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CELLULAR AND MOLECULAR BIOLOGY

Study of *Melipona quadrifasciata* brain under operant learning using proteomic and phosphoproteomic analysis

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Abstract: Learning to anticipate events based on the predictive relationship between an action and an outcome (operant conditioning) is a form of associative learning shared by humans and most of other living beings, including invertebrates. Several behavioral studies on the mechanisms of operant conditioning have included Melipona quadrifasciata, a honey bee that is easily manipulated due to lack of sting. In this work, brain proteomes of Melipona bees trained using operant conditioning and untrained (control) bees were compared by two-dimensional gel electrophoresis analysis within pI range of 3-10 and 4–7; in order to find proteins specifically related to this type of associative learning.One protein was detected with differential protein abundance in the brains of trained bees, when compared to not trained ones, through computational gel imaging and statistical analysis. This protein was identified by peptide mass fingerprinting and MS/MS peptide fragmentation using a MALDI-TOF/TOF mass spectrometer as one isoform of arginine kinase monomer, apparently dephosphorylated. Brain protein maps were obtained by 2-DE (Two-dimensional gel electrophoresis) from a total proteins and phosphoproteins extract of the bee Melipona quadrifasciata. One isoform of arginine kinase, probably a dephosphorylated isoform, was significantly more abundant in the brain of trained bees using operant conditioning. Arginine kinase has been reported as an important enzyme of the energy releasing process in the visual system of the bee, but it may carry out additional and unexpected functions in the bee brain for learning process.

Key words: 2-DE, arginine kinase isoform, brain proteome, MALDI-TOF/TOF, mass spectrometry, operant conditioning.

INTRODUCTION

The complexity of the vertebrate brain, in which stimuli are processed and connected in several hierarchical steps, makes it difficult to discern the circuits that are responsible for learning and behavior. Complex vertebrate brains and less complex animal brains, including those of invertebrates, are able to take advantage of two forms of associative learning that appeared early in evolution and have been indispensable since: classical (Pavlovian) (Pavlov & Anrep 1927) and operant conditioning (Skinner 1938, Thorndike 1911).

The honeybees have efficient sensorial and nervous systems that are responsible for their adaptation (Giurfa 2003). In addition to presenting a complex social behavior, honeybees have been employed as a model system in several behavioral and ethological studies, addressing navigation, social organization and

learning (Hammer & Menzel 1995, Menzel & Muller 1996, Giurfa et al. 1999, Reinhard et al. 2004, Stach et al. 2004).

Learning to anticipate events based on the predictive relationship between an action and an outcome (operant conditioning) is a form of associative learning shared by vertebrate and invertebrate brains (Brembs 2003). Bees exhibit an extraordinary capacity to learn and discriminate patterns, colors, odors and shapes (Giurfa 2007, Srinivasan 2010, Avargues-Weber et al. 2011). These learning abilities have been investigated mostly in bumblebees and in the common honeybee *A. mellifera* (Gumbert 2000, Dyer & Chittka 2004, Riveros & Gronenberg 2009) as well as in new-world stingless bees, such as *M. quadrifasciata* and *M. rufiventris* (Pessotti 1967, Menzel et al. 1989, Mc Cabe et al. 2007).

Seminal work by Giurfa et al. (2001) showed that *A. mellifera* can learn matching-to-sample and non-matching-to-sample relations between visual properties of color stimulus when bees are presented with a sample stimulus followed by two choice stimuli. In addition, they revealed that *A. mellifera* is able to transfer the concepts of ''sameness'' and ''difference'' from colors to novel stimuli such as patterns and vice versa, and even cross-modally; from odors to colors (González et al. 2003).

Another particularly interesting study, conducted on visual operant conditioning in some species of stingless Brazilian bees (Pessotti & Le'Senechal 1981) evidence that the association of color with food happens within the genera *Melipona* and *Scaptotrigona*. Interestingly, *Scaptotrigona* bees discriminated very well between blue and yellow (Moreno et al. 2005). In addition, Moreno et al. (2012) showed that the small brain of insects is capable of learning arbitrary relationships between visual stimuli or colors and odors, which can be species-specific skills. The contrast observed on the relational learning performance between stingless bees *M. rufiventris* and honeybees *A. mellifera* could be linked to their specific foraging and recruitment strategies, which might have developed in adaptation to different environments (Santos et al. 2010).

Behavioral proficiency in learning and memorization by honeybees may be associated with several factors, ranging from changes in brain structure, endocrine activity, gene expression and neurochemistry. However, despite the importance of genomic and transcriptomic data, it is mainly at the proteomic level that one can grasp wide biochemical diversity and functionality of the relevant signaling pathways underlying the processes of learning and memory. Proteomic analysis becomes necessary for achieving a more comprehensive view of the differential expression of bee brain proteins in trained vs. untrained conditions. This study presents a comparative proteomic and phosphoproteomic analysis of the impact of operant conditioning in the brains of *M. quadrifasciata* bees using two-dimensional gel electrophoresis (2-DE) and mass spectrometry.

MATERIALS AND METHODS

Learning by operant conditioning

Bees of *M. quadrifasciata* species forager workers underwent discriminative learning training (Figure 1). Initially, the bees were taught, through modeling, to fly from the hive to the training equipment, located 1.5 m away. This occurred with the equipment originally located in the hive exit, with exposed sucrose syrup on the cover; this food source was gradually pushed away until it was completely removed. When the bee was systematically flying to the source, the syrup was removed, and bees underwent color discrimination training by response modelling. Previous studies showed that bees discriminate

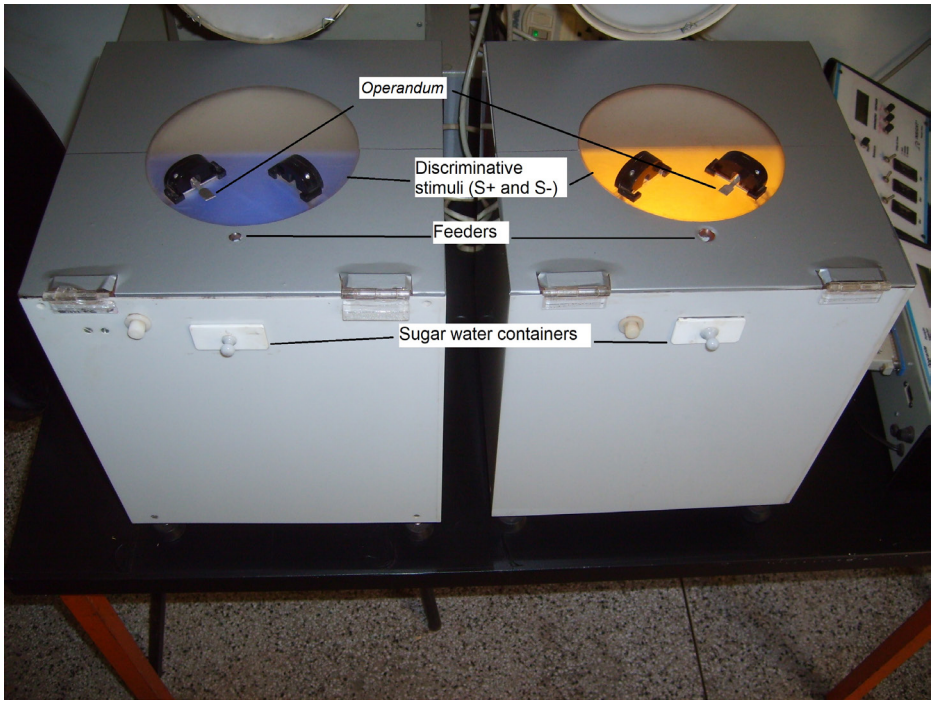

Figure 1. Setup used in the discriminative training with bees. Two experimental boxes were connected to a computer that controlled all events and recorded data. Each box had one bar (operandum) and one feeder located on the open surface on top of it. Two light stimuli (blue and yellow) were displayed simultaneously (one on each box), one S+, one S-.

very well between blue and yellow. For this phase, two equal experimental sets were used. In each one of them, a 12 cm-diameter bright circle was employed, in one case blue and in the other one a yellow circle enclosing the response bars. The syrup was presented in association with one color only (always in the blue circle, never in the yellow one). The position of the colors followed a pseudo-random order over trials, in order to control for position association. Incorrect responses were followed by an interval without color signaling, during which there was no syrup reward available. The learning criterion was of at least 15 successful successive attempts (e.g. 15 consecutive correct answers for the color stimulus, no response in the incorrect response bar). The results were analyzed in terms of cumulative frequency to the presence of each stimulus, discrimination index and number of trials to achieve the criterion of discrimination acquisition. The bees leaving the hive in the morning were used as controls.

Brain dissection

M. quadrifasciata forager trained (using an operant conditioning paradigm) and untrained worker bees were provided by the Federal University of São Carlos, Brazil. Bees were anesthetized using chloroform. For dissection, every brain was washed thoroughly for 2 to 3 s in ice-cold lysis buffer containing 7 M urea, 2M thiourea, 1% DTT, 2% Triton X-100, and 0.5% Pharmalyte 3-10 or 4-7, a protease inhibitors cocktail (cOmplete Mini-Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Mannheim, Germany). After soaking with cold lysis buffer, brains were immediately immersed in liquid N_2 and stored at - 80 °C.

Sample preparation

Every biological replicate was prepared by out of 10 pooled brains for each condition. Brains were lysed by manual homogenization using a micropestle with 200 µL of cold lysis buffer, in ice. Then, followed by 1h incubation for at room temperature in an orbital shaker, samples were

centrifuged at 13,000 rpm for 15 min at room temperature. The resulting supernatants were submitted to protein concentration assay using 2D Quant kit (GE Healthcare, Uppsala, Sweden).

Two-dimensional gel electrophoresis

To compare the experimental groups trained and untrained three extracts of 10 brains of each group were prepared for 2-DE. Two 2-DE gel replicates were run for each extract, yielding six gels per experimental group. Also, two preparative gels were run for each extract. 2-DE was optimized, and experiment carried out using 18 cm IPG strips (GE Healthcare) for isoelectrofocusing (IEF), pH range 4-7 or 3-10, for the first dimension, and 10% T polyacrylamide SDS-PAGE for the second dimension. IPG strips were rehydrated for 12 h in 370 µL of lysis buffer supplemented with 10% (v/v) isopropanol containing 50 µg and 100 µg of proteins for analytical gels and preparative gels, respectively. IEF was carried out at 20 °C using an IPGphor II equipment (GE Healthcare) under the following conditions: 500 V gradient for 1 h, 1 000 V for 1 h and 8 000 V for 4 h and 30 min. Proteins in the IPG gel strips were reduced and alkylated as following before the second dimension: Strips were soaked for 20 min in a solution containing 6 M urea, 30% glycerol, 2% SDS, and 125 mM DTT and for additional 20 min in the same buffer containing 300 mM acrylamide instead of DTT. SDS-PAGE was performed on 10% T polyacrylamide gels run on a Protean II system (Bio-Rad, Hercules, CA) connected to a Multitemp II cooling bath (GE Healthcare). The electrophoresis second dimension was effectuated at 20 °C and 25 mA constant current for 6 h. Proteins were visualized using a mass spectrum (MS) compatible silver staining procedure as described elsewhere (Jensen et al. 1999).

Gel image and Statistical analyses

Acquisition of gel images was carried through a Sharp JX-330 scanner (Tokyo, Japan) and using 300 dpi as resolution parameter. Digitalized images were imported to the Image Master 2D Platinum software (GE Healthcare), version 5.0. An automatic image detection of stained proteins was achieved under minimal user interference as recommended by the manual and, whenever needed, manual spot edition. The protein spot was matched automatically using 10 evenly distributed and well-defined spots. The amount of protein presented by each spot in every gel was estimated based on the total amount of proteins loaded to gel. All comparison between the two experimental conditions was made taking into account the average percentage of the total spot volume per condition. Protein spots that were present in at least five of the six replicates and had volume difference higher than two folds change between control and trained were accepted to the Image Master Student's t test statistical analysis. After the volume analysis, spots displaying statistically significant differential abundance ($p < 0.01$) were selected for protein identification. Spots with low intensity or in doubtful zones had their data disregarded.

Protein digestion and MS

Selected protein spots were digested using sequencing grade modified trypsin (Promega, Madison, WI) (González et al. 2003). Tryptic peptides were desalted and concentrated by means of ZipTips C18 (Millipore, Bedford, MA). Each sample with 1 µL was disposed onto the MALDI-TOF sample plate followed by equal volume of matrix solution consisted of 10 mg/mL R-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid (TFA) and 50% acetonitrile (ACN). The samples mixture with matrix were left to dry before acquisition of data by MALDI-TOF. All

spectra were obtained by a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using delayed extraction mode and reflector. For the equipment internal calibration, trypsin autolysis and keratin sequences (842.50 and 1475.77, respectively) was used. Data acquisition covered peptide masses (MH+) from 800-3000 Da range. Spectra acquisition were done through the software FLEX Control v. 1.1.0.0 (Bruker Daltonics) and raw spectra was handled by XTOF v. 5.1.1 (Bruker Daltonics) to output a list of peaks.

Protein identification

Proteins were searched for its identity by the use of peak lists generated through BioTools v. 2.0 (Bruker Daltonics) in line with Mascot (Perkins et al. 1999) and having as database the NCBI protein database (National Center for Biotechnology Information, Bethesda, MD). The methodology chosen to identify proteins were the Peptide Mass Fingerprinting (PMF), using the monoisotopic masses of tryptic peptides. Searches were done using error tolerance lower than 100 ppm and no phylogenetic restrictions. Searches that did not return protein identification with a significate Mascot scores were applied to a second round of identification using taxonomy restriction metazoa. Additional parameters applied were oxidation of methionine and propionamide cysteine (acrylamide alkylation) as variable and fixed modification respectively. No missed cleavages were allowed for in most of the searches. Protein result were considered as identified when Mascot search engine exceeded its threshold score assuming p- value < 0.05.

RESULTS AND DISCUSSION

Preliminary experiments with 2-DE allowed us to observe that when using 3-10 IPG strips for IEF the protein fractionation was limited in the basic area. To solve that restriction, 10% isopropanol was added to 2-DE buffer as reported elsewhere (Görg et al. 1997) to improve resolution in the basic pH region. For SDS-PAGE second dimension, 10% T polyacrylamide concentration was used to allow that high molecular mass polypeptides, which comprised the majority of spots in the 2-DE maps, would migrate in the electrophoresis for a better resolution.

Maps of bee brain proteins trained using operant conditioning and control bee brains were first performed in pH 3-10 range (Figure 2). The 2-DE profile showed relatively few differences between the compared samples. By visual inspection, only one or two spots showed differences in the acidic range (signaled in a square). In order to broaden the pH range and achieve higher 2-DE resolution, IPG strips were used in the pH 4-7 range (Figure 3). Additionally, the image and statistical analysis conducted on the gel images showed that only one spot has differential abundance, signaled by the arrow in Figure 3.

Maps 2-DE revealed few differences between the samples (data not shown). This might be the relatively short learning time (8 hours). This relatively short time lapse was probably not enough for the syntheses of new proteins associated with this kind learning process. Another possibility could be given by the potential limitations of the methodology; for example, the low sensitivity to detect proteins that are expressed in low number of copies per cell, such as regulatory proteins, and the use of whole brain extracts. The use of extracts of mushroom body or other specific regions could circumvent or clarify this problem.

Table I shows spots 1 and 2 of Figure 3 that were identified as the same protein. Hence, these two spots could be arginine kinase isoforms.

Arginine kinase was identified in two different spots, spots 1 and 2 (Figure 3), however

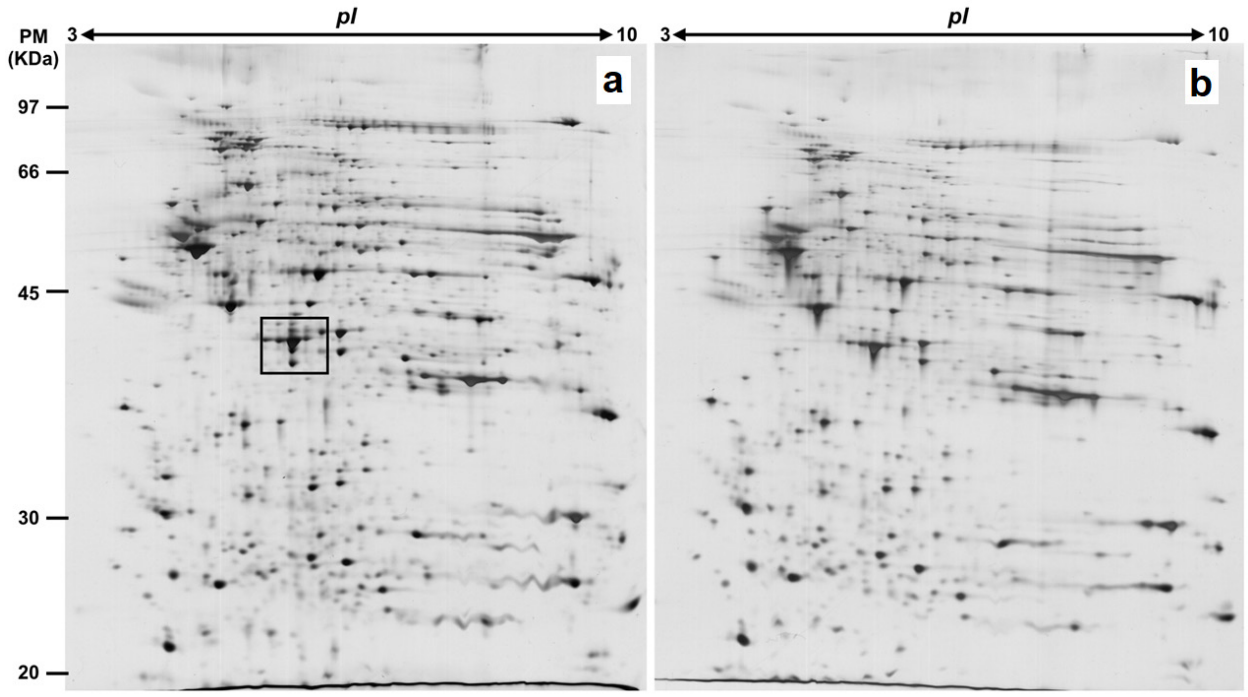

Figure 2. 2-DE gels (pI 3-10) comparison between operant conditioning trained and not trained control bees *Melipona quadrifasciata*, using three biological replicates for each group, and two technical replicates for each sample. a: Bees trained by operant conditioning, b: Control. Spot that showed differences by visual inspection is signaled in a square.

only the spot 2 exhibited statistical difference higher than two folds change in its abundance when compared trained and control bees (Supplementary Material – Figures S1, S2).

Isoforms are visible as groups of spots distributed along the Mr and pI axis, this could be caused by genetic polymorphism or due to post-translational modifications (PTM) such as phosphorylation, acetylation, glycosylation or sialic acid addition among others. In previous proteomic studies on profiling the *Polybia paulista* wasp venom, two forms of arginine kinase were glycosylated (Santos et al. 2010). Phosphorylation modification was demonstrated for arginine kinase using ProQ Diamond phosphoprotein staining (Dawson & Storey 2011). This result exhibit a 64% greater the amount of phosphate associated to arginine kinase in anoxic crayfish muscle when compared with aerobic controls. Data presented elsewhere (Old et al. 2005) brought the information that arginine kinase from crayfish tail muscle also respond to a phosphor-regulation and such PTM modify its adaptability to aerobic and anoxic conditions, suggesting that arginine kinase reversible phosphorylation may adjust enzyme function in response to changing oxygen conditions.

Further analysis must be carried out to arrive to more detailed information on the identities, occurrence, cellular locations and functions of putative PTM on the target protein.

Two-dimensional gel electrophoresis is a high-resolution method allowing separating intact proteins, displaying their PTM (Rabilloud et al. 2010). Individual spots of interest that are resulting from PTM were taken into account for analysis. In the case of "label free" quantitative analysis by "Shotgun" proteomics, all isoforms are in unison considered without same

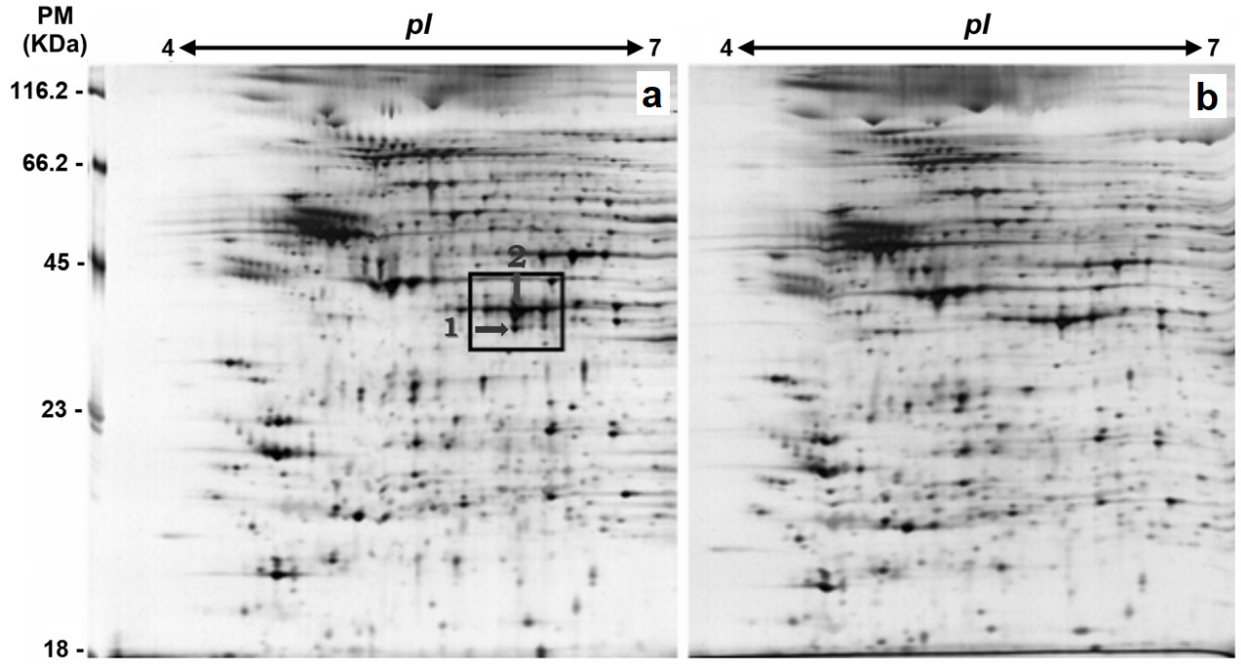

Figure 3. 2-DE gel profile of *Melipona quadrifasciata* bee brain proteins using IPGs of 4-7 acidic pH range using three biological replicates for each group, and two technical replicates for each sample. a: Bees trained by operant conditioning, b: Control. Arrows indicates the proteins isoform identified by PMF, according to Table I.

distinctiveness as the 2-DE has. A previous report from our group (Garcia et al. 2009) presents various isoforms of α-glucosidase by two dimensional electrophoresis while MudPIT was unable of such discernment (Hernandez et al. 2012). Analysis by 2-DE also allowed the detection of all MRJP1 (major royal jelly protein 1) isoforms (Cruz et al. 2011). On the other hand, spectral count method can be limiting for proteins with higher expression levels as the MS detector can be saturated. For each HPLC run and for each protein spot, a saturation effect for the spectral counting was 30 spectra (Old et al. 2005). Therefore, the isoform analysis by 2-DE can be a complementary approach.

To confirm the protein identification, one of the most intense peaks from the MS was selected, fragmented and analyzed by MS/MS. This peak represented a peptide mass of 1731.9 Da.

Arginine kinase (L-arginine N-phosphotransferase) is an enzyme with important role in cellular energy metabolism in invertebrates. It catalyzes the transference of the reversible phosphoric group of adenosine triphosphate (ATP) to arginine, yielding adenosine diphosphate (ADP) and phosphoarginine. Previous studies have shown that in bees there are three isoforms of arginine kinase, transcribed from a single gene in its major tissues (Kucharski & Maleszka 1998).

The arginine kinase mRNA can be found with remarkable abundance in the central nervous system and the antenna and is one of the essential proteins of the signal transduction pathways of the honey bee involved in learning and memory (Münch et al. 2008). Moreover, mRNA expression is two to three times greater in the compound eyes, indicating that arginine kinase would be an important constituent of the

Table I. Proteins identification properties from spots 1 and 2 that shows both as the same protein, arginine kinase isoforms.

Spot	Protein	Taxonomy	Coverage	Peptides	pI	Mr
1	Arginine kinase	*Apis mellifera*	14%	6	5.66	40.4 kDa
2	Arginine kinase	*Apis mellifera*	16%	7	5.66	40.4 kDa

energy release mechanism in the visual system (Old et al. 2005). This protein also has a potential calmodulin-binding site (Calabria et al. 2008), it is a phosphagen kinase highly conserved and, in invertebrates, it is the major guanidine kinase identified to date (Mühlebach et al. 1994). Ubiquitous distribution of arginine kinase might suggest its involvement in a synthetic redox/ shuttle mechanism that is common to many tissue types (Wolschin & Amdam 2007) and in response to adverse abiotic and biotic stresses in *Apis cerana cerana* (Chen et al. 2015).

A study in the kinetoplastid parasite *Trypanosoma brucei* showed that there are multiple isoforms of arginine kinase with different subcellular localization (Voncken et al. 2013). Phosphoarginine- and arginine-kinase system of the *T. brucei* are composed by three isoforms of arginine kinase (TbAK1-3) with high similarity of primary sequence. In that study, using myc-tagged protein for immunofluorescence microscopy experiment reveled that each isoform has subcellular compartment specificity: TbAK1 was found in the flagellum, TbAK2 was grounded to glycosome, while the TbAK3 was observed in *T. brucei* cytosol. As for the trypanosome flagellum, the TbAK1 flagellar location is resulted by its 22 amino acid along N-terminal sequence that is target by GFP-fusion protein. For TbAK2, it has a conserved peroxisomal targeting signal for gycosomal location, the C-terminal tripeptide 'SNL'. The TbAK3 is located into the parasite cytosol and seems to not have any targeting sequences.

In the present study, arginine kinase exhibiting differential protein abundance between trained and untrained bees was the one with lowest molecular mass. This isoform perhaps triggers a mechanism associated with the type of learning by operant conditioning in bees, and it would of great interest to study the interactions of this protein with other proteins. Li et al. (2010) investigated the specific time and the influences of primary proteins on post embryonic caste determinations in honeybee by using proteomic analysis in combination with bioinformatics and revealed thar worker larvae over-expressed arginine kinase as compared to the queen larvae.

Additionally, assuming learning can be caused by protein PTM, particularly phosphorylation, and given the phosphoproteins are involved in all biological processes, and specifically in regulation processes; comparative studies of phosphoproteomic profile were carried out between the two bees' brain proteins samples (Figure 4). Phosphoprotein detection was conducted using ProQ-Diamond, which is specific for phosphoproteins and allows high sensitivity detection.

Computer analysis performed to determine relevant differences in the concentration of phosphoproteins between the control and trained groups revealed that five spots resulted in significant changes, but the identification was not possible because these silver stained proteins were not visually accessible. The arginine kinase isoform previously identified was not detected with ProQ-Diamond. Thus, it is likely to be a non-phosphorylated isoform (Figure 4, signalized with arrow).

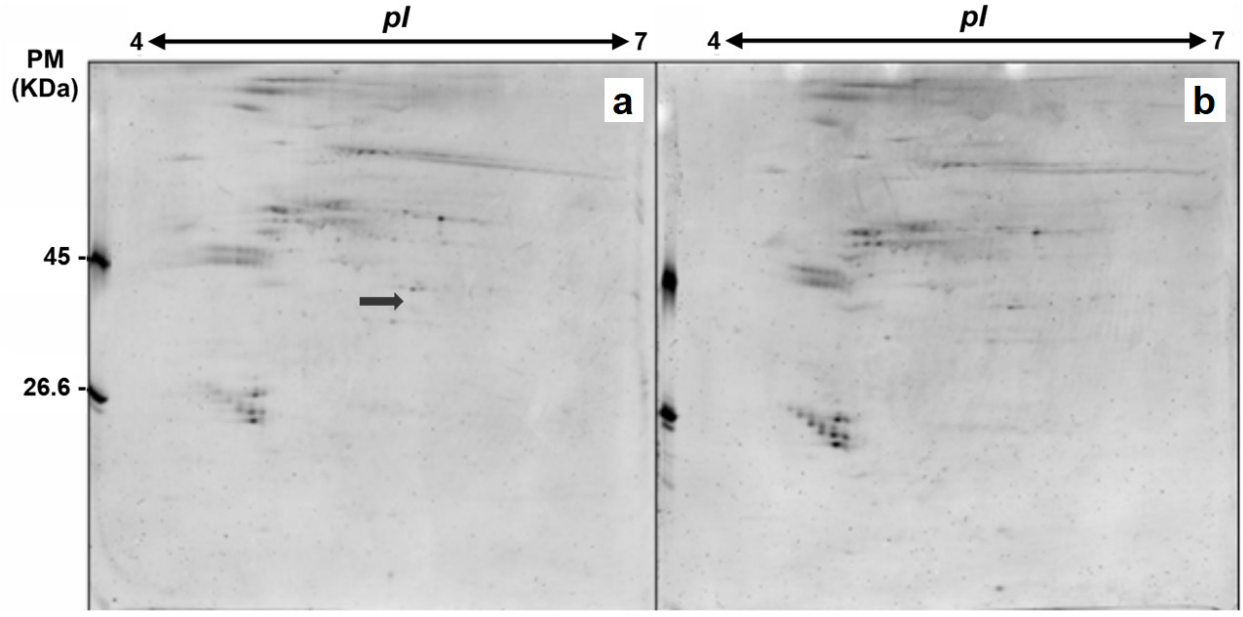

Figure 4. 2-DE profile of *Melipona quadrifasciata* bee brain phosphoproteins using IPGs of 4-7 acidic pH range. The profile was elaborated through the use of three biological replicates for each experimental group and two technical replicates for each of them. a: Bees trained by operant conditioning, b: Control. Arrow indicates area of arginine kinase isoform when silver staining was applied.

In conclusion, brain protein maps were obtained by 2-DE of an extract of proteins and total phosphoproteins from the bee *M. quadrifasciata*, submitted to operant conditioning training. The results showed the arginine kinase isoform, probably dephosphorylated, more abundant in the brain of trained bees. Future studies should test in bee or *Drosophila melanogaster* whether the expression of arginine kinase and its phosphorylation modify operant learning. Therefore, the present study using the brain of an invertebrate animal model such as that of the bee *M. quadrifasciata* may contribute to experimental and behavioral neuroscience in future investigations with more complex animal models.

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SUPPLEMENTARY MATERIAL

Figures S1, S2.

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MVS, DGS, FSE designed the study and critically revised the manuscript. LGH, CHSG, JMFS, GCNC, LKC, AMM contributed to the main experiment. All authors read and approved the final manuscript.

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