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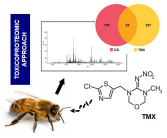

Research article

Using a toxicoproteomic approach to investigate the effects of thiamethoxam into the brain of *Apis mellifera*

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Abstract

Neonicotinoids have been described as toxic to bees. In this context, the *A. mellifera* foragers were exposed to a sublethal concentration of thiamethoxam ($LC_{50/100}$: 0,0227 ng de thiamethoxam/μL^{-1} diet), a neurotoxic insecticide, for 8 days; and it was decided to investigate the insecticide effect on the brain by a shotgun proteomic approach followed by label-free quantitative-based proteomics. A total of 401 proteins were identified in the control group (CG); and a total of 350 proteins in the thiamethoxam exposed group (TMX). Quantitative proteomics data showed up 251 proteins with significant quantitative values in the TMX group. These findings demonstrated the occurrence of shared and unique proteins with altered expression in the TMX group, such as ATP synthase subunit beta, heat shock protein cognate 4, spectrin beta chain-like, mushroom body large-type Kenyon cell-specific protein 1-like, tubulin alpha-1 chain-like, arginine kinase, epidermal growth factor receptor, odorant receptor, glutamine synthetase, glutamate receptor, and cytochrome P450 4c3. Meanwhile, the proteins that were expressed uniquely in the TMX group are involved mainly in the phosphorylation, cellular protein modification, and cell surface receptor signalling processes. Interaction network results showed that identified proteins are present in five different metabolic pathways - oxidative stress, cytoskeleton control, visual process, olfactory memory, and glutamate metabolism. Our scientific outcomes demonstrated that a sublethal concentration of thiamethoxam can impair biological processes and important metabolic pathways, causing damage to the nervous system of bees, and in the long term, can compromise the nutrition and physiology of individuals from the colony.

Keywords: bee brain; neonicotinoid; insecticide; shotgun proteomics; label-free quantitation

1. Introduction

Bees are important pollinating agents in natural ecosystems and agricultural plantations (Boyle et al., 2019). Although bumblebees, stingless bees, and solitary bees also play an important role in pollination, honeybees are used on a large scale to meet crop pollinator demands (Garibaldi et al., 2014; Winfree et al., 2007). The population density of *Apis mellifera* L., 1758 (Hymenoptera, Apidae) has been reduced to levels that could harm natural and agricultural ecosystems, affecting the ability of plants to reproduce (Potts et al., 2010). The causes of the population reduction of honeybees have been the subject of studies in recent decades. Despite the various factors associated with the disappearance of bees, colony collapse disorder (CCD) is the focus of several studies. This syndrome was observed in colonies of *A. mellifera* in the United States in 2006, and according to van Engelsdorp et al. (2009), it was characterized by the rapid loss of worker bees, resulting in weak or dead colonies with excess brood populations. Sometimes the queen is present, together with young worker bees. Another symptom is the absence of dead bees inside or near the hive, and late pest invasion and kleptoparasitism of other bees (Vanengelsdorp et al., 2011). Furthermore, there is no relationship between infectious agents and CCD (Cox-Foster et al., 2007).

Several factors have been identified as possible causes of CCD, such as climate changes, diseases and parasites, genetically modified crops, bee forage, poor queens, socio-political factors affecting managed colony populations and, pesticides with direct our sublethal effects (Ciarlo et al., 2012; Connolly, 2013; Decourtye et al., 2011; Johnson et al., 2010; Vanengelsdorp et al., 2011; VanEngelsdorp and Meixner, 2010). According to Connolly (2013), the resources collected by bees can be contaminated by the use of systemic insecticides in crops, such as phenylpyrazoles and neonicotinoids, and their toxicity is related to the occurrence of CCD. Besides, many pesticides and insecticides may have sublethal effects on forager bees, causing behavioral changes (Aliouane et al., 2009; Yang et al., 2008), as well as changes in cellular physiology on the brain (Catae et al., 2017; Friol et al., 2017; Oliveira et al., 2013; Roat et al., 2013), midgut (Catae et al., 2014; Friol et al., 2017; Oliveira et al., 2019, 2013) and, Malpighian tubules (Catae et al., 2014; Friol et al., 2017; Rossi et al., 2013) due to the stress caused by such agents (Gregorc and Ellis, 2011). Approximately 90% of insecticides are neurotoxic, such as thiamethoxam. This insecticide is an acetylcholine agonist and binds to

the nicotinic acetylcholine receptors of insects (nAChRs), which are located in post-synaptic neurons (Buckingham et al., 1997; Suchail et al., 2003). Thus, unlike acetylcholine, which is hydrolysed by acetylcholinesterase, this compound is not degraded immediately and, because of this the nerve impulses are continuously transmitted and lead to hyperexcitation of the insect's nervous system (Gallo et al., 2002). Among the symptoms resulting from neonicotinoid intoxication, nervous system collapse, tremors, and death stand out (Faria, 2009).

The knowledge about the physiology of the nervous system of the bees and using new approaches have become increasingly important to investigate the impact of pesticides on bees; and the genome sequencing (Weinstock et al., 2006) of the species *Apis mellifera* has allowed the development of comparative studies of proteomics. Since then, some studies aim to evaluate specific regions such as mushroom bodies, antennal lobes, optical lobes in the bee brain (Meng et al., 2018) and hypopharyngeal gland development (Feng et al., 2009), comparison between the calyx region of the mushroom body with the central brain (Wolschin et al., 2010), differences between the brain of nurses with forager bees (Garcia et al., 2009; Hernández et al., 2012) and different approaches using insecticides exposure (Al Naggar and Baer, 2019; Balsamo et al., 2019; Catae et al., 2018; Decio et al., 2019; Roat et al., 2014; Wang et al., 2020; Zaluski et al., 2020).

Thus, considering the importance of bees as pollinator insects, investigation of the effects of thiamethoxam on the brain of *A. mellifera* becomes relevant. Research into which brain proteins are affected by the exposure of bees to the insecticide can provide important data regarding the effects of this compound on bee behaviour and physiology. In this scenario, the use of a gel-free shotgun proteomic approach combined with the label-free quantitative-based proteomics to investigate the honeybee brain becomes extremely elucidative in proteomic analyses; as demonstrated in the present study. The findings of the current investigation may contribute to improving our understanding of the biological processes that are affected when bees are exposed to thiamethoxam; in addition, these results contribute to broadening our understanding of the insecticide action mechanism in the nervous system of *A. mellifera.*

2. Materials and Methods

2.1. Honeybees

Forager bees of the species *A. mellifera* Africanized were obtained from the apiary of the Biosciences Institute, Department of Biology, São Paulo State University (UNESP), at Rio Claro, SP, Brazil. All experiments followed the recommendations of the guidelines for xenobiotic assessments on bees (OECD, 1998a, 1998b). Thus, some adaptation on this method was made because these guidelines are for European honeybee and the current investigation is working with Africanized honeybees, as described before by Catae et al. (2017). The active ingredient of the insecticide thiamethoxam (Thiamethoxam PESTANAL®, analytical standard. Sigma-Aldrich) was used. Furthermore, three hundred newly emerged honeybees were marked with non-toxic ink on the thorax and they were taken back to the colony. After 21 days, only marked foragers bees were collected.

2.2. Intoxication bioassays via contaminated food

From three different colonies, 120 bees carrying pollen were collected arriving from the field were collected in plastic cages (250 mL). During the collection, the bees received food prepared with sugar and honey to prevent dying from starvation. In the laboratory, they were kept in the biochemical oxygen demand (BOD) chamber at 32 ± 2ºC and 70 ± 10% RH. The food was changed for a liquid sucrose solution supplied in 2 mL microtubes. The treated group consisted of 60 foragers of *A. mellifera* that received a diet containing an aqueous sugar solution contaminated with 0.0227 ng thiamethoxam/µL-1 diet, that match with concentrations of residues found in the field. A stock solution was prepared using thiamethoxam and acetone as solvent. From this solution, a serial dilution was performed to obtain the final concentration of the insecticide, however, dilutions were made using a sugary solution. Thus, the percentage of acetone present in this sublethal concentration was 0.00021%. Considering that in the field residues of thiamethoxam in pollen and nectar range from 1 to 53 ppb and from 1 to 12 ppb, respectively (Krupke et al., 2012; Mullin et al., 2010; Pilling et al., 2013; Stoner and Eitzer, 2012), this study tested one concentration equivalent to 22.7 ppb. The control group (60 bees) received food without contamination. This bioassay was done in triplicate. Both experimental groups received food *ad libitum* for 8 days. The assay was considered valid since the mortality

of the control group was lower than 10%. For the proteomic shotgun, the bees were collected 8 days after the beginning of exposure to insecticide - the thiamethoxam exposed group, named TMX, and the control group, named CG.

2.3. Brain protein extraction

For each experimental group, 45 bees were submitted to anesthesia by a -20ºC freezer before dissection; and their brains were removed. Triplicate samples of 15 brains were conditioned in vials containing protein extraction solution (7 M urea, 2 M thiourea, Triton X-100, 10 mM 1,4-dithioerythritol and protease inhibitor cocktail 0.2% v/v), and were processed immediately for protein extraction. The samples were gently pressed, thereby releasing the brain contents; after that, samples were centrifuged at 4ºC, 12000 x g, for 10 min, and the supernatant was collected in a tube; this procedure was performed 3 times. The protein concentration was estimated using the Qubit® Protein Assay Kit (Life Technologies - Qubit® 3.0 fluorometer) according to the manufacturer's instructions.

2.4. Mass spectrometry-based proteomic analyses

For the in-solution digestion (Shotgun proteomic approach*)*, three replicate samples (400 µg) from the control group (CG) and thiamethoxam exposed group (TMX) were processed as described previously by dos Santos-Pinto et al. (2016). The tryptic peptides from digested samples were solubilized in 50% ACN/0.5% TFA and subjected to mass spectrometry analysis. An HPLC system (Shimadzu) coupled to a µLC-ESI-micrOTOF-Q-III (Bruker Daltonics Bremen, Germany) was used for peptide analyses. The mass spectrometer was calibrated with a Tune-Mix Electrospray Calibrant (Agilent) solution. A C18 column (150 x 2.1 mm) (Aeris Widepore 3.6u XB, Phenomenex, Allcrom) was used in a gradient of 5-95% ACN for 180 min. The analysis and data acquisition control were performed by HyStar v3.2 software (Bruker Daltonics) as described by de Souza et al (2019).

2.5. Database search

Database-proteomics searches were performed by using MASCOT 2.3 (Matrix Science, London, UK) against the latest available *Apis* protein sequences deposited in the NCBInr

183 database (http://blast.ncbi.nlm.nih.gov). Thus, all 106,621 entries contained in the *Apis*
184 database were selected. Search parameters were set as follows: taxonomy, enzyme selected
185 as trypsin, 2 maximum missing cleavage sites allowed, peptide mass tolerance was 0.5 Da for
186 the MS and 0.8 Da for the MS/MS spectra, carbamidomethyl (C) was specified as a fixed
187 modification, while methionine oxidation was specified as variable modification. Afterwards, the
188 identified proteins were submitted to Scaffold 4.3.2 (Proteome Software Inc., Portland, OR) for
189 additional filtering to validate peptide identification. It was required significant matches to at
190 least 2 different sequences using a calculated false discovery rate (FDR) of less than 1%. The
191 peptide probability identification was set to a minimum of 99%, meanwhile, the protein
192 probability identification was set to 95%, according to the Scaffold Local FDR algorithm.
193
194 *2.6. Quantitative-based proteomics*
195 Label-free quantitation was implemented using the Scaffold 4.3.2 (Proteome Software Inc.,
196 Portland, OR). The analyzes were performed using three replicate data acquired in the MS^n
197 analyzes. The following parameters were used: maximum retention time alignment of 10 min.
198 with a minimum S/N of 5 for feature linking mapping. Abundances were based upon
199 precursor/peptide (TIC) total ion current. Normalization was performed to ensure that the total
200 sum of the abundance was the same for all samples. Imputation was performed by replacing
201 the missing values with random values from the lower 5% of the detected values.
202
203 *2.7. Functional and Gene Ontology (GO) analysis*
204 The Blast2GO algorithm (https://www.blast2go.com/) (Conesa et al., 2005) was used to report
205 the biological process categorization of the identified proteins considering the biological
206 process, molecular function and cellular component terms in the Gene Ontology
207 (http://www.geneontology.org). For this purpose, it was set the nonexclusive category for each
208 classification, which means that a number of candidates were found to be localized in more than
209 one cellular compartment.
210

2.8. Network analysis

In order to reveal functional interactions among proteins, the identified protein groups were submitted to STRING V10.0 (http://string-db. org); a Search Tool for the Retrieval of Interacting Genes/Proteins analysis (Franceschini et al., 2013). It was set the following parameters, with high confidence (0.7), for the active prediction methods used in our analysis: co-expression, co-occurrence, neighbourhood, gene fusions, experiments, databases, and text mining. The proteins are represented by each node; and the interaction are represented by each edge.

3. Results

After mass spectrometry analyses and data processing, it was possible to identify a total of 350 proteins in the thiamethoxam exposed group (TMX) and a total of 401 proteins in the control group (CG). Supplementary Tables S1 and S2 show all the proteins identified in the present study, classified according to their functions attributed by analyses in the GO database (cellular component, biological process and molecular function). The data are presented in the format of a Venn diagram to verify the similarities and differences in proteomes between the two groups, CG and TMX. As shown in the Venn diagram (Fig. 1A), both groups shared 191 proteins; while the TMX group contained 159 expressed unique proteins.

Since this investigation is focusing the TMX effects on the bee brain, the expressed unique proteins of TMX group was analysed using the Blast2go tool to categorize the proteins according to the biological processes (level 2) and compared to the expressed unique proteins in CG. These results are demonstrated in Figure 1B-C, where we can note that the expressed unique proteins in TMX are involved mainly in the following biological processes: transmembrane transport (20%), regulation of transcription (18%), oxidation-reduction process (16%), phosphorylation (15%), others (12%), cellular protein modification process (9%), cell surface receptor signalling process (5%), cellular component organization (3%), and small-molecule metabolic process (2%). Despite phosphorylation, cellular protein modification process and cell surface receptor signalling process are not the most common biological processes in the TMX, we can note that these biological processes do not occur in the CG; and they are related to each other. Phosphorylation as an effective way to regulate proteins contributes to the cellular protein modification process and metabolism. Any disorders in the phosphorylation

process are likely to drive the inappropriate activation or deactivation of the cellular processes, resulting in the break of the delicate balance in the cells (Ardito et al., 2017). Phosphorylation-mediated signalling networks essentially regulate the entire biology of cells and organisms in normal and abnormal states; it occurs a signal amplification, enabling communication among different cellular compartments, triggered by a phosphorylation cascade. Thus, receptors and enzymes are activated and deactivated by phosphorylation and dephosphorylation (Choudhary and Mann, 2010).

The interactions of identified unique proteins in the TMX were investigated using the STRING V10.0 Search Tool with an *A. mellifera*-specific database to obtain more reliable results. Thus, Figure 2A-E shows the interaction networks of some identified unique proteins of the TMX group, present in five different metabolic pathways. The networks identified are related to (i) oxidative stress (Fig. 2A), (ii) cytoskeleton control (Fig. 2B), (iii) visual process (Fig. 2C), (iv) olfactory memory (Fig. 2D), and (v) glutamate metabolism (Fig. 2E). The first process, (i) oxidative stress, comprises cytochrome c oxidase subunit I, cytochrome oxidase subunit I and PRKC apoptosis WT1 regulator protein-like isoform X5; in addition to these unique proteins, we can also mention the presence of ATP synthase subunit beta and heat shock protein cognate 4 in both groups. In the second process, (ii) cytoskeletal control, unique proteins such as spectrin beta chain-like, histone deacetylase Rpd3, mushroom body large-type Kenyon cell-specific protein 1-like, talin-2-like, myelin expression factor 2, and beta-1-syntrophin-like were identified in the TMX group. While the tubulin alpha-1 chain-like and similar to -Tubulin at 56D CG9277-PB, isoform B were identified in both groups. The third process, (iii) visual process, included arginine kinase, sterile alpha and TIR motif-containing protein 1-like, ATP-dependent helicase brm, and ESF1 homolog as unique proteins identified in the TMX group. Another metabolic pathway, (iv) olfactory memory, identified in the map of protein interactions comprises epidermal growth factor receptor, arrestin homolog isoform 1, protein trapped in endoderm-1 isoform X1, G protein-coupled receptor kinase 1-like, PREDICTED: similar to Guanine nucleotide-binding protein G(i) subunit alpha 65A, and putative chemosensory receptor 2 - odorant receptor as unique proteins from the TMX group. In addition to these proteins, protein such as ras-specific guanine nucleotide-releasing factor 1-like was also identified in both groups; and the interesting finding was the presence of the gustatory receptor for sugar taste as unique protein from the

CG and not in the TMX. Finally, in the fifth pathway identified, (v) glutamate metabolism process, included cytochrome c-type heme lyase, cytochrome b5 reductase 4, isocitrate dehydrogenase [NAD] subunit beta, glutamate decarboxylase-like isoform 1, similar to Cytochrome P450 4c3 (CYPIVC3), long-chain-fatty-acid--CoA ligase ACSBG2, probable enoyl-CoA hydratase, glutamate receptor, ionotropic kainate 2-like, and glutamate receptor-like as unique proteins in the TMX group. Moreover, the glutamine synthetase was identified in both groups.

Assuming the relevance of the five metabolic pathways mentioned above, the expression of the identified proteins, from both groups, was quantitatively verified by a label-free strategy based upon to total ion current (TIC) values of all spectra assigned to a protein. According to quantitation analysis parameters adopted in the Scaffold algorithm, a total of 200 proteins in the CG and a total of 251 proteins in the TMX presented significant quantitative values associated with the MS/MS sample. Due to the large amount of data generated, these results are shown in Supplementary Table S3; and all proteomic data generated from this analysis are given in Supplementary Table S4. A Venn analysis was adopted to verify the shared proteins between CG and TMX. As shown in Figure 3A, 24 proteins were shared by both groups; in our comparative analysis, only proteins with the same accession code were designated as shared proteins between the two groups. Figure 3B shows the heat map intensity of 24 shared proteins highlighting the expression abundance in each group (Supplementary Table S3). From this total, 6 shared proteins are involved in the metabolic pathways mentioned above; and Table 1 contains some quantitative proteomic data related to these proteins and unique proteins identified in the TMX according to interaction network analyses. Figure 4A-C is representing these results showing the expression abundance of the proteins in both groups when they are present. Thus, Figure 4A shows the protein expression abundances from oxidative stress and cytoskeleton control interaction networks; while Figure 4B shows the protein expression abundances from the visual process and olfactory memory; and Figure 4C shows the protein expression abundances from glutamate metabolism interaction network. All proteins identified in the TMX were expressed in considerable levels being most abundant when compared to CG. However, in the olfactory memory metabolic pathway (Figure 4B), the

gustatory receptor protein for sugar taste (designated by “L”) is present in higher abundance in the CG, but not in the TMX.

Considering the involvement of thiamethoxam in damage to the visual process and olfactory memory, the Figure 5A-B shows the potential relationships among some most abundant proteins present in the visual process interaction network; and the potential relationships among some most abundant proteins present in the olfactory memory interaction network. As can be seen in Figure 5A, there is a close relationship among arginine kinase (red node, designated by “a”) and others proteins such as disulfide-isomerase (designated by “b”), DNA-directed RNA polymerase subunit (designated by “c”), elongation factor 1-alpha F2 (designated by “d”), elongation factor 1-alpha F1 (designated by “e”), and glyceraldehyde-3-phosphate dehydrogenase (designated by “f”) involved in the visual process interaction network. While the Figure 5B shows a close relationship among olfactory receptor 2 (designated by “a”), sensory neuron membrane protein 1 (designated by “b”), uncharacterized protein that belongs to the glutamate-gated ion channel (designated by “c” and “d”), gustatory receptor, which mediates acceptance or avoidance behaviour, depending on its substrates (designated by “e” and “h”), gustatory receptor, which plays a role in the sugar gustatory response (designated by “f”), and NMDA-type glutamate receptor 1 (designated by “g”) involved in the olfactory memory interaction network. The quantitative findings demonstrated the occurrence of unique proteins and shared proteins with altered expression in the TMX group, contributing to different metabolic pathways, which will be discussed soon; for while Figure 6 shows the schematized representation of the possible general effects of a sub-lethal dose of thiamethoxam on the bee brains, according to interaction network analyses of some most abundant identified proteins in the thiamethoxam exposed group (TMX).

4. Discussion

The current investigation evaluated through biochemical analysis the toxic effect of thiamethoxam on the honeybee brain. Proteins identified uniquely in the control group (CG) were involved in the following biological processes: transport, modification process of cellular proteins, oxidation-reduction process, and intracellular signal transduction. According to the Menegasso et al. (2016) study, these processes demonstrate that in the CG, unique proteins

are also required for the development of biological processes necessary for the normal performance of bee brain activities. However, the toxicity of thiamethoxam in the bee brain is evidenced by observing the proteins identified exclusively in the TMX group. These proteins are involved in the following biological processes: phosphorylation, cellular protein modification process, and cell surface receptor signalling process. The presence of these biological processes suggests the expression of proteins with abnormal conformations that most likely compromise the performance of neuronal functions. It is likely that exposure to thiamethoxam for 8 days induces a series of important neuroproteomic changes in the brain of *A. mellifera*; and these changes could be related to the decreased numbers of relevant expressed proteins in the TMX group. To support this, Tavares et al. (2019) demonstrated that sublethal concentrations of TMX decrease the phosphoprotein levels, such as synapsins, in the bee brain structures (mushroom bodies and the antennal lobes), which are involved in stimuli reception, learning, and memory consolidation. These proteins are involved in the transmission of the nervous signal related to the communication among neurons (Humeau et al., 2011). In addition, several studies have demonstrated the effects of TMX on the nervous system neurophysiology including cellular alterations (Friol et al., 2017), alterations in neural activity of acetylcholinesterase (Tavares et al., 2017) and in-memory ability of honeybees (Papach et al., 2017; Peng and Yang, 2016). In the toxicogenomic study by Christen et al. (2018) to verify the effects of neonicotinoid exposure, including thiamethoxam, in the brain of bees demonstrated significant alteration of genes expression; mainly genes related to metabolism and detoxification processes. It is important to mention that those results got by Christen et al. (2018) are from the genomic approach; thus, it was not considered the post-translational modifications such as phosphorylation in the understanding of the study as it was in the current investigation.

According to the interaction network analysis and quantitative findings, it was demonstrated that expressed unique proteins from the TMX participate in processes such as (i) oxidative stress, (ii) cytoskeleton control, (iii) visual process, (iv) olfactory memory and (v) glutamate metabolism. Related to oxidative stress, studies have demonstrated that cytochrome c oxidase is involved in the intrinsic apoptosis pathway, where the mitochondrial pathway is activated, involving mitochondrial membrane permeability changes and release of cytochrome c to the cytosol, forming the apoptosome complex (Desagher and Martinou, 2000; Green and

Martin, 1995; Herr and Debatin, 2001). The activation of caspase-9 mediated by cytochrome c serves as a mechanism of signal amplification during the apoptotic process (Anazetti et al., 2003; Kuida, 2000; Li et al., 1997; Slee et al., 1999). Moreover, studies have demonstrated a relationship between cytochrome c and heat shock proteins (HSP), since it was reported that HSPs inhibited apoptosis in non-neuronal cell lines (Beere et al., 2000; Klein and Brune, 2002; Saleh et al., 2000). According to these studies, the overproduction of HSPs suppressed the release of cytochrome c to the cytosol after apoptotic stimuli (Creagh et al., 2000; Klein and Brune, 2002). It is known that HSPs are cellular markers of the stress response, besides to promote the degradation of abnormal proteins, they can prevent cell apoptosis (Bierkens, 2000; Malaspina and Zacarin, 2006). Despite that, our quantitative experimental results show that due exposure to thiamethoxam, HSPs were not capable to prevent apoptosis pathway in the honeybee brain; thus, the findings suggest increased expression of cellular stress proteins that signal possible cerebral ischaemia, with a consequent loss of neuronal functions.

Considering the cytoskeleton control process, studies performed by Catae et al. (2017) and Friol et al. (2017) with the honeybee brain exposed to sublethal concentrations of imidacloprid and thiamethoxam, respectively, evidenced the loss of cell-cell contact. The data obtained at the present study corroborated the participation of cytoskeleton in the tissue disorganization for worker honeybee and this tissue disorganization was observed in the adult brain demonstrating a late effect of this neurotoxic insecticide. In the case reported by Friol et al. (2017), honeybees were exposed to thiamethoxam during the larval phase. During apoptosis, the morphological changes, according to Hacker (2000), are the change in shape, the reorganization of the outer limits of the cell, and the rupture of contact with the neighbouring cells in the tissue. Thus, the structure of the mushroom bodies can be altered by the death of the *Kenyon* cells, and this can result in some effects considered harmful to the bees such as loss of memory and alterations in olfactory sensing and learning (Aliouane et al., 2009; Decourtye et al., 2004a, 2004b; Desneux et al., 2007; Kevan, 1999). In the current investigation proteins related to cytoskeleton organization and structure, cell adhesion, and synapse organization were identified in higher abundance in the TMX. These findings suggest that the over-expression of these proteins could be related to a way to avoid the damage and tissue

disorganization due to thiamethoxam action in the brain, characterizing a possible compensatory response to neurotoxic effects.

The involvement of thiamethoxam in damage to the visual process can be evidenced through protein interactions from the visual process and proteins from the neuronal damage and apoptosis processes, which are present in the same metabolic route. Arginine kinase, identified in considerable level in the TMX when compared to CG, is relatively abundant in the central nervous system and the antennas, but this protein is present at the highest level of expression in the compound eyes of *A. mellifera* (Kucharski and Maleszka, 2003). Thus, this protein is considered an important component of energy release in the visual system in bees (Kucharski and Maleszka, 2003). Another unique protein from the TMX, ATP-dependent helicase brm, is a transcriptional regulatory protein and has been reported to be related to axonal degeneration, apoptosis process, neuronal disease, signal transduction and actin cytoskeleton, transcriptional regulation and DNA repair signal (Tamkun et al., 1992). Thus, the presence of these proteins in the TMX, in considerable levels, may be involved in possible damage to visual information processing in the brains of bees exposed to thiamethoxam.

Insects can detect olfactory signals and cues via olfactory sensory neurons located on the antenna, and it is the interaction of volatile odorant molecules with chemosensory receptor proteins that initiates the signal transduction and transmission toward the olfactory centres in the insect brain (Dobritsa et al., 2003; Vosshall et al., 2000). It was demonstrated that In *Drosophila,* the epidermal growth factor receptor promotes cell proliferation and differentiation (Kurada and White, 1999), while arrestin proteins have been described as important components for the desensitization of G protein-coupled receptor cascades that mediate neurotransmission, as well as olfactory and visual sensory reception (Merrill et al., 2002). Thus, this metabolic route shows that the exposure of bees to thiamethoxam for 8 days triggers a signalling pathway in the brain activated by protein G (the first protein of this route), which can alter the neurotransmission involved in olfactory and visual sensory reception, causing damage in learning and memory processes. Moreover, in the current study the odorant receptor protein was identified in considerable level in the TMX when compared to CG. Honeybees present behavioural plasticity and can change their behaviour according to the environment and by stimuli with events important for survival (Sialana et al., 2019; Smith and Burden, 2014). It's

known that the odorant proteins are involved in the recognition of chemical stimuli by the insect olfactory system (Leal, 2013), and the expression of this protein in abundance in the TMX suggests that olfactory system is avoiding the impairment in the stimuli perception due thiamethoxam effects. In contrast, the gustatory receptor for sugar taste, another protein related to olfactory memory interaction network showed up in higher abundance in the CG, but not in the TMX. Some studies have demonstrated that gustatory receptor gene expression is generally higher in foragers, and thus, the gustatory sensitivity increases following the transition from in-hive tasks to foraging (Amdam et al., 2006; Simcock et al., 2017). Thus, the quantitative result for this protein in the CG is totally consistent; while in the TMX, the gustatory receptor is not present in a considerable amount to be quantified due to the effects of nutritional state caused by thiamethoxam. These results suggest an impairment in the nutritional mechanism associated with feeding regulation in the honeybee; since the insect is not capable to detect the presence of nutrients and/or insecticide when it is in foraging work (Kessler et al., 2015).

The glutamate metabolism process is signalled primarily by the presence of glutamine synthetase and glutamate decarboxylase-like proteins, which are most abundant in the TMX. In the brain, glutamine synthetase is extremely important in the process of reducing ammonia concentration (detoxification) and in regulating glutamate levels (Rowbottom et al., 1996; Suárez et al., 2002), while glutamate decarboxylase-like protein is involved in the synthesis of γ-aminobutyric acid (GABA) in GABA-producing neurons (Fox and Larsen, 1972). Cytochrome P450 4c3 belongs to a broad and diverse superfamily of proteins responsible for oxidizing a large number of substances to make them more polar and water-soluble (Zhang and Scott, 1996). Since glutamate is an important neurotransmitter in bees and a glutamate precursor (Leboulle, 2012), our quantitative findings may be indicative of a compensatory response of the glutamatergic system to the damage caused to the cholinergic system in bee brains. In addition, hyperexcitation in the brain caused by thiamethoxam exposure is signalled by the production of GABA, an inhibitory neurotransmitter of the nervous system, showing an imbalance in the nervous impulse transmission system in the brain. However, this imbalance in pulse transmission, induced by increased glutamate production, probably induces toxicity to the brain, as evidenced by the presence of proteins related to cell death and stress (Leboulle, 2012) in this metabolic pathway.

Taking account that quantitative proteomics results demonstrated the alteration of protein expression in different metabolic pathways, which are impaired in the bees exposed to thiamethoxam, it is possible to suggest that these proteins are located amongst the different bee brain structures - mushroom bodies, optic lobes, and antennal lobes. The mushroom bodies are described as acting in the integration of all the sensory inputs of the bees; and are involved in the processing of high-order information in the central nervous system of insects, such as sensory integration, learning, memory and spatial orientation (Fahrbach, 2006; Farris and Sinakevitch, 2003; Kiya et al., 2007). While, the optical lobes are characterized by being the main olfactory nerve centre (primary olfactory centre), responsible for processing information on odours captured by the antennas; this region is also considered one of the major nerve centres involved in olfactory learning (Cruz-Landim, 2009; Fahrbach, 2006); and antennal lobes, which are related to the processing of information coming from the compound eyes (Cruz-Landim, 2009; Galizia et al., 1999; Menzel and Giurfa, 2001) and therefore has functions involved with the vision of the insect.

5. Conclusions

The scientific outcomes demonstrated here suggest intense brain activity in the visual centres, olfactory centres, and centres of sensorial and motor integration in the TMX group. The investigation of the cerebral structures affected by the insecticide is of paramount importance since, through this knowledge, it is possible to demonstrate the behavioural and physiological consequences of this exposure in the insects. Thus, the proteomic findings presented here indicate that bees exposed to thiamethoxam can suffer memory and olfactory loss, as well as visual deficiencies. Overall, our toxicoproteomic data may contribute to improving our understanding on the effects of neurotoxic insecticides in the bee brain and may also be relevant for scientists in environmental chemistry and toxicology, by forming a database and facilitating the design of future studies in the area.

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Conflicts of interest

The authors declare no conflicts of interest in relation to this manuscript.

Supporting information

Supporting information may be found in the online version of this article.

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828 **Figures**
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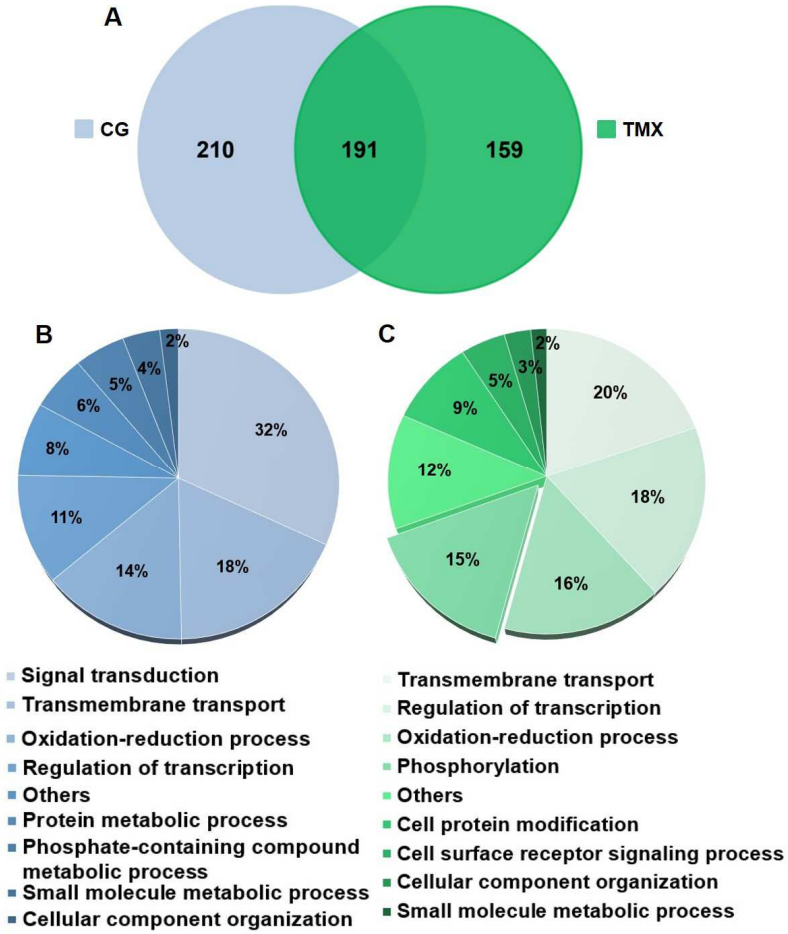

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832
833 **Fig. 1. A -** Venn diagram of identified total proteins in the bee brains highlighting the number of
834 proteins uniquely identified in the thiamethoxam exposed group (TMX). Biological process
835 categorization of the unique proteins identified in each group: **B -** control group (CG) and **C -**
836 thiamethoxam exposed group (TMX).
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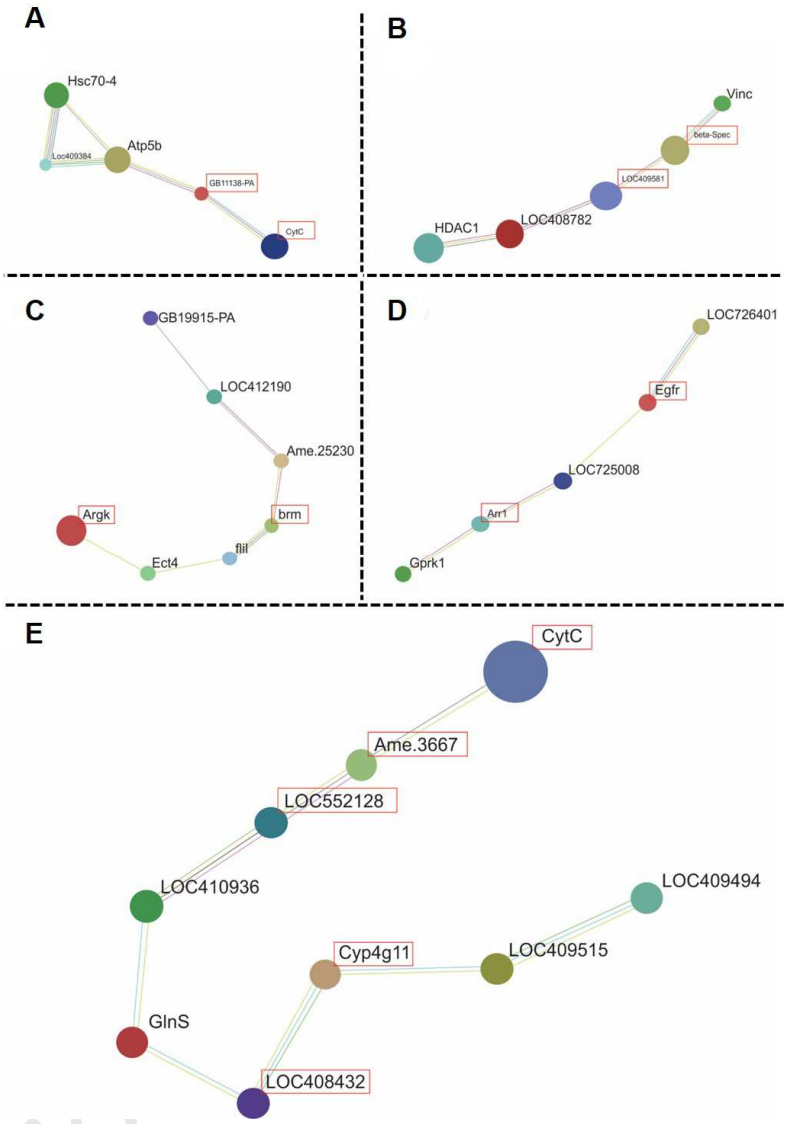


Fig. 2. Protein-protein interaction network of the proteins uniquely identified in the thiamethoxam exposed group (TMX). **A -** (i) Oxidative stress: CytC - cytochrome c; GB11138-PA - cytochrome c oxidase subunit 3; Atp5b - ATP synthase subunit beta; LOC409384 - 60 kDa heat shock protein; Hsc70-4 - heat shock protein cognate 4. **B -** (ii) Cytoskeleton control: beta-Spec - beta spectrin; LOC409581 - f-actin-capping protein subunit beta-like; LOC408782 - tubulin beta-1; HDAC1 - histone deacetylase 1. **C -** (iii) Visual process: Argk - Arginine kinase; Ect4 - sterile alpha and TIR motif-containing protein 1; flil - flightless I; brm - ATP-dependent helicase brahma; Ame.25230 - ESF1 homolog; LOC412190 - probable ATP-dependent RNA helicase DDX47-like; GB19915-PA dimethyladenosine transferase. **D -** (iv) Olfactory memory: Egfr - epidermal growth factor receptor; Arr1 - arrestin 1; LOC725008 - protein trapped in endoderm-1-like; Gprk1 - G protein-coupled receptor kinase 1; LOC726401 - ras-specific guanine nucleotide-releasing factor 1-like. **E -** (v) Glutamate metabolism: CytC - cytochrome c; Ame.3667 - cytochrome c-type heme lyase-like; LOC410936 - cytochrome b5 reductase 4-like; LOC552128 - isocitrate dehydrogenase [NAD] subunit beta; GlnS - glutamine synthetase; LOC408432 - glutamate decarboxylase-like; Cyp4g11 - cytochrome P450 monooxygenase; LOC409515 - long-chain-fatty-acid--CoA ligase 3-like; LOC409494 - enoyl-CoA hydratase domain-containing protein 2, mitochondrial-like. The network was constructed by the STRING v10.0 Search Tool.

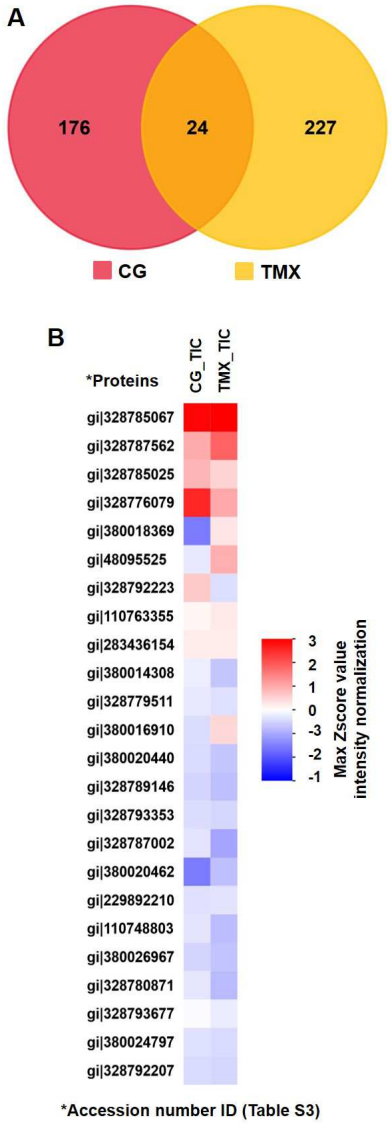


Fig. 3. Quantitative proteome differences in the control group (CG) and thiamethoxam exposed group (TMX). **A -** Venn diagram of most abundant identified proteins in the bee brains by quantitative label-free analysis. **B -** Heat map of shared most abundant proteins between CG and TMX. TIC - Total ion current.

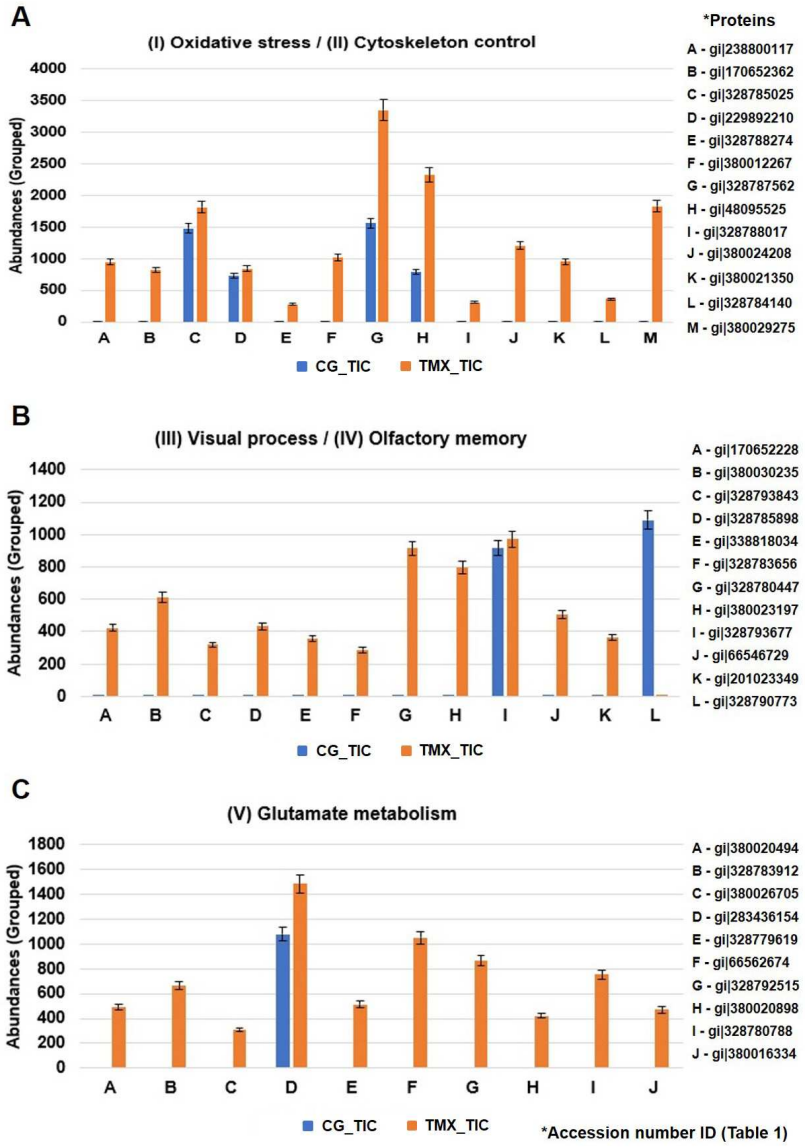
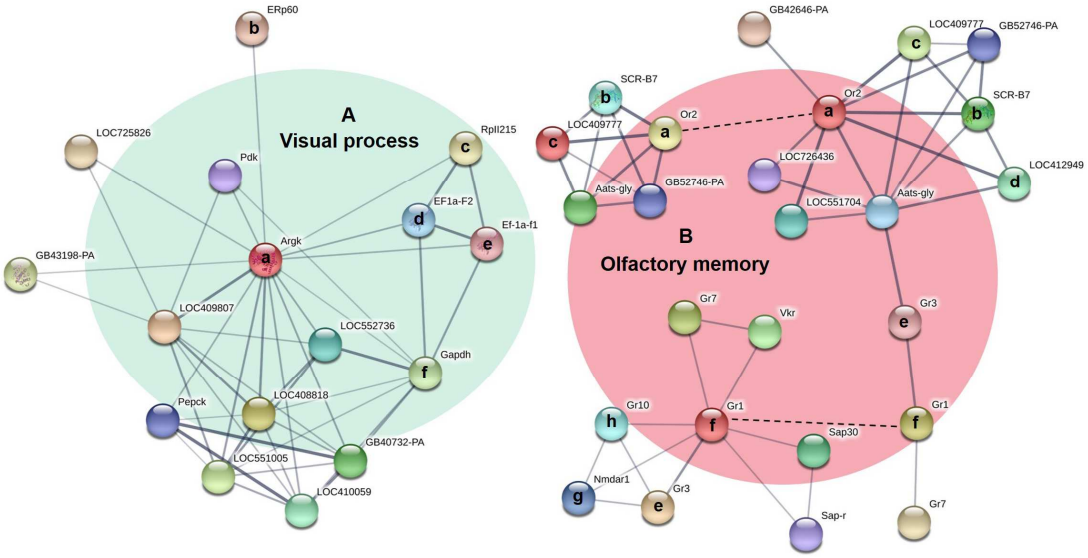


Fig. 4. Quantitative label-free proteomic analysis of the most abundant proteins, highlighting the proteinaceous related to different metabolic pathways in the bee brains according to interaction network analyses. **A -** (I) oxidative stress and (II) cytoskeleton control, **B -** (III) visual process and (IV) olfactory memory, **C -** (V) glutamate metabolism. Abundances were based upon precursor/peptide (TIC) total ion current. CG - control group; TMX - thiamethoxam exposed group.



884
885 **Fig. 5.** Protein interaction network analyses of some most abundant identified proteins related
886 to interaction network analyses of visual process and olfactory memory. The protein network in
887 the confidence view generated by the STRING database is demonstrated. **A -** visual process: **a**
888 **-** arginine kinase; b - protein disulfide-isomerase; **c -** DNA-directed RNA polymerase subunit; **d -**
889 elongation factor 1-alpha F2; **e -** elongation factor 1-alpha F1; **f -** glyceraldehyde-3-phosphate
890 dehydrogenase. **B -** olfactory memory: **a-** olfactory receptor 2; **b -** sensory neuron membrane
891 protein 1; **c -** uncharacterized protein that belongs to the glutamate-gated ion channel; **d -**
892 uncharacterized protein that belongs to the glutamate-gated ion channel; **e -** gustatory receptor,
893 which mediates acceptance or avoidance behaviour, depending on its substrates; **f -** gustatory
894 receptor, which plays a role in the sugar gustatory response; **g -** NMDA-type glutamate receptor
895 1; **h -** gustatory receptor, which mediates acceptance or avoidance behaviour, depending on its
896 substrates. Stronger associations are represented by thicker lines.
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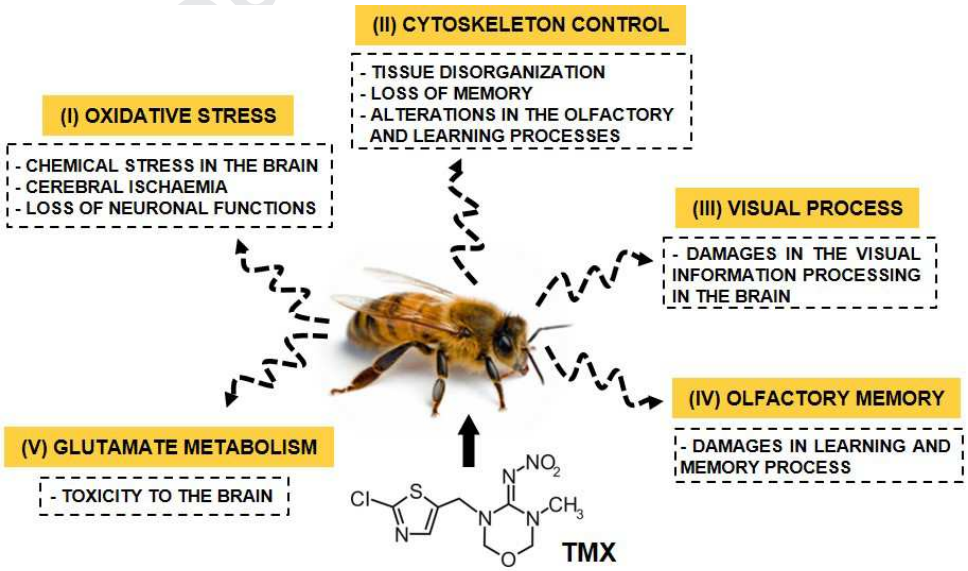

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902 **Fig. 6.** Schematized representation of the possible general effects of a sub-lethal dose of
903 thiamethoxam on the bee brains, according to interaction network analyses of some most
904 abundant identified proteins from the thiamethoxam exposed group (TMX).

Table 1. Quantitative label-free proteomic analysis of the most abundant proteins, highlighting the proteinaceous related to different metabolic pathways in the bee brains according to interaction network analyses. Abundances were based upon precursor/peptide (TIC) total ion current. CG - control group; TMX - thiamethoxam exposed group; *PIP % - score, protein identification probability.

Accession number	Identified proteins	Taxonomy	MW	Folding expression	CG_TIC	TMX_TIC	SC_*PIP	TMX_*PIP
	(I) Oxidative stress							
gi|238800117	Cytochrome c oxidase subunit I	*Apis cerana*	59 kDa	630000	1,5	945,68	-	100%
gi|170652362	Cytochrome oxidase subunit I	*Apis koschevnikovi*	40 kDa	550000	1,5	821,67	-	100%
gi|328785025	ATP synthase subunit beta, mitochondrial	*Apis mellifera*	55 kDa	1.2	1478,70	1810,50	100%	100%
gi|229892210	Heat shock protein cognate 4	*Apis mellifera*	71 kDa	1.2	731,87	842,50	100%	100%
gi|328788274	PRKC apoptosis WT1 regulator protein-like isoform X5	*Apis mellifera*	35 kDa	190000	1,5	278,26	-	99%
	(II) Cytosqueleton control							
gi|380012267	Spectrin beta chain-like	*Apis florea*	278 kDa	680000	1,5	1016,70	-	100%
gi|328787562	Tubulin alpha-1 chain-like	*Apis mellifera*	53 kDa	2.1	1556,90	3346,00	100%	99%
gi|48095525	Similar to -Tubulin at 56D CG9277-PB, isoform B	*Apis mellifera*	50 kDa	3.0	784,58	2319,00	100%	100%
gi|328788017	Histone deacetylase Rpd3	*Apis mellifera*	56 kDa	200000	1,5	304,77	-	99%
gi|380024208	Mushroom body large-type Kenyon cell-specific protein 1-like	*Apis florea*	161 kDa	800000	1,5	1203,60	-	100%
gi|380021350	Talin-2-like	*Apis florea*	292 kDa	630000	1,5	949,70	-	100%
gi|328784140	Myelin expression factor 2	*Apis mellifera*	59 Kda	240000	1,5	359,11	-	99%
gi|380029275	Beta-1-syntrophin-like	*Apis florea*	32 kDa	1200000	1,5	1824,10	-	100%
	(III) Visual process							
gi|170652228	Arginine kinase	*Apis andreniformis*	17 kDa	280000	1,5	422,87	-	100%
gi|380030235	Sterile alpha and TIR motif-containing protein 1-like	*Apis florea*	157 kDa	410000	1,5	613,12	-	100%
gi|328793843	ATP-dependent helicase brm	*Apis mellifera*	218 kDa	210000	1,5	319,84	-	100%
gi|328785898	ESF1 homolog	*Apis mellifera*	86 kDa	290000	1,5	434,23	-	100%
	(IV) Olfactory memory							

gi|338818034	Epidermal growth factor receptor	*Apis mellifera*	144 kDa	240000	1,5	359,03	-	100%
gi|328783656	Arrestin homolog isoform 1	*Apis mellifera*	42 kDa	190000	1,5	286,49	-	99%
gi|328780447	Protein trapped in endoderm-1 isoform X1	*Apis mellifera*	38 kDa	610000	1,5	913,99	-	100%
gi|380023197	G protein-coupled receptor kinase 1-like	*Apis florea*	73 kDa	530000	1,5	794,72	-	100%
gi|328793677	Ras-specific guanine nucleotide-releasing factor 1-like	*Apis mellifera*	158 kDa	1.1	916,76	971,17	100%	100%
gi|66546729	Similar to Guanine nucleotide-binding protein G(i) subunit alpha 65A	*Apis mellifera*	40 kDa	340000	1,5	508,34	-	100%
gi|201023349	Putative chemosensory receptor 2, odorant receptor	*Apis mellifera*	54 kDa	240000	1,5	362,64	-	100%
gi|328790773	Gustatory receptor for sugar taste	*Apis mellifera*	53 kDa	0.000001	1089,90	1,5	100%	-
	(V) Glutamate matabolism							
gi|380020494	Cytochrome c-type heme lyase	*Apis florea*	32 kDa	330000	1,5	491,16	-	100%
gi|328783912	Cytochrome b5 reductase 4	*Apis mellifera*	65 kDa	440000	1,5	663,55	-	100%
gi|380026705	Isocitrate dehydrogenase [NAD] subunit beta,	Apis florea	41 kDa	210000	1,5	310,93	-	100%
gi|283436154	Glutamine synthetase	*Apis mellifera*	41 kDa	1.4	1079,30	1483,70	100%	100%
gi|328779619	Glutamate decarboxylase-like isoform 1	*Apis mellifera*	61 kDa	340000	1,5	512,26	-	100%
gi|66562674	Similar to Cytochrome P450 4c3 (CYPIVC3)	*Apis mellifera*	58 kDa	700000	1,5	1049,00	-	100%
gi|328792515	Long-chain-fatty-acid--CoA ligase ACSBG2	*Apis mellifera*	73 kDa	580000	1,5	863,51	-	100%
gi|380020898	Probable enoyl-CoA hydratase, mitochondrial	*Apis florea*	35 kDa	280000	1,5	422,37	-	100%
gi|328780788	Glutamate receptor, ionotropic kainate 2-like	*Apis mellifera*	101 kDa	500000	1,5	754,36	-	100%
gi|380016334	Glutamate receptor-like	*Apis florea*	66 kDa	310000	1,5	469,20	-	100%

Highlights

1. Neurotoxic insecticide affected the bee biological processes and metabolic pathways.
2. The exposed group to thiamethoxam presented altered expression proteins.
3. The altered proteins identified are involved in the nervous system of bees.
4. The data suggest intense brain activities in the exposed group to the insecticide.
5. Bees can suffer memory loss, olfactory and visual deficiencies.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: