3$ was added to the 25 μg trypsin (final concentration 10 ng/μl); 10 μl of this solution was pipetted on each dried protein spot and incubated for 60 min at 4 °C. The supernatant was discarded to minimize auto digestion of trypsin. Then the Eppendorf tube was turned upside down and 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 were added and incubated for 60 min at 37 °C. Thereafter, 20 μl of 50% (v/v) acetonitrile [containing 2.5% (v/v) TFA] acid were added to the gel and incubated for 60 min at 30 °C. After each step the supernatants were pooled and dried using a Speed-Vac system.

Mass spectrometry and protein identification

Before obtaining the mass spectra of the peptide mixture, the digested peptides were desalted and cleaned with ZipTip C18 pipette tips (Millipore Corp., Bedford, MA, USA) according to the manufacturer's instructions. All analyses were performed using a Bruker Daltonics Autoflex (Bruker Daltonics, Billerica, Mass. USA) operated in the delayed extraction of 190 ns and reflector mode with an accelerating voltage of 20 KV. The peptide mixture was analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics) in 50% acetonitrile/0.1% trifluoroacetic acid. External calibration was performed with a peptide calibration standard (Bruker Daltonics, Part No.: 206 195) and internal calibration with trypsin autoproteolytic fragments. Finally, the masses of proteolytic peptide fragments, were obtained by peptide mass fingerprinting (PMF), a mass spectrometry based protein identification technique. To interpret the mass spectra of protein digests, the generated peaks lists of the tryptic peptide masses were queried against all entries in the NCBI nr (non-redundant) database using the MASCOT search engine of Matrix Science Ltd (http://www.matrixscience.com/search_form_select.html) and Xproteo (http://xproteo.com:2698). Search parameters for MASCOT were: trypsin cleavage; allow up to one missed cleavage; no restriction on protein mass; peptide mass tolerance 0.2 Da; variable modification: oxidation (M); and search parameters for Xproteo were: trypsin cleavage; allow up to one missed cleavage; protein mass 0–300 kDa; protein pI 1–14; peptide mass tolerance 0.2 Da. Only proteins identified with a Mowse score greater than 81 (significant at 95% confidence interval) and $d' \geq 4$ (significant at 99% confidence interval) are reported in the honeybee genome, the score for the top match the score for the highest ranked hit to a non-homologous was not reported. The results of the two different search engines integrated and Mascot was used in the first place. If a protein was identified by one and the other was not included.

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