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Profiling the proteomics in honeybee worker brains submitted to the proboscis extension reflex

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ABSTRACT

The proboscis extension reflex (PER) is an unconditioned stimulus (US) widely used to access the ability of honeybees to correlate it with a conditioned stimulus (CS) during learning and memory acquisition. However, little is known about the biochemical/genetic changes in worker honeybee brains induced by the PER alone. The present investigation profiled the proteomic complement associated with the PER to further the understanding of the major molecular transformations in the honeybee brain during the execution of a US. In the present study, a quantitative shotgun proteomic approach was employed to assign the proteomic complement of the honeybee brain. The results were analyzed under the view of protein networking for different processes involved in PER behavior. In the brains of PER-stimulated individuals, the metabolism of cyclic/heterocyclic/aromatic compounds was activated in parallel with the metabolism of nitrogenated compounds, followed by the up-regulation of carbohydrate metabolism, the proteins involved with the anatomic and cytoskeleton; the down-regulation of the anatomic development and cell differentiation in other neurons also occurred.

Significance: The assay of proboscis extension reflex is frequently used to access honeybees' ability to correlate an unconditioned stimulus with a conditioned stimulus (such as an odor) to establish learning and memory acquisition. The reflex behavior of proboscis extension was associated with various conditioned stimuli, and the biochemical/genetic evaluation of the changes occurring in honeybee brains under these conditions reflect the synergistic effects of both insect manipulations (training to answer to an unconditioned stimulus and training to respond to a conditioned stimulus). Little or no information is available regarding the biochemical changes stimulated by an unconditioned stimulus alone, such as the proboscis extension reflex. The present investigation characterizes the proteomic changes occurring in the brains of honeybee workers submitted to proboscis extension reflex. A series of metabolic and cellular processes were identified to be related to the reflex of an unconditioned stimulus. This strategy may be reproduced to further understand the processes of learning and memory acquisition in honeybees.

1. Introduction

Despite a small brain size, honeybees have been used as important models in neurobiology; these insects exhibit a wide repertoire of behaviors and a remarkable ability to learn [1]. Honeybees can identify odors and colors [2], as well the shapes and the relationships between objects such as sameness/differences [3,4], the order of the objects [5], numerical attributes associated with the objects [6], and relative positioning above/bellow [7]. According to Zhang et al. (2006) [8] and Pahl et al. (2007) [9], these insects can learn and make decisions regarding where/when to go within a temporal scale; they even have the ability to associate elements of the circadian cycle.

Their outstanding olfactory memory is important in the search for food and may be developed in association with a series of various clues that are important in foraging [10,11]. These abilities appear to be important in ensuring that worker bees develop navigational routes, establish landmarks for these routes, and identify their forage sources [1,12,13].

The proboscis extension reflex (PER) represents an experimental strategy to assess honeybees' ability to exhibit an unconditioned stimulus (US) [14]. This behavior represents an effective paradigm for analyzing the physiology and psychological rules underlying the behavioral repertoire of these insects [15]. Notably, PER is an aspect of honeybee feeding behavior; when the antenna is stimulated by contact with a solution of carbohydrates, the insects automatically extend their proboscis

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to drink the sugars solution, thereby constituting a reflex response to a US [16,17]. Honeybees acquire/develop an olfactory memory from the food source during visits to flowers for collecting nectar and transfer this memory to the paradigm in the laboratory during experiments [17]. The PER paradigm is frequently used to investigate learning and memory in honeybees, especially in evaluating associative learning between US and different types of CS, such as exhibiting PER each time that an identified odor is associated with a reward offered to the bees (such as drinking a solution of carbohydrates), a classic method of Pavlovian conditioning [18].

The molecular mechanisms of olfactory learning and memory in honeybees have been thoroughly investigated to improve understanding at the biochemical and physiological levels. Thus, a correlation between a reduced level of expression of protein kinase A and *N*-methyl-D-aspartate receptors and the impairment of the long-term memory of workers of *Apis mellifera* was found [19,20]. Other studies also reported the involvement of various protein types in learning and memory acquisition in the honeybee brain; thus, the expression levels of the receptors for acetylcholine, octopamine, dopamine, and glutamate [21,22,23,24] and calcium/calmodulin-dependent kinase II [25] are correlated with the abovementioned processes. Honeybees submitted to training for olfactory memory presented the down-regulation of many protein-coding genes [26,27]. A similar conclusion was also reported in a study of differentially expressed mRNA in honeybees after maze learning assays [28]. Hadar and Menzel (2010) [29] demonstrated that excitatory and inhibitory learning occur through different molecular circuits and that the blocking of protein synthesis inhibited the consolidation of the excitatory learning, indicating that this process is directly dependent on the synthesis of proteins in the honeybee brain.

Despite the large number of studies related to the learning and memory acquisition using PER as an experimental strategy to assess the honeybees' ability to exhibit a US, very few studies have examined the genetic, biochemical, and physiological processes demanded by the PER alone. The studies generally focus on the effects of a PER associated with a CS; therefore, even the major biochemical processes involved with this reflex behavior are unknown. Thus, the main objective of the present investigation is to profile the proteomic complement associated with PER, to further understand the major molecular transformations occurring in the honeybee brain during the execution of a US. In the present study, a shotgun approach combined with a label-free strategy for protein quantification was applied to determine the proteomic complement of the honeybee brain. The results were analyzed in light of the proteins networking for different processes involved with PER reflex behavior.

2. Experimental section

2.1. Materials

A Qubit® Protein Assay Kit and RapiGest SF acid-labile surfactant were purchased from Invitrogen (Carlsbad, CA) and Waters Corp. (Milford, MA), respectively. The sequencing-grade modified trypsin was purchased from Promega (San Luis Obispo, CA, USA). All other laboratory reagents were acquired from Sigma–Aldrich (St. Louis, MO).

2.2. PER test

Honeybees (*A. mellifera*) were maintained in the apiary of the Bioscience Institute of the University of Sao Paulo State, Campus of Rio Claro, SP, Brazil. The colony used in the experiments was well formed, free of disease, well fed, and in possession of a laying queen. Initially, a honeycomb containing larvae (free of bees) was collected from the colony and maintained for 24 h inside a Biochemical Oxygen Demand incubator (ELECTROlab) previously set to 32 °C and 70% relative humidity. Newly emerged workers were marked (day 0) on the thorax using a nontoxic paint and returned to the colony for later capture when they reached 20 days of age.

The behavior of "Proboscis Extension Reflex" (PER) was used as an input reference for neuroproteomic studies. PER is the proboscis extension by an insect as a reflex to antennal stimulation. Individual bees were securely attached to the tube with small pieces of tape to restrain the bees without harming them. Their heads protruded from the tube, and only the antennae and mouthparts could move freely. Then, each bee was subjected to sucrose odor (PER group). Each trial involved one stimulation with sucrose solution and one stimulation with water with an interval of 1 min between the stimulations; the interval between one trial and another was 5 min. The bees were submitted to a total of five trials.

The PER group was composed of individuals that responded to stimuli with sucrose (80% v/v) but did not respond to water in any of the trials, and the control group was submitted to the same conditions applied to the PER group, with the exception of not exposing the insects to the sucrose odor. After collection, the bees were immediately frozen in liquid nitrogen where they remained until the head was dissected in a cocktail of protease inhibitor for sample preparation.

2.3. Sample preparation and in-solution digestion

Proteins were extracted using RapiGest surfactant (0.2% final) and macerated for 5 min. Subsequently, the samples were centrifuged for 15 min at 20,000 × *g* to remove insoluble material. Afterwards, the total protein concentration was determined with the Qubit® Protein Assay Kit together with the Qubit® 3.0 fluorometer according to the manufacturer's instructions. Two hundred micrograms of proteins were reduced with 10 mM (final) of dithiothreitol for 30 min at 60 °C and, after cooled to room temperature, the proteins were alkylated with 30 mM (final) of iodoacetamide for an additional 35 min at room temperature. Finally, the proteins were digested with sequencing-grade modified trypsin added at an enzyme/substrate ratio of 1:50 (w/w). The digestion was performed for 18 h at 37 °C. The reaction was stopped with trifluoeacetic acid to a final concentration of 0.4% and incubated at 37 °C for an additional 40 min to enhance the hydrolysis of RapiGest. Subsequently, the sample was centrifuged for 10 min at 20,000 × *g* at 4 °C to remove insoluble material. The digested peptide mixture was equally divided into three aliquots of 10 μg, which were individually desalted over stage tip in-house columns that were packed with SPE disks (C_{18}, 47 mm) and dried in a vacuum.

2.4. LC–MS/MS acquisition

The samples were subjected to LC–MS/MS analysis with a Thermo Scientific Easy-nLC 1000 ultra-high-performance liquid chromatography (UPLC) system coupled with a LTQ-Orbitrap XL ETD mass spectrometer (Mass Spectrometry Facility-RPT02H PDTIS/Carlos Chagas Institute-Fiocruz Paraná), as follows. The peptide mixtures were loaded onto a column (75 μm i.d., 15 cm long) packed in-house with a 3.2 μm ReproSil-Pur C_{18}-AQ resin (Dr. Maisch) with a flow of 500 nL/min and subsequently eluted with a flow of 250 nL/min from 5% to 40% ACN in 0.5% (v/v) formic acid and 0.5% (v/v) DMSO in a 120 min gradient [30]. The mass spectrometer was set in a data-dependent mode to automatically switch between MS and MS/MS (MS_2) acquisition. Survey full-scan MS spectra (from m/z 300–2000) were acquired in the Orbitrap analyzer with the resolution $R = 60{,}000$ at m/z 400 (after accumulation to a target value of 1,000,000 in the linear trap). The ten most intense ions were sequentially isolated and fragmented in the linear ion trap using collisional induced dissociation at a target value of 10,000. Previous target ions selected for MS/MS were dynamically excluded for 90 s. The total cycle time was approximately 3 s. The general mass spectrometric conditions were spray voltage, 2.4 kV; no sheath and auxiliary gas flow; ion transfer tube temperature 175 °C; collision gas pressure, 1.3 mTorr; normalized energy collision energy using wide-band activation mode;

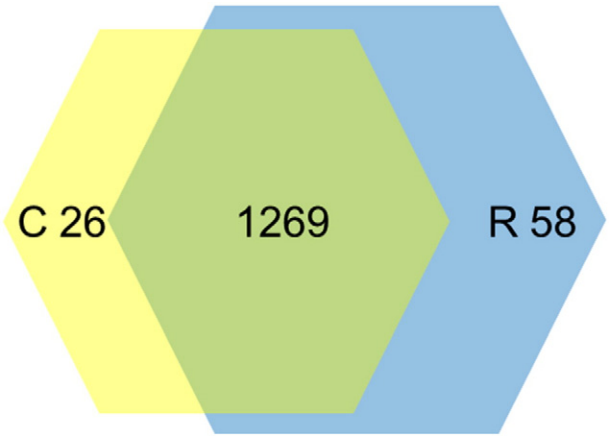

Fig. 1. Venn Diagram of identified proteins. C—number of proteins uniquely identified in the control group; R—number of proteins uniquely identified in the PER group.

35% for MS_2. The ion selection threshold was 250 counts for MS_2. An activation q = 0.25 and activation time of 30 ms were applied in MS_2 acquisitions. Technical replicates were performed with and without multistage activation.

2.5. Mass spectrometry data analysis

The reference proteome set of *Apis*, composed of 72,781 sequences, was downloaded from the NCBI (http://www.ncbi.nlm.nih.gov/) on February 06, 2015. PatternLab v3.2 [31] was used for generating a target-decoy database by grouping subset sequences, adding the sequences of 127 common mass spectrometry contaminants, and, for each sequence, including a reversed version of it. The search was performed using PatternLab's integrated Comet [32] search engine. Briefly, the search parameters included fully tryptic and semi-tryptic peptide candidates with masses between 600 and 6000 Da. The modifications were the carbamidomethylation of cysteine and the oxidation of methionine as fixed and variable, respectively. The precursor tolerance was 40 ppm, and we used bins of 1.0005 Da for analyzing the fragment ion scans. The validity of the peptide sequence matches (PSM) was assessed using PatternLab's Search Engine Processor module (SEPro) [33]. Briefly, identifications were grouped by charge state (+2 and ≥+3) and then by tryptic status (tryptic and semi-tryptic), resulting in four distinct subgroups. For each group, Comet's XCorr, DeltaCN, and Secondary score values were used to generate a Bayesian discriminating function. A cutoff score was established to accept a false discovery rate (FDR) of 1% based on the number of decoys. This procedure was independently performed on each data subset, resulting in a false-positive rate that was independent of tryptic status or charge state. Proteins identified with a single mass spectrum required the identification to have an XCorr greater than 2.5. A minimum sequence length of 6 amino acid residues was required. The results were post-processed to accept PSM with less than 7 ppm. The proteins were grouped according to maximum parsimony using the bipartite graph approach [34].

2.6. Label-free quantitation

A label-free protein quantitative analysis was performed according to the normalized spectral abundance factor (NSAF) [35] provided by Search Engine Processor (SEPro). Differentially abundant proteins were pinpointed using PatternLab's [Referencia] TFold Module [Referencia]. Briefly, the later relies on a theoretical FDR estimator (Benjamini–Hochberg [Referencia]) to maximize the number of identifications that satisfy both a fold-change cutoff that varies with the *t*-test *p*-value as a power law and a stringency criterion that aims to detect lowly abundant proteins that could yield false positives.

Table 1
Proteins identified uniquely in the control group. Results below listed only proteins obtained for *p*-values < 0.01.

Accession code	Protein	Function
572316394	Gephyrin-like isoform X1 [*Apis dorsata*]	Establishment of synaptic specificity at neuromuscular junction
572309684	Cytochrome *c* oxidase subunit 5B, mitochondrial-like [Apis dorsata]	Mitochondrial electron transport; gene expression
820861115	Cell cycle control protein 50A isoform X1 [*Apis florea*]	Transport
572266110	Eukaryotic peptide chain release factor subunit 1-like isoform X1 [Apis dorsata]	Translation; gene expression; directs the termination of nascent peptide synthesis (translation) in response to the termination codons UAA, UAG, and UGA
820864455	E3 ubiquitin-protein ligase SMURF2 isoform X1 [Apis florea]	Gene expression; protein ubiquitination involved in ubiquitin-dependent protein catabolic process
572307607	Microsomal triglyceride transfer protein large subunit-like [Apis dorsata]	Lipid transport
820865977	Alpha-*N*-acetylglucosaminidase [Apis florea]	Carbohydrate metabolic process
28201825	Melittin	It increases the permeability of cell membranes to ions, particularly Na + and indirectly Ca2 +
48101413	tRNA N6-adenosine threonylcarbamoyltransferase [*Apis mellifera*]	tRNA processing
571572819	85/88 kDa calcium-independent phospholipase A2-like [Apis mellifera]	Lipid metabolic process; Fc-gamma receptor signaling pathway involved in phagocytosis
380021437	cGMP-dependent protein kinase, isozyme 2 forms cD5/T2 [Apis florea]	Signal transduction; neuron migration
380017597	Putative ATP-dependent RNA helicase Pl10 [Apis florea]	Translational initiation; cell differentiation
571501059	Importin subunit alpha-3 [Apis mellifera]	Cytokine-mediated signaling pathway
380028389	Uncharacterized protein LOC100866826 [Apis florea]	Unknown
820858777	Acetyl-CoA acetyltransferase, cytosolic [Apis florea]	Response to starvation; ketone body biosynthetic process; brain development
66508242	Cysteine sulfinic acid decarboxylase-like isoform X3 [Apis mellifera]	Carboxylic acid metabolic process; taurine biosynthetic process
820865075	Uncharacterized protein LOC100872391 [Apis florea]	Unknown
572318265	Metaxin-2-like isoform X2 [Apis dorsata]	Protein targeting to mitochondrion
820838108	Type-2 histone deacetylase 1 [Apis florea]	Transcriptional regulation
110755983	Coiled-coil domain-containing protein 58-like [Apis mellifera]	Unknown
571577446	Ubiquitin carboxyl-terminal hydrolase isoform X1 [Apis mellifera]	DNA repair; protein deubiquitination
380013771	ATP-binding cassette subfamily E member 1 [Apis florea]	May protect against heart failure under conditions of tachycardic stress; transport
373203037	Dehydrogenase/reductase SDR family member 7 [*Apis cerana*]	Oxidation-reduction process
572306641	DNA mismatch repair protein Mlh3-like [Apis dorsata]	Mismatch repair; protein localization
571507718	nesprin-1-like [Apis mellifera]	May be involved in the maintenance of nuclear organization and structural integrity
572305886	Methionine–tRNA ligase, cytoplasmic-like [Apis dorsata]	Gene expression; translation

Table 2
Proteins identified uniquely in the PER group. Results below listed only proteins obtained for p-values <0.01.

Accession code	Protein	Function
572303892	Tropomyosin-1, isoforms 9 A/A/B-like isoform X34 [Apis dorsata]	Muscle contraction; dendrite morphogenesis
20138953	Full = Major royal jelly protein 3	Nutrition
572308465	Troponin I-like isoform X21 [Apis dorsata]	Muscle contraction
373211472	40S ribosomal protein S23 [Apis cerana]	Tanslation; gene expression
380030096	60S ribosomal protein L37a [Apis florea]	Translation; focal adhesion
572297073	Protein FAM162B-like [Apis dorsata]	Integral component of membrane
328782283	Tumor suppressor candidate 3-like [Apis mellifera]	Protein *N*-linked glycosylation; transport; cognition
380014359	Scavenger receptor class B member 1 [Apis florea]	Receptor-mediated endocytosis; cell adhesion
380028021	Bifunctional purine biosynthesis protein PURH [Apis florea]	Purine biosynthesis; brainstem development; cerebral cortex development
328777128	Putative peptidyl-tRNA hydrolase PTRHD1-like isoformX2 [Apis mellifera]	Aminoacyl-tRNA hydrolase activity
571574382	Chymotrypsin inhibitor-like isoform X3 [Apis mellifera]	Peptidase inhibitor activity
820848042	dnaJ homolog subfamily C member 22 [Apis florea]	Protein refolding
380015267	Persulfide dioxygenase ETHE1, mitochondrial isoform X1 [Apis florea]	hydrogen sulfide catabolism in the mitochondrial matrix; glutathione metabolic process.
380017778	Programmed cell death protein 5 [Apis florea]	Apoptotic process; cellular response to transforming growth factor beta stimulus;
571527764	Papilin-like isoform X2 [Apis mellifera]	Cell rearrangements; modulating metalloproteinases action during organogenesis
571573872	Hydroxymethylglutaryl-CoA lyase, mitochondrial-like isoform X1 [Apis mellifera]	Ketogenesis (alternative source of energy to glucose); mitochondrion organization
66514532	Guanine nucleotide-binding protein subunit beta-2-like [Apis mellifera]	Recruitment, assembly and/or regulation of a variety of signaling molecules
572314172	Nucleolar protein 58-like isoform X2 [Apis dorsata]	rRNA modification; cell growth
572271381	Putative ferric-chelate reductase 1 homolog isoform X1 [Apis dorsata]	Oxidation-reduction process
820844091	Asparagine synthetase [glutamine-hydrolyzing] isoform X1 [Apis florea]	*L*-asparagine biosynthesis; response to nutrient levels
820866486	Small ubiquitin-related modifier 3 [Apis florea]	Nuclear transport, DNA replication and repair, mitosis and signal transduction; post-translational protein modification
571576375	Long-chain fatty acid transport protein 4 [Apis mellifera]	Response to nutrient; transmembrane transport
572317990	PX domain-containing protein kinase-like protein-like isoform X1 [Apis dorsata]	Binds to and modulates brain Na,K-ATPase subunits ATP1B1 and ATP1B3 and may thereby participate in the regulation of electrical excitability and synaptic transmission.
572297844	GDP-Man:Man(3)GlcNAc(2)-PP-Dol alpha-1,2-mannosyltransferase-like [Apis dorsata]	Biosynthetic process; post-translational protein modification
380024704	Dihydroorotate dehydrogenase (quinone), mitochondrial [Apis florea]	Response to starvation; oxidation-reduction process
820844631	Small nuclear ribonucleoprotein Sm D3 [Apis florea]	Splicing of cellular pre-mRNAs; central nervous system development; neuron differentiation
820839774	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7 isoform X2 [Apis florea]	Transfer of electrons from NADH to the respiratory chain; respiratory electron transport chain
373204224	Septin-2 [Apis cerana]	Cytokinesis; neurogenesis; regulation of *L*-glutamate transport
380014988	Apolipophorins [Apis florea]	Transport for various types of lipids; Wnt signaling pathway; transport
572298373	Apolipophorins-like [Apis dorsata]	Transport for various types of lipids; Wnt signaling pathway; transport
820841040	Coatomer subunit beta [Apis florea]	Mediate biosynthetic protein transport from the ER, via the Golgi up to the trans Golgi network
380017696	Mitochondrial import receptor subunit TOM40 homolog 1-like [Apis florea]	Channel-forming protein essential for import of protein precursors into mitochondria
380022501	Endocuticle structural glycoprotein SgAbd-1-like [Apis florea]	Structural constituent of cuticle
380022594	Endocuticle structural glycoprotein SgAbd-2-like isoform X1 [Apis florea]	Structural constituent of cuticle
373208644	Protein lethal(2)essential for life [Apis cerana]	Protein lipidation; regulation of translational initiation by eIF2 alpha phosphorylation
571540960	Non-specific lipid-transfer protein-like isoform X1 [Apis mellifera]	May play a role in regulating steroidogenesis
571576907	26S protease regulatory subunit 7 [Apis mellifera]	ATP-dependent degradation of ubiquitinated proteins; protein polyubiquitination
300593245	Unnamed protein product [Apis mellifera]	Unknown
572305981	Mitochondrial import inner membrane translocase subunit Tim8-like [Apis dorsata]	Import and insertion of some multi-pass transmembrane proteins into the mitochondrial inner membrane; nervous system development;
380020436	Regucalcin-like isoform X2 [Apis florea]	Modulates Ca^{2+} signaling, and Ca^{2+}-dependent cellular processes and enzyme activities
110758964	Regucalcin-like [Apis mellifera]	Modulates Ca^{2+} signaling, and Ca^{2+-}dependent cellular processes and enzyme activities
572302484	Solute carrier family 25 member 46-like [Apis dorsata]	Transport
572307192	Neuronal calcium sensor 2-like isoform X3 [Apis dorsata]	Neuronal calcium sensor, regulator of G protein-coupled receptor phosphorylation in a calcium dependent manner
571514423	Twitchin [Apis mellifera]	Regulator of muscle contraction and relaxation; protein phosphorylation
571506597	Histone H1.2-like [Apis mellifera]	Histones H1 are necessary for the condensation of nucleosome chains into higher-order structures
572313551	Protein lethal(2)essential for life-like [Apis dorsata]	protein lipidation; regulation of translational initiation by eIF2 alpha phosphorylation
572304388	Probable small nuclear ribonucleoprotein Sm D1-like [Apis dorsata]	Essential for pre-mRNA splicing; mRNA processing
66523683	Aspartate-tRNA ligase, cytoplasmic	Translation; tRNA

Table 2 (*continued*)

Accession code	Protein	Function
	[Apis mellifera]	aminoacylation for protein translation
380015451	Tetra-peptide repeat homeobox protein 1-like [Apis florea]	DNA binding
572313862	PDZ and LIM domain protein Zasp-like isoform X1 [Apis dorsata]	Regulator of cell matrix adhesion; act as an adapter that brings other proteins (like kinases) to the cytoskeleton
571525756	Uncharacterized protein LOC408779 [Apis mellifera]	Unknown
66532125	Eukaryotic translation initiation factor 3 subunit J isoform 1 [Apis mellifera]	Required for several steps in the initiation of protein synthesis; neurogenesis
66511652	Asparagine–tRNA ligase, cytoplasmic-like [Apis mellifera]	Translation; tRNA aminoacylation for protein translation
571505185	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial-like [Apis mellifera]	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase
449,310,784	Dehydrogenase/reductase SDR family member 4 [Apis mellifera]	Alcohol metabolic process; protein tetramerization; receptor binding
571546137	Pupal cuticle protein-like [Apis mellifera]	Chitin-based cuticle development
328783881	slit homolog 2 protein-like isoform X1 [Apis mellifera]	Nervous system development; axonogenesis; axon guidance; olfactory bulb development
571532852	UDP-glucuronosyltransferase 2B13-like [Apis mellifera]	Flavonoid biosynthetic process; xenobiotic metabolic process

2.7. Functional and Gene Ontology (GO) analysis

The Blast2GO algorithm (https://www.blast2go.com/) [36] was used to report the biological process categorization of the identified proteins according to Gene Ontology, which contains ontologies and a subset of the terms found in the entire GO (http://www.geneontology.org) [37]. The categories for each classification are nonexclusive (i.e., a number of candidates were found to be localized in more than one cellular compartment). Proteins were classified into functional categories using Blast2GO, and the representation of statistically enriched gene ontology was performed with the algorithm Gene Ontology Enrichment Analysis Software (GOEAST—http://omicslab.genetics.ac.cn/GOEAST/) [38]. The proteins annotated with Blast2GO were submitted as background list to GOEAST, configured to run as a hypergeometric test, adjusted for raw *P*-values into false discovery rate (FDR) using the Banjamini-Yekutieli method [39].

2.8. Network analysis

The list of the identified proteins was subjected to Search Tool for the Retrieval of Interacting Genes/Proteins (STRING V10.0, http://string-db.org) [40] analysis to reveal functional interactions between proteins. Active prediction methods used in our analysis were neighborhood, co-expression, gene fusions, experiments, co-occurrence, databases, and text mining, all with high confidence (0.7). Each node represents a protein, and each edge represents an interaction.

3. Results

The experimental approach using a shotgun proteomic analysis of the honeybee brain permitted the reliable identification of 1713 proteins, satisfying a 1% false discovery rate considering the redundancies in the sequence database (Table S1 in the online version at http://dx.doi.org/10.1016/j.jprot.2016.05.029.); after applying the maximum parsimony criterion on this list, the number of proteins was reduced to 1353. When the proteomic complements of both groups (control and PER-stimulated) were compared and represented using a Venn diagram (Fig. 1), 1269 proteins were found to be common to both groups, whereas the control group presented 26 unique proteins (Table 1) and the PER group presented 58 unique proteins (Table 2). Among the proteins common to both groups, the quantitative analysis revealed 44 differentially abundant proteins, 18 of which were up-regulated (Table 3) and 26 of which were down-regulated (Table 4) in the PER group.

All the identified proteins were then analyzed using the Blast2GO algorithm for categorizing the proteins according to biological process at the fourth level (Table S2 in the online version at http://dx.doi.org/10.1016/j.jprot.2016.05.029.), and a summary of this analysis is presented in Fig. 2. This result shows that the major biological processes in the honeybee brain are related to transport, the processing of small

Table 3
Proteins that presented up-regulation in PER group, when compared to the control group. Results below listed only proteins obtained for *p*-values <0.01.

Accession code	Fold Change	Protein	Function
20138892	−5.202536	Major royal jelly protein 2; Short = MRJP-2	Plays an important role in honeybee nutrition
572264329	−3.732665	Muscle-specific protein 20-like [Apis dorsata]	Maintenance of the neuromuscular junction; synapse; phosphorylation
380017591	−2.683406	60S ribosomal protein L8 [Apis florea]	Translation; centrosome duplication
20138866	−2.206654	Major royal jelly protein 1; short = MRJP-1	Caste determination, defense against microorganism infection
572315191	−1.981185	60S ribosomal protein L30-like [Apis dorsata]	Translation; focal adhesion
571544763	−1.953545	Putative acyl-CoA-binding protein-like [Apis mellifera]	Energy metabolism; transport; learning and memory; long-term synaptic potentiation
66552169	−1.949275	60S ribosomal protein L28 [Apis mellifera]	Translation; gene expression; neurogenesis
571500813	−1.863367	40S ribosomal protein S17 [Apis mellifera]	Translation; gene expression; focal adhesion
572302099	−1.773173	60S ribosomal protein L4-like [Apis dorsata];	Translation; centrosome duplication; gene expression; focal adhesion
571500154	−1.740841	Alkyldihydroxyacetonephosphate synthase-like [Apis mellifera]	Ether lipid biosynthesis; oxidation-reduction process
380027232	−1.643647	40S ribosomal protein S4 [Apis florea]	Translation; focal adhesion
571550343	−1.579705	Fatty acid synthase-like [Apis mellifera]	Regulation of muscle contraction; troponin complex
572299096	−1.506677	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type-like isoform X4 [Apis dorsata]	Glutamine metabolic process
284812514	−1.480842	MRJP5 [Apis mellifera]	Caste determination, influence by environmental factors
78101800	−1.471263	TPA_exp: troponin T isoform 4 [Apis mellifera]	Signal peptide; peripheral nervous system axon regeneration; calcium ion binding
380029418	−1.461579	Uncharacterized protein LOC100871673 [Apis florea]	Structural constituent of ribosome; translation
94400893	−1.43053	Troponin T, skeletal muscle [Apis mellifera]	Structural constituent of ribosome; translation; RNA binding
820837885	−1.355229	40S ribosomal protein S6 [Apis florea]	Mitochondrion degradation; regulation of autophagy; multicellular organismal aging; protein homodimerization activity

Table 4
Proteins that presented down-regulations in PER group, when compared to the control group. Results below listed only proteins obtained for *p*-values <0.01.

Accession code	Fold change	Protein	Function
66514595	1.25493	Ras-related protein Rab-3 isoformX2 [Apis mellifera]	Multicellular organismal development;
571554588	1.259689	2-oxoglutarate dehydrogenase, mitochondrial-like isoform X5 [Apis mellifera]	Tricarboxylic acid cycle; oxidation-reduction process
335892820	1.383799	Pyruvate dehydrogenase E1 component , mitochondrial [Apis mellifera]	Protein transport; signal transduction; GTP binding
571575167	1.431369	NADP-dependent malic enzyme isoform X1 [Apis mellifera]	Proteasome-mediated ubiquitin-dependent protein catabolic process
507418852	1.439915	Rab escort protein [Apis mellifera]	Transferase activity; cell cycle
380028013	1.466512	ATP-dependent RNA helicase WM6 [Apis florea]	mRNA export
380026816	1.496064	Mitochondrial fission 1 protein [Apis florea]	Apoptotic process; peroxisome fission
571537241	1.517303	Transcriptional activator protein Pur-beta-B-like isoform X2 [Apis mellifera]	Cell differentiation; cell proliferation
295422209	1.532515	Unnamed protein product [Apis mellifera]	Intracellular signal transduction; protein ubiquination
66561330	1.539567	Phosphoglucomutase isoform X3 [Apis mellifera]	Breakdown and synthesis of glucose; cell adhesion
820856961	1.552771	EH domain-containing protein 3 [Apis florea]	Endocytic transport
572263147	1.565997	Proton-coupled amino acid transporter 1-like isoform X1 [Apis dorsata]	Neutral amino acid/proton symporter; transport
328779221	1.571725	UDP-glucuronosyltransferase 1–3-like [Apis mellifera]	Elimination of potentially toxic xenobiotics and endogenous compounds
380030413	1.575212	Failed axon connections [Apis florea]	Neurogenesis
380016147	1.614437	Uncharacterized protein LOC100867355 [Apis florea]	Unknown
571514456	1.724201	RNA-binding protein squid-like [Apis mellifera]	mRNA splicing, via spliceosome
66546988	1.744358	*D*-arabinitol dehydrogenase 1-like [Apis mellifera]	Unknown
571573277	1.836049	CDGSH iron-sulfur domain-containing protein 2 homolog isoform X1 [Apis mellifera]	Depression of endoplasmic reticulum Ca^{2+} stores during autophagy
373205486	1.84741	Ras-related protein Rab-8 A [Apis cerana]	Regulators of intracellular membrane trafficking; transport
572316658	1.915055	Proteasome subunit alpha type-5-like [Apis dorsata]	Proteasome-mediated ubiquitin-dependent protein catabolic process; apoptotic process.
66547760	1.971948	Dihydropteridine reductase isoform X2 [Apis mellifera]	Cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases
571550590	1.982327	Glutaminase; mitochondrial isoformX1 [Apis mellifera]	Regulates the levels of the neurotransmitter glutamate in the brain
571518500	2.022802	40S ribosomal protein S21-like isoform X1 [Apis mellifera]	Translation initiation factor; gene expression
380024395	2.219811	Glutaredoxin 3 [Apis florea]	Cell redox homeostasis
66506276	2.350153	Uncharacterized protein LOC551541 [Apis mellifera]	Unknown
380025630	2.702238	Calumenin [Apis florea]	Peripheral nervous system axon regeneration

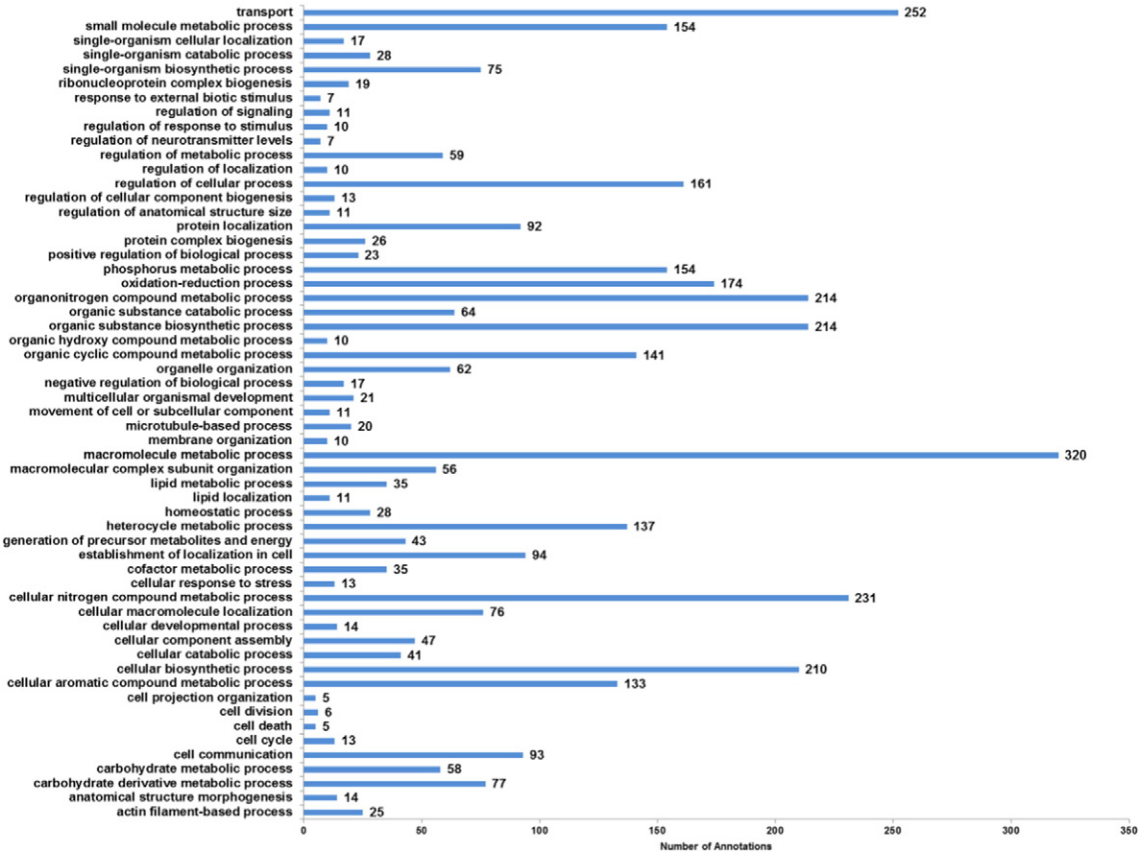

Fig. 2. Categorization of all proteins identified in the honeybee brain (considering both the control and PER groups) in biological process at the fourth level, provided by the Blast2GO algorithm.

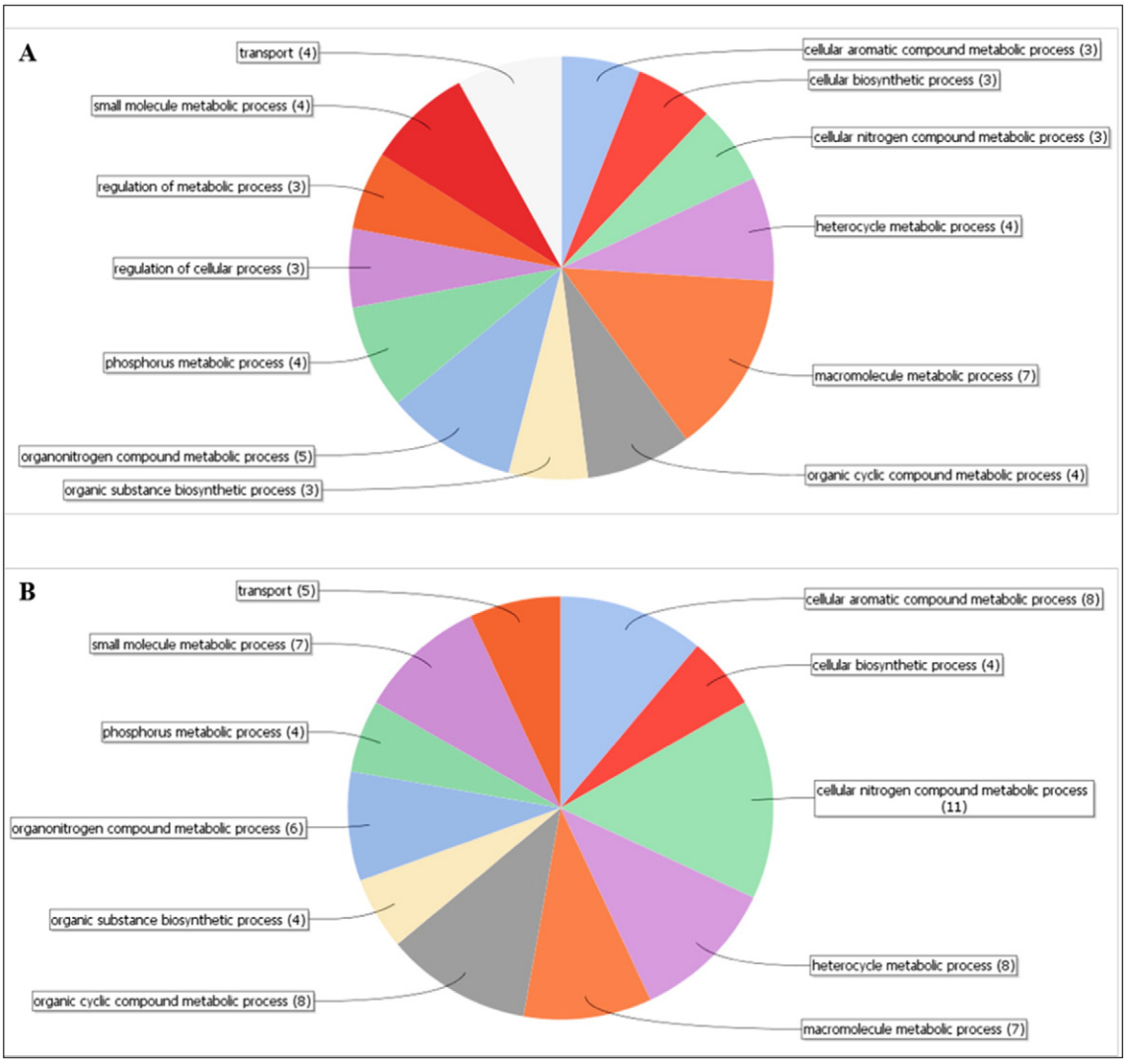

Fig. 3. Categorization according to biological process at the fourth level using the Blast2GO algorithm for the proteins unique to each group: (*A*) proteins identified only in the control group; (*B*) proteins identified only in the PER group.

molecules, the regulation of cellular processes, phosphorus metabolism, ox–redox processes, the biosynthesis/catabolism of organic substances, the biosynthesis of proteins, and the metabolism of nitrogenated compounds. The Blast2GO analysis for the unique proteins is shown in Fig. 3A (for the control group) and 3B (for the PER group). This analysis revealed that the 26 unique proteins in the control group belong to 13 categories of biological processes, whereas the 58 unique proteins from the PER group belong to 11 different biological processes. The Blast2Go analysis for the differentially expressed proteins is shown in Fig. 4A (for the proteins down-regulated in the PER group) and 4B (for the proteins up-regulated in the PER group); this analysis revealed that the 26 down-regulated proteins belong to 11 categories of biological processes, whereas the 18 up-regulated proteins belong to six categories of processes.

The analysis of enriched GO terms for the unique proteins revealed 5 and 4 different biological and/or molecular processes in the brains of the individuals both of the control group (naive) and in the PER group, respectively. The enriched GO terms of the control group were protein import into nucleus (GO:0006606), ATP metabolic process (GO:0046034), methionine metabolic process (GO:0006555), protein ubiquination (GO:0016567), and protein deubiquitination (GO:0016579) (Fig. S1 in supplementary information). Meanwhile, among the unique proteins from the brains of individuals of PER group, the enriched GO terms were RNA processing (GO:0006396), translation (GO:0006412), nucleotides metabolic process (GO:0009117), and protein macromolecular assembly (GO:0034622) (Fig. S2 in supplementary information).

The analysis enriched of GO terms detected 5 and 4 biological and molecular processes among the down-regulated and up-regulated proteins of PER group, respectively. The enriched GO terms of the down-regulated proteins were intracellular transport (GO:0046907), signal transduction (GO:0007165), tricarboxylic acid catabolic process (GO:0072352), regulation of purine nucleobase metabolic process (GO:0006141), and modification-dependent catabolic process (GO:0019941) (Fig. S3 in supplementary information). Meanwhile, among the up-regulated proteins the enriched GO terms were intracellular transmembrane proteins transport (GO: 0006725), regulation of mRNA metabolic process (GO:1903311), and innate immune response (GO: 0045087) (Fig. S4 in Supplementary information).

The proteins establish regulatory networks by interacting with each other [36]; thus, the proteomic complement of the honeybee brain was analyzed using the String V10.0 algorithm and searching for protein-protein interactions, specifically for *A. mellifera*; the results are shown in Figs. 5–8. Thus, when the 26 proteins unique to the control group were submitted to the analysis with the String algorithm, 9 were identified to participate with protein networks, as shown in Fig. 5A; the networks identified are related to the regulation of cell death (Fig. 5B), differentiation of nervous system (Fig. 5C), and protein catabolism (Fig. 5D). When the 58 proteins unique to the PER group were analyzed with

Fig. 4. Categorization by biological process at level four according to the algorithm Blast2GO, considering the comparison between the PER vs. control group; (*A*) proteins down-regulated in PER group and (*B*) proteins up-regulated in the PER group.

String, 19 were identified to participate in protein networks (Fig. 6A); these networks were related to the regulatory circuits of biosynthetic processes (Fig. 6B), carbohydrate metabolism (Fig. 6C), and nervous system differentiation (Fig. 6D), general metabolism (Fig. 6E), and a response specific to the stimulus (perception of the odor of sucrose) (Fig. 6F).

Proteins down-regulated in the brains of bees in the PER group were submitted to a search in String DB, and 12 of these proteins were identified to participate in protein networks (Fig. 7A); the networks identified were related to the regulation of anatomical development and cell differentiation (Fig. 7B), general biosynthetic processes (Fig. 7C), and general biological regulation (Fig. 7D). The 18 proteins up-regulated in the PER group were also analyzed with String, and 10 participated in protein networks (Fig. 8A); the networks identified were related to the regulation of anatomical structure development (Fig. 8B), cytoskeleton organization (Fig. 8C), and general biosynthetic processes (Fig. 8D).

4. Discussion

The PER is considered a US in honeybees and is frequently used in behavioral experiments to evaluate the ability of these insects to establish associations between the US and CS; bees are generally rewarded with a carbohydrate solution [14,15,16,17]. These experiments usually evaluate biochemical and/or genic changes at the final of two types of insect manipulations, i.e., the individuals are trained for the PER in association with a second behavior (such as recognizing the size/color of an object or even an odor); if an association between the processes is established by the bees, the individuals extend their proboscis to be rewarded with a carbohydrate solution. Thus, the biochemical and/or genetic variables observed reflect the overall changes induced by both insect manipulations. PER alone is an important reflex in part of the experimental strategy to access honeybees' ability to learn and acquire memory; thus, it is necessary to identify the biochemical changes induced in honeybee brains by this reflex.

When investigating this process, it is important to consider that the olfactory memory of worker honeybees was initially acquired during visits to the flowers for collecting nectar; this memory is then transferred to the paradigm in the laboratory during the experiments [17]. Therefore, a component of the olfactory memory in the brain of worker bees is present at the application of the paradigm in the laboratory. To access the major biochemical processes occurring during the execution of PER in the brains of honeybee workers, the present investigation profiled the proteomic complement of 20-day-old bees, trained for the execution of PER and compared it with untrained bees.

A total of 1353 proteins (maximum parsimony) were reliably identified in the brains of both groups (1295 proteins were identified in the control group; 1327 proteins were identified in the PER group). Among these proteins, 26 were unique to the control group (Table 1), whereas 58 were unique to the PER group (Table 2). The interrogation

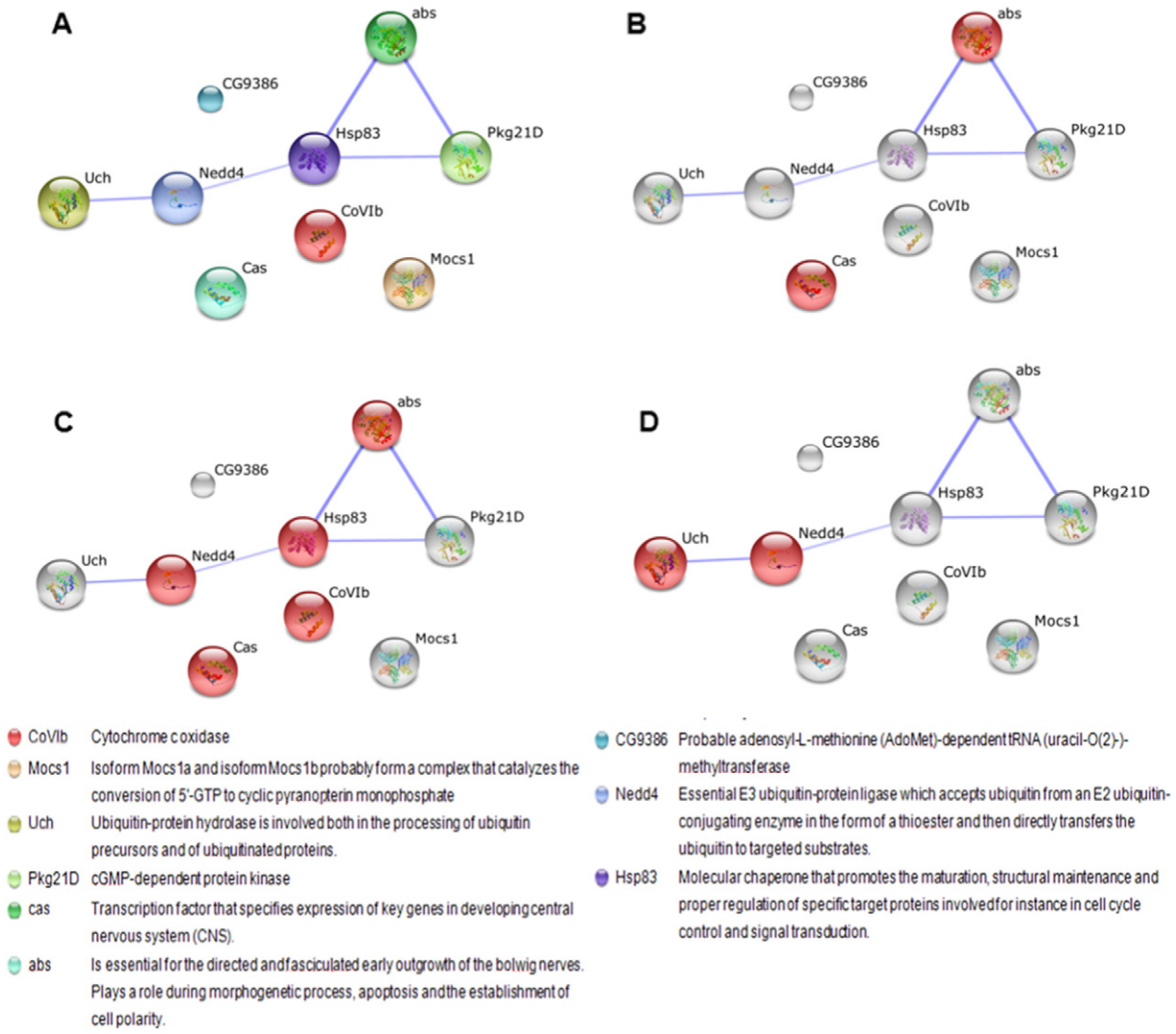

Fig. 5. Protein–protein interaction network constructed using STRING v10.0 to (*A*) proteins identified only in the control group; (*B*) cell death, (*C*) nervous system development, and neuron differentiation and (*D*) proteolysis involved in the cellular protein catabolic process. Proteins with stronger associations are linked with thicker lines.

of Blast2GO DB for these unique proteins revealed interesting metabolic features, as shown in Fig. 3:

i) 11 proteins were related to the metabolism of nitrogenated compounds in the PER group (versus only 3 proteins in the control group).
ii) 8 proteins were related to the metabolism of heterocyclic compounds in the PER group, whereas only 4 proteins were related to this metabolism in the control group.
iii) 8 proteins were related to the metabolism of aromatic compounds in the PER group, whereas only 3 proteins were related to this metabolism in the control group.
iv) 8 proteins were related to the metabolism of cyclic compounds in the PER group, whereas only 4 proteins were related to this metabolism in the control group.

Therefore, 35 of the 58 proteins unique to the brains of the individuals of PER group are related to the metabolism of cyclic/heterocyclic/aromatic compounds; however, 14 of the 26 proteins unique to the control group are related to these transformations, suggesting that the metabolic transformations of compounds containing ringed structures are particularly important during for the PER. These observations include the metabolism of some neurophysiologically important classes of compounds such as pyridines (niacin), pyrroles (pyrrole-2-carboxylate), purines (adenosine), pyrimidines (uridine-5′-monphosphate), indoles (serotonin, tryptamine), imidazoles (biotin), and phenylated compounds (dopa and dopamine).

Among the 1269 proteins common to both groups, 18 were up-regulated in the brains of individuals in the PER group (Table 3), 26 were down-regulated in the individuals of the PER group (Table 4). When the Blast2GO DB was prompted with these proteins, some interesting metabolic features were observed (as shown in Fig. 4):

i) A total of 16 of the up-regulated proteins were related to metabolic processes of nitrogenated compounds, whereas only 3 down-regulated proteins belonged to this process.
ii) 3 down-regulated proteins were related to the metabolism of phosphorylated metabolites.

Apparently, the observation that there are more down-regulated proteins than up-regulated ones in the PER assay appears to be consistent with previous reports in which the use of the PER was associated with different CS resulted in more down-regulated than up-regulated genes [25,26,27]. We highlight that among the major up-regulated brain PER group, the royal jelly proteins (Table 3), the proteins MRJP-1 and -2, were more abundant in the brain of nurse bees when compared to the foragers ones [41].

The complex phenotypic traits in honeybees appear to result from an organized clusterization of proteins to form interactive networks that activate and regulate a series of functional circuits [42]; the

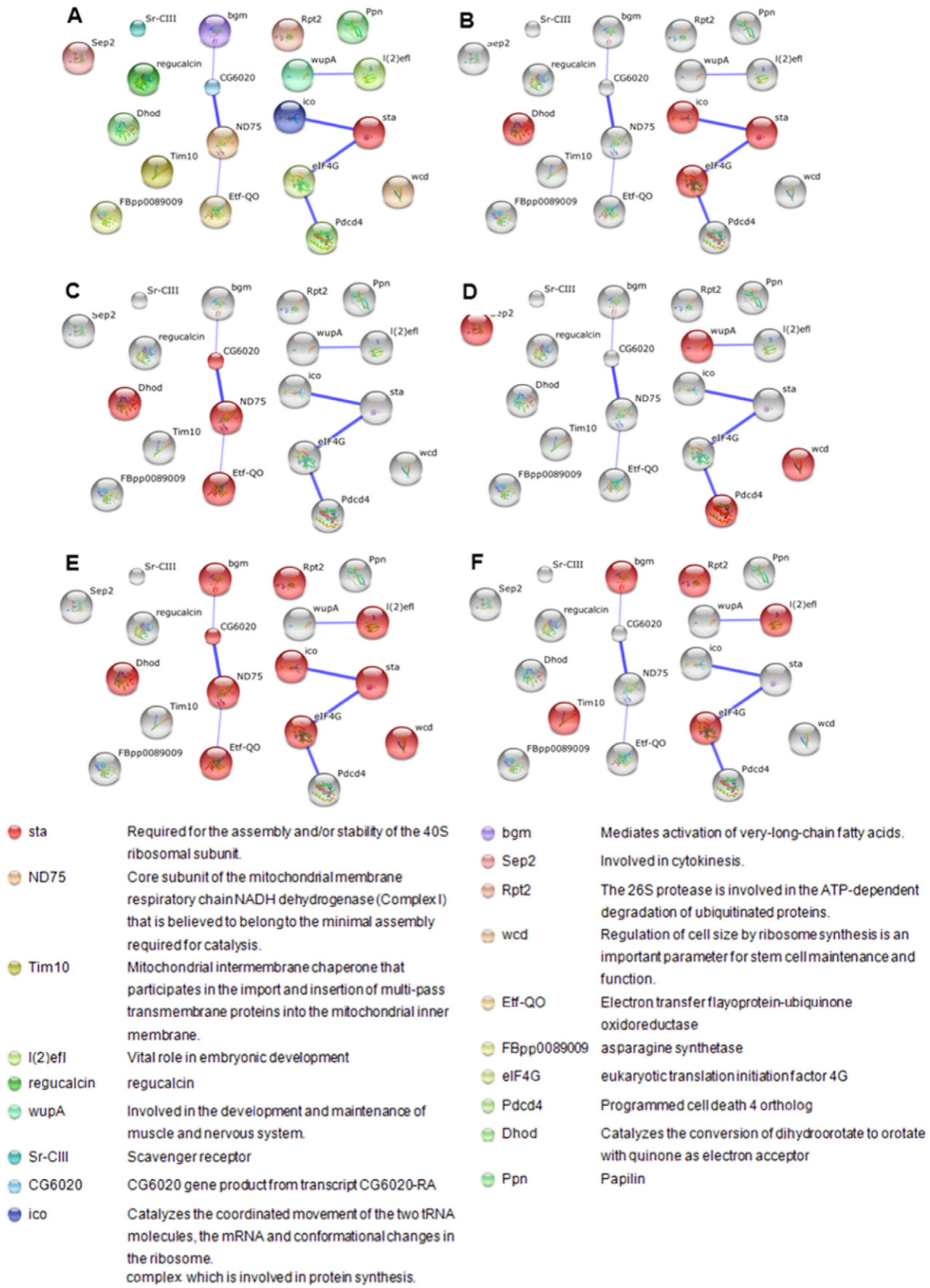

Fig. 6. Protein–protein interaction network constructed using STRING v10.0 to (*A*) proteins identified only in the PER group; (*B*) biosynthetic process, (*C*) carbohydrate derivative metabolic process, (*D*) nervous system development, and neuron differentiation, (*E*) primary metabolic process, and (*F*) response to stimulus. Proteins with stronger associations are linked with thicker lines.

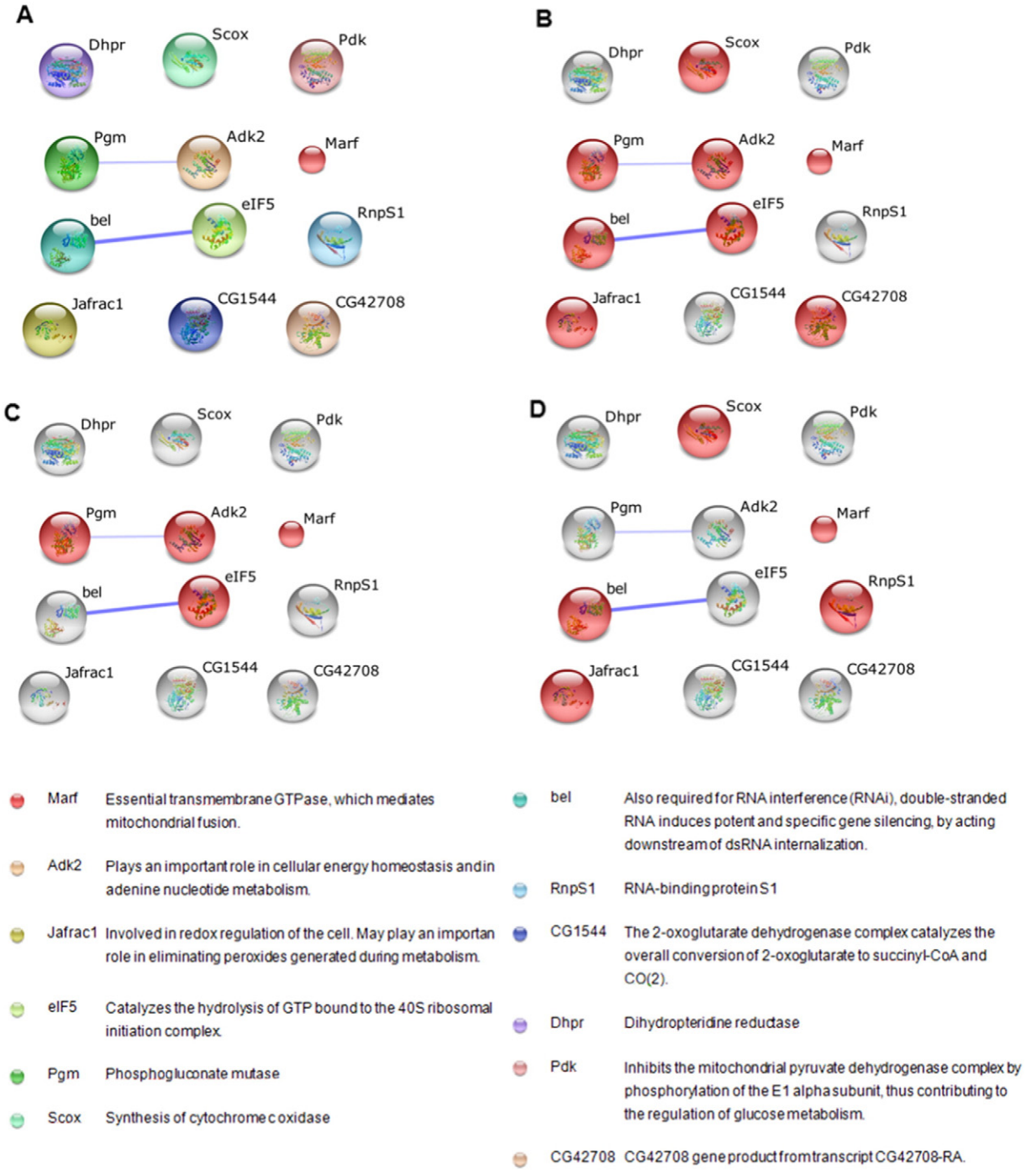

Fig. 7. Protein–protein interaction network constructed using STRING v10.0 to (*A*) proteins that showed down-regulation in the PER group compared with the control group; (*B*) developmental system, anatomical structures development and cell differentiation, (*C*) biosynthetic process, and (*D*) biological regulation. Proteins with stronger associations are linked with thicker lines.

complexity of these networks may increase due to the multiple functions presented by the most proteins. To investigate the potential formation of proteomic networks, the unique proteins in addition to those differentially expressed were analyzed with the algorithm String v10.0; the results are summarized in Figs. 5–8. Notably, there is no specific databank of protein–protein interactions for honeybees in other algorithms used for simulating these interactions. The data available in String DB for the *A. mellifera* taxa are reduced and the number of specific networks formed was also relatively reduced, but these networks simulations are consistent. We did not simulate any interaction using data from taxa other than *A. mellifera*. An interpretation of the results shown in Fig. 5 reveals that nine of the unique proteins of the control group formed a small network related to the control of cell death (Fig. 5B), and nervous system differentiation (Fig. 5C), and proteolysis associated with protein catabolism (Fig. 5D). These processes constitute normal regulatory circuits necessary to the brain plasticity of the worker bees, which are primarily related to the temporal polyethism characteristics of these insects. When the proteins unique to the brain of the individuals of the PER group were analyzed with String, they formed a network involved in the regulation of five different regulatory circuits (Fig. 6B–F): general biosynthetic processes (6B), the metabolism of carbohydrates (Fig. 6C), nervous

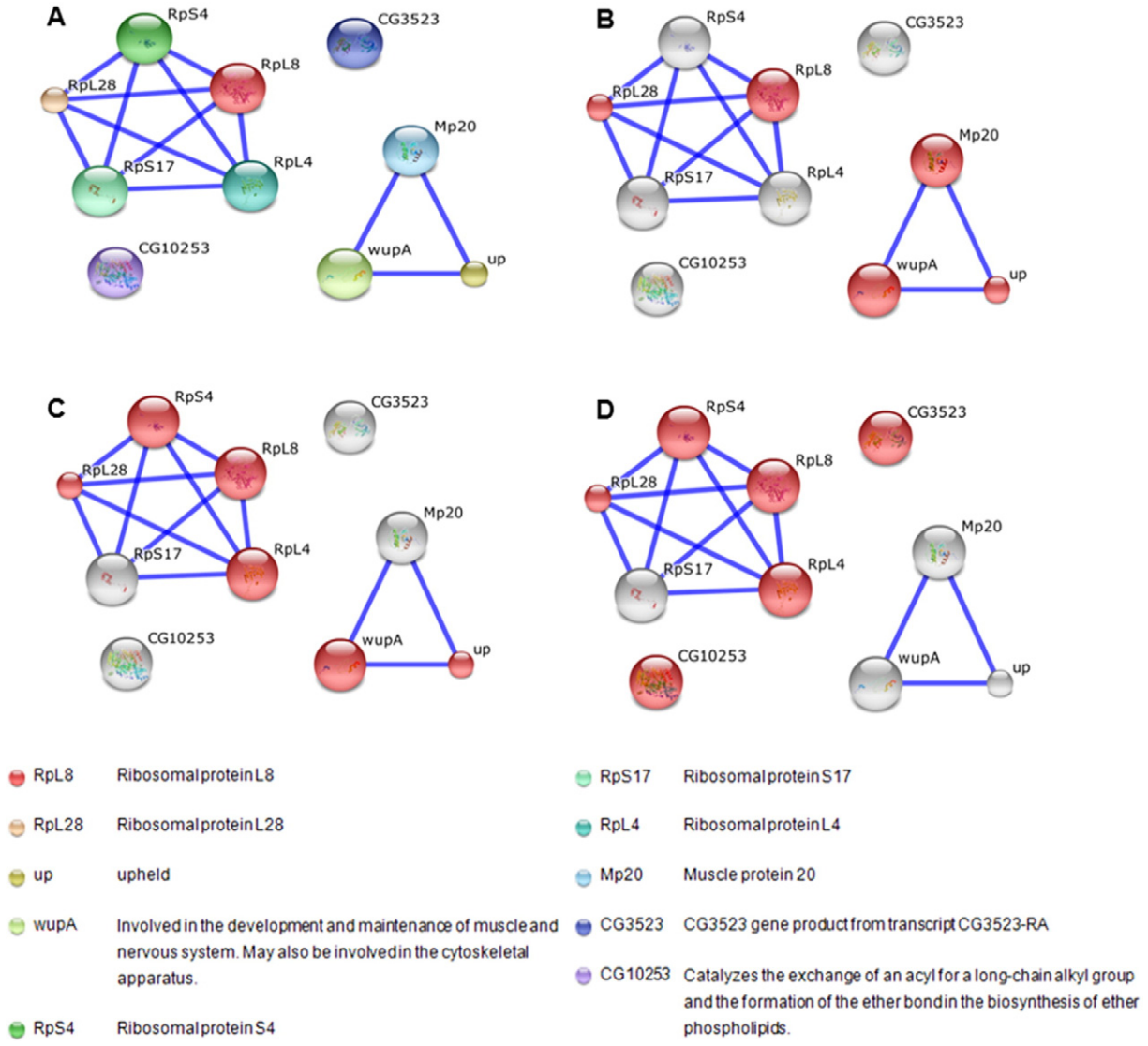

Fig. 8. Protein–protein interaction network constructed using STRING v10.0 to (*A*) proteins that showed up-regulation in the PER group compared with the control group, (*B*) developmental process and anatomical structure development, (*C*) cytoskeleton organization, (*D*) biosynthetic process. Proteins with stronger associations are linked with thicker lines.

system development (Fig. 6D), primary metabolism (Fig. 6E), and a specific response to the stimulus (Fig. 6F). Among these regulatory circuits, i) *the metabolism of carbohydrates*, ii) *the development of nervous system*, and iii) *specific response to the stimulus* warrant emphasis. The honeybee brain appears to adapt to the PER, requiring energy via carbohydrate metabolism. Regarding the specific response to the stimulus (recognizing the odor of sucrose and extending the proboscis), the proteins involved in this response (mitochondrial chaperone, protein vital embryonic development, proteins responsible for the activation of fatty acids, and translation factors) appear to be related to the synthesis of growth factors. These processes are apparently related to the brain adaption to a specific US.

The results of the analysis of enriched GO terms are indicating that the training of honeybee workers for PER reflex behavior seems to trigger different responses in honeybee brain, with different types of regulation of protein expression.

The networking analysis performed for the down-regulated proteins in the brains of the individuals of the PER group (Fig. 7B–D) revealed three regulatory circuits: i) *anatomic development and cell differentiation* (Fig. 7B), ii) *a general biosynthetic process* (Fig. 7C), and iii) *general biological process regulation* (Fig. 7D). The analysis with the String algorithm of the up-regulated proteins in the brains of individuals in the PER group revealed the formation of a small network related to the regulation of three processes (Fig. 8B–D): i) *anatomic development* (Fig. 8B), ii) *cytoskeleton development* (Fig. 8C), and iii) *general biosynthetic processes* (Fig. 8D). The up-regulation of anatomic and cytoskeleton development is of notable importance.

The results may indicate some degree of cell differentiation. Some regions of the honeybee brain are known to have special importance in terms of odorant memory, especially the mushroom bodies, which have a remarkable structural plasticity according to the age and/or specific environment of the honeybees [43].

In the present investigation were observed different protein networks related to the differentiation of nervous system, but no process of neurogenesis was reported. It was reported in the literature that the neurogenesis is uncommon in the brain of adult honeybees and that the behavioral development must depend of a series of other developmental processes [44], such as those reported above in the present study. The results above demonstrate that brain proteomics is linked to PER behavior and that some changes in brain proteins expression may mediate changes in the US-related behavior, as similarly proposed for other types of behaviors in honeybees [45].

5. Conclusions

The brains of honeybee workers submitted to a PER assay underwent a series of specific metabolic transformations and various biological processes at the cellular level to adapt the insects' brains to coordinate odor recognition and to associate this odor with a reflex behavior to perform a US (proboscis extension). Thus, the comparative analysis of the proteomic profiles for the brains of honeybee workers submitted and not submitted to PER assay revealed that the brains of PER-stimulated individuals demonstrated an activation of the metabolism of cyclic/heterocyclic/aromatic compounds in parallel with the metabolism of nitrogenated compounds; this was followed by the down-regulation of proteins involved in the metabolism of phosphorylated metabolites and the up-regulation of the proteins related to the metabolism of carbohydrates. This likely occurred to supply metabolic energy for the cellular processes necessary to adapt the brain to the PER. Regarding cellular processes, the proteomic profile was stimulated by the PER, which indicates a series of various stimuli to the differentiation of the nervous system; however, there was a down-regulation in the anatomic development and cell differentiation in other neurons, followed by the up-regulation of proteins involved with anatomic and cytoskeleton development.

List of abbreviations

PER	Proboscis extension reflex
US	Unconditioned stimulus
CS	Conditioned stimulus
ACN	Ecetonitrile
SPE	Solid-phase extraction
LC	Liquid chromatography
MS	Mass spectrometry
ETD	Electron-transfer dissociation
DMSO	Dimethyl sulfoxide

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2016.05.029.

Conflicts of interest

The authors declare no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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