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Proteins from eggs of the spittlebug *Mahanarva spectabilis* (Hemiptera: Cercopidae) reveal clues about its diapause regulation

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Research Paper

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Abstract

Embryo development in eggs of the spittlebug *Mahanarva spectabilis* (Distant) (Hemiptera: Cercopidae) passes through four phases (known as S1 to S4) being stopped at S2 during diapause. Studies about the molecular basis of diapause in spittlebugs are nonexistent. Here, we analyzed proteins from non-diapausing (ND), diapausing (D) and post-diapausing (PD) eggs of the spittlebug *M. spectabilis*. In total, we identified 87 proteins where 12 were in common among the developmental and diapause phases and 19 remained as uncharacterized. Non-diapausing eggs (S2ND and S4ND) showed more proteins involved in information storage and processing than the diapausing ones (S2D). Eggs in post-diapausing (S4PD) had a higher number of proteins associated with metabolism than S2D. The network of protein interactions and metabolic processes allowed the identification of different sets of molecular interactions for each developmental and diapause phases. Two heat shock proteins (Hsp65 and Hsp70) along with two proteins associated with intracellular signaling (MAP4K and a serine/threonine-protein phosphatase) were found only in diapausing and/or post-diapausing eggs and are interesting targets to be explored in future experiments. These results shine a light on one key biological process for spittlebug survival and represent the first search for proteins linked to diapause in this important group of insects.

Introduction

Spittlebug is the common name given to sucking-sap insects (Hemiptera: Cercopidae) whose nymphs produce foam while feeding on the roots of host plants. Among the different species of spittlebugs in Brazil, *Mahanarva spectabilis* (Distant) (Hemiptera: Cercopidae) has received attention due to its broad occurrence in the Brazilian territory and its major impact on productivity and quality of forages grasses (Alvarenga *et al.*, 2019). After reaching adulthood, *M. spectabilis* feed by sucking sap of forage plants and, in general, each female lays an average of near 100 eggs (Valério, 2009). Embryogenic development in the eggs passes through four different phases (named as S1 to S4) where each phase has specific attributes such as appearance of the hatching line (S1), operculum black in color becoming evident (S2), exposure of the black surface of the operculum (S3) and embryo exhibiting red ocelli and abdominal spots (S4) (Peck, 2002). Under normal conditions in Brazil, the development of *M. spectabilis* egg takes about 20 days (Auad and Carvalho, 2009; Auad *et al.*, 2011). However, environmental conditions are not always optimal for egg development and survival.

Most of the area cultivated with tropical forages in Brazil is under well-defined wet and dry seasons. In Central Brazil, a rainy season for most of spring and summer (October to March) commonly occurs followed by a period of lower precipitation in most of autumn and winter (from April to September) (Pereira *et al.*, 2019). Around 80–90% of the annual precipitation is observed during the wet season (Assad *et al.*, 1993) which enables the forage species to reach significantly higher yield and quality. On the other hand, the dry season is marked by the low productivity and quality of the forages. It has been reported that the dry and wet seasons have consequences to the biology of *M. spectabilis*. For instance, nymphs and adults are most frequently observed in the field during the rainy season (Valério, 2009). During the dry season the survival of *M. spectabilis* eggs requires a mechanism known as diapause (Valério, 2009; Auad *et al.*, 2011), which is a process where the development is delayed, cell division is slowed or stopped and stress tolerance is enhanced (King and MacRae, 2015). As a consequence, the organism enters in a physiological condition that allows its survival under unfavorable conditions. As a multivoltine species with facultative diapause (Silveira Neto *et al.*, 1976), near all eggs laid by *M. spectabilis* during the wet season are non-diapausing but the diapausing

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eggs become the predominant ones when the wet season is ending (Auad *et al.,* 2011). Diapausing eggs of *M. spectabilis* stop the embryonic development at S2 which results in non-diapausing and diapausing eggs spending different amounts of time to finish their development (19–21 days for non-diapausing eggs in comparison with 138–154 days for diapausing eggs) (Auad and Carvalho, 2009; Auad *et al.,* 2011). The duration of diapause is important for *M. spectabilis* to synchronize its occurrence in the field with the beginning of the next wet season. Interestingly, the stimuli to initiate or terminate diapause in *M. spectabilis* seems not to be associated with some climate conditions, such as temperature, relative humidity and photoperiod, since eggs kept in a controlled environment respond similarly as expected under field conditions (Auad *et al.,* 2011).

There are three typically described phases of diapause known as initiation, maintenance and termination (Koštál, 2006). The molecular mechanisms necessary to enter and progress through the phases of diapause probably require regulatory elements to drive the process (MacRae, 2010). This assumption has led several groups to endeavor toward a better understanding of the molecular response to diapause and post-diapause (King and MacRae, 2015; Bryon *et al.,* 2017). In this context, studies regarding the molecular aspects of diapausing eggs of spittlebugs must be performed to increase our knowledge about the diapause process, better understand the biology of spittlebugs and potentially establish new strategies to control these insects. In the past few years, a number of studies have identified proteins associated with diapause in insects from different taxonomic groups as coleopterans (Ren *et al.,* 2016; Tan *et al.,* 2017; Ma *et al.,* 2019), dipterans (Zhang *et al.,* 2019), lepidopterans (Zhang *et al.,* 2012; Fan *et al.,* 2013) and orthopterans (Tu *et al.,* 2015; Hao *et al.,* 2017; Cui *et al.,* 2019). Although there are a few studies regarding diapause-related proteins in hemipterans (Wolschin and Gadau, 2009; Colinet *et al.,* 2012), this type of investigation is nonexistent for spittlebugs.

Based on the lack of information regarding the proteins involved in diapause of spittlebugs, this paper aims at identifying and characterizing proteins from non-diapausing, diapausing and post-diapausing eggs of *M. spectabilis*. This is the first study about proteins associated with diapause in spittlebugs, which not only increases our knowledge about the biology of this interesting group of insects but could led to new strategies to reduce their impact on forage plants.

Materials and methods

Selection of non-diapausing, diapausing and post-diapausing eggs

The probability of obtaining non-diapausing and diapausing eggs of *M. spectabilis* depends on the time of the year where the adults are collected in the field (Auad *et al.,* 2011). Thus, the adults of *M. spectabilis* were collected at the experimental field of Embrapa Dairy Cattle (21°33′22″ S and 43°16′15″ W) in two seasons: spring, where there is a low probability of finding diapausing eggs, and autumn, where there is a high probability. The insects were kept in cylindrical acrylic cages ($30 \times 30 \times 60$ cm^3), lined with moist gaze and distilled water. The eggs deposited on the substrate were collected, washed with water jet on a series of sieves, placed in Petri dishes (10 cm diameter) lined with filter paper and incubated in BOD type climatic chamber (28 ± 2°C, 70 ± 10% relative humidity, 14 h photophase). Every day the filter paper was moistened with distilled water. The identification of non-diapausing (ND), diapausing (D) and post-diapausing (PD) eggs was based in the physical features of the eggs as well as in the time required to reach the developmental phase. The physical features were evaluated under a stereomicroscope where specific attributes from each phase were evaluated (Peck, 2002), as appearance of the hatching line in developmental phase S1, operculum black in color becoming evident in phase S2, exposure of the black surface of the operculum in phase S3 and embryo exhibiting red ocelli and abdominal spots in phase S4. Upon reaching the phases S2 and S4, with eggs showing the physical features of these phases within the estimated time for non-diapausing development (4 and 12 days after laying the eggs, respectively), non-diapausing eggs (S2ND and S4ND) were collected and frozen at −20°C. The eggs that kept the physical features of the phase S2 for 30 days after laying were classified as diapausing (S2D). The S2D eggs that advanced to the S4 phase after about 150 days were classified as post-diapausing (S4PD). Both S2D and S4PD eggs were collected and stored at −20°C. A schematic representation of the egg development and the diapausing phases studied here is provided (fig. 1).

Sample preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

One hundred and twenty eggs from each of the phases S2ND, S4ND, S2D and S4PD were placed in tubes and mechanically homogenized in 50 µl of 4% SDS buffer. The samples were then placed in liquid nitrogen for 5 s, disrupted in Tissuelyser II (QIAGEN) for 2 min at 30 rpm and sonicated for 30 s in an ice bath. This procedure was repeated five times with a 30 s interval between each repetition. After the five cycles, the samples were centrifuged (13,000 ***g***) at 4°C for 10 min, the supernatants were collected and subjected to protein quantification by the Bradford method (Bradford, 1976). A total of 50 µg of protein was analyzed on polyacrylamide gel (12%) under denaturing conditions (SDS-PAGE), as described previously (Laemmli, 1970). The process was performed at 70 V, allowing the sample to go through 2 cm in the resolution gel. The gel was stained with Coomassie G-250 blue solution and digitized using ImageScanner III (GE Healthcare).

Liquid chromatography–mass spectrometry (LC-MS)/MS analysis and protein identification

The protein bands were manually excised by cutting the gel in strips of about 2 mm each (fig. 2) and subjected to trypsin digestion (Shevchenko *et al.,* 2006). The gel fragments were decolorized due to successive washes in 50 mM ammonium bicarbonate solution and 50% methanol, followed by acetonitrile for dehydration. Proteins were reduced in 200 mM DTT solution, prepared in 100 mM ammonium bicarbonate, for 30 min at 56°C using dry bath. They were then alkylated in 200 mM of iodoacetamide and in 100 mM of ammonium bicarbonate solution, for 30 min in the dark and at room temperature. The gel pieces were washed with 100 mM ammonium bicarbonate for 10 min, dehydrated in acetonitrile for 5 min and dried in speed vac for 15 min. For the enzymatic digestion, the gels were rehydrated with solution containing trypsin (from porcine pancreas, proteomics grade – Sigma-Aldrich), 20 ng ml^{-1} of 40 mM ammonium bicarbonate solution, pH 8.0 and 10% acetonitrile; and then incubated at 37°C for 16 h. The digested peptides were extracted using 50%

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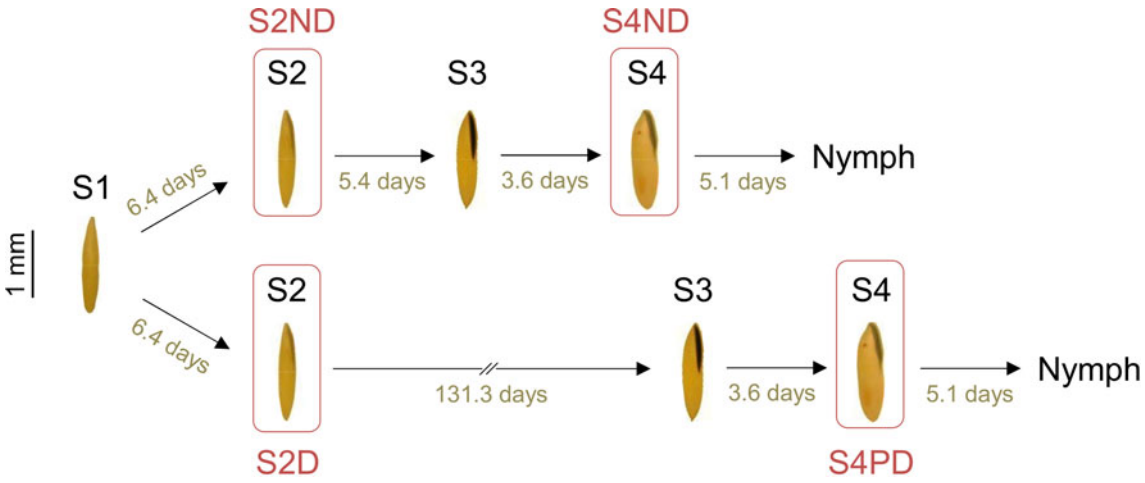

Figure 1. Phases of egg development (S1 to S4) and diapause of *M. spectabilis*. The duration of each phase in non-diapausing eggs is based on an *in vitro* cultivation where eggs were daily watered (Auad and Carvalho, 2009). For the diapausing eggs, the duration of S2 was calculated by subtracting the expected number of days of S1, S3 and S4 from 146.4 days, which is the mean diapause duration of *M. spectabilis* based on two seasons (Auad *et al.*, 2011). One hundred and twenty eggs from each phase denominated as S2ND and S4ND (non-diapausing), S2D (diapausing) and S4PD (post-diapausing) were used in this study. We aimed to compare proteins among the phases S2ND, S4ND, S2D and S4PD.

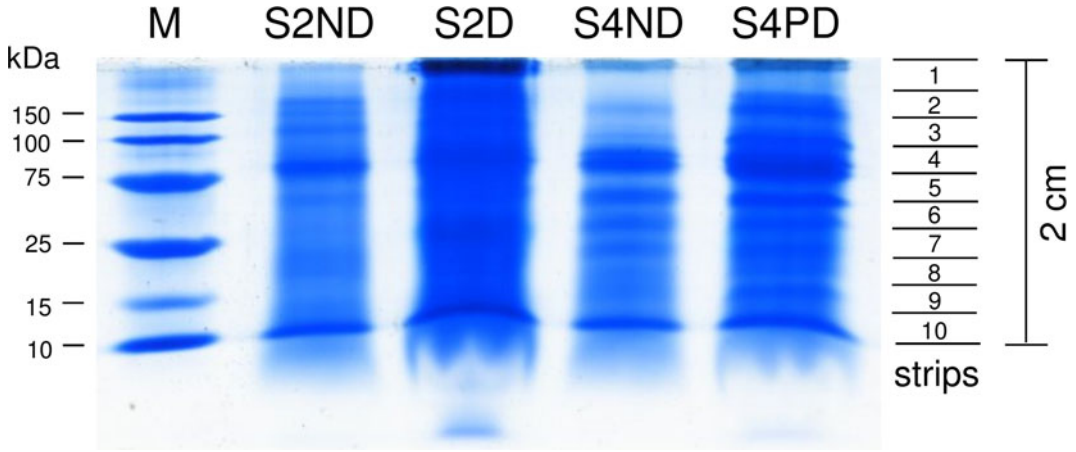

Figure 2. SDS-PAGE of unfractionated proteins from non-diapausing (S2ND and S4ND), diapausing (S2D) and post-diapausing (S4PD) eggs of *M. spectabilis*. The samples were separated on 12% polyacrylamide gel under denaturing conditions and, after the samples were electrophoresed through 2 cm, the gel was manually cut in strips of about 2 mm each. The proteins extracted from the gel were then subjected to LC-MS/MS analysis.

ethyl acetate extraction buffer and 5% formic acid, dried by vacuum centrifugation and resuspended in 0.1% formic acid.

The samples of *M. spectabilis* eggs were subjected to the chromatographic analysis in trap column and C18 BEH130 capillary column (1.7 µm × 100 µm × 100 mm) operating at a flow rate of 0.400 µl min^{-1}. The eluted peptides were automatically injected into an Ion-trap mass spectrometer (Amazon-Bruker), acting in the online mode, using a nanoESI ionization needle. The ion scans performed by the mass spectrometer were between 300 and 1500 *m*/*z* in positive mode and the data were acquired for 60 min in each LC-MS/MS analysis. The mass spectrometer was operated in auto-MSn mode. The data acquisition was managed by Hystar software, version 3.2 (Bruker Daltonics) and the spectra were processed by Data Analysis, version 4.0, software (Bruker Daltonics) using the standard configurations for proteomics.

The spectra were analyzed by PEAKS software, version 7.0 (Bioinformatics Solutions Inc.) with a local client license, connected to a remote server. Protein identification was performed by comparing the lists of masses generated against the Insecta protein database (downloaded on 19 January 2017, with 2,560,922 entries) deposited with Uniprot (https://www.uniprot.org/). The parameters used in the program were: oxidation of methionine as a variable modification, cysteine carbamidomethylation as fixed modification, a lost cleavage, charge states of 2+, 3+, 4+, trypsin as a cleavage enzyme and mass error of 0.15 Da. False discovery rate (FDR) identifications of less than 1% were considered to be true positives.

Functional annotations, protein comparisons among developmental and diapause phases and ontology analysis

Proteins were subjected to similarity search using BLASTp against the *Phytozome* protein database, GI numbers were extracted and sent to UniprotRetrived/ID to generate the functional characterization (GO). After that, 36 uncharacterized proteins were found. Genes were identified using *Drosophila* annotations

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deposited in the euKaryotic Ortholog Groups (KOG) database (table S1). A second round of annotation was performed by subjecting the sequences of the 36 uncharacterized proteins to BLASTp against *Insecta* database. BLASTp results with *E*-value higher than 1×10^{-3} were not considered and 19 proteins remained as uncharacterized (table S2). All proteins were used in cluster analysis to identify which are specific or common, between different developmental and diapause phases (S2ND, S4ND, S2D and S4PD). Interaction networks between proteins were predicted using STRING 10.0 software (http://stringdb.org/). The accession numbers for each protein generated by UniProt were loaded into the software, which was configured to search the *Drosophila ananassae* database deposited in STRING 10.0 software. The required minimum interaction score was set to 0.900 (high confidence) and no more than 20 interactions were allowed.

Results

Identification of proteins in non-diapausing, diapausing and post-diapausing eggs of M. spectabilis

We were able to identify 87 proteins (table S1) with high confidence using PEAKS 7 software (FDR < 5%). Among these proteins, 41 were identified in non-diapausing eggs (19 in S2ND and 22 in S4ND), 20 in diapausing eggs (S2D) and 26 in post-diapausing eggs (S4PD). The uncharacterized proteins represented 18.2% of the proteins specifically found in S2ND, 26.7% in S4ND, 38.5% in S2D and 16.7% in S4PD (tables S1 and S2). Most proteins were specific to one of the phases, including a mitogen-activated protein kinase kinase kinase kinase (MAP4K) found only in S2D and one heat shock protein (Hsp65) along with a serine/threonine-protein phosphatase that were found only in S4PD (table S1). However, 12 proteins were identified as common among the phases (fig. S1). Among the common proteins, two were found as common to all phases (two vitellogenins) and two in common among three phases (one uncharacterized protein in S2ND, S4ND and S2D and one vitellogenin-like in S2ND, S2D and S4PD). Moreover, proteins were found in common between the same developmental phase in non-diapausing and diapausing eggs: tubulin alpha chain in S2ND and S2D and tubulin alpha chain and myosin heavy chain in S4ND and S4PD. We also found proteins in common between eggs from different developmental and diapause phases: vitellogenin and putative hemoglobin subunit alpha in S4ND and S2D and vitellogenin, putative hemoglobin subunit alpha and one uncharacterized protein in S2ND and S4PD (fig. 3, tables S1 and S2). Although some proteins do not have the same code and consequently were not counted as common in the Venn diagram (fig. S1), proteins with the same function were also found as common among phases. That includes histones (H2A, H2B or H4) among all phases, vitellogenin in S2ND and S4ND, and one Hsp70 in S2D and S4PD (fig. 3). Although some proteins were in common among different phases, the number of peptide sequences for each annotated protein was different (with the maximum of eight peptide sequences for an uncharacterized protein in S4ND).

When the functional categorization of the proteins was analyzed, the eggs in S2ND were found to have a higher number of proteins involved in information storage and processing (55%). That number is twice as many when compared with diapausing eggs in the same developmental phase (S2D). The same trend was observed with eggs from a more advanced developmental phase (S4), in which the non-diapausing eggs had 3.8 times more proteins involved in the information storage and processing than the diapausing ones (fig. S2). Eggs in S4 had higher percentage of proteins associated with cellular process and signaling (46% in S4ND and 50% in S4PD) when compared to S2 (27% in S2ND and 38% in S2D) (fig. S2). Eggs in post-diapause (S4PD) showed higher number of proteins associated with metabolism (35%) when compared with eggs in diapause (S2D) (23%) (fig. S2).

Protein–protein interaction and biological significance of proteins identified in non-diapausing, diapausing and post-diapausing eggs of M. spectabilis

The annotated proteins from the non-diapausing and diapausing eggs were subjected to STRING 10.0 software, which allowed visualizing the network of protein interactions and metabolic processes (fig. S3). Among the proteins identified in the non-diapausing eggs, we identified histones (His2B and His4 in S2ND and His2A in S4ND), ribosomal protein (in S2ND) and elongation factor 1-alpha (in S4ND) (table S1), that comprise proteins involved in macromolecule biosynthesis processes and regulation of gene expression. In the diapausing eggs (S2D), we identified vitellogenins (Yp1, Yp2 and Yp3), heat shock proteins (Hsp68, Hsp70Ab and Hsp70Bc) and DNA binding proteins (His2A, His2B, His4, Fbpp0085281 and CG31613), which was the group with the larger number of protein interactions (fig. S3). Based on their molecular functions, the predominant group of proteins in S2D is related to mechanisms of phosphorylation and proton transport. In post-diapausing eggs (S4PD), we also identified DNA-binding proteins, vitellogenins and thermal shock proteins. Additionally, this phase contains proteins related to the generation of metabolites energy precursors (Eno, Tpi, PyK, Pglym78, Ald, Gapdh1, Gapdh2, blw, Oscp, ATPsyn-d, CG7813, CG5389, CG7610, CG1746 and I(1)G0230), comprising glycolytic processes and ATP (adenosine triphosphate) metabolism. In S4PD the predominant group of proteins is related to molecular activity and ribosomal constituents. This result is consistent with the higher percentage of proteins associated with metabolism found in S4PD eggs in comparison with S2D (fig. S2).

Discussion

In this study, proteins were identified from non-diapausing, diapausing and post-diapausing eggs of *M. spectabilis*, at embryo developmental phases S2 and S4. We observed the presence of common proteins such as tubulin alpha chain, vitellogenin and histones. Among the common proteins, the Hsp70 found in the diapausing eggs (S2D) and post-diapausing eggs (S4PD) can be highlighted (table S1). Among the proteins that were found as specific to each phase, a MAP4K was found only in the diapausing eggs (S2D) and an Hsp65 along with a serine/threonine-protein phosphatase were detected only in the post-diapausing eggs (S4PD). Our analysis of the network of the possible protein-protein interactions clearly indicated different sets of molecular interactions for each phase (fig. S3).

The molecular aspects of diapause have been studied in a number of insects (King and MacRae, 2015; Bryon *et al.*, 2017; Ragland *et al.*, 2019). These insects avoid adverse conditions either imposed by cold winter, which are usually faced in regions with a temperate climate, or dry seasons, which are faced by spittlebugs in Central Brazil (Valério, 2009; Auad *et al.*, 2011). Depending on the methodology and tools used, the number of proteins detected in studies investigating diapause varies largely

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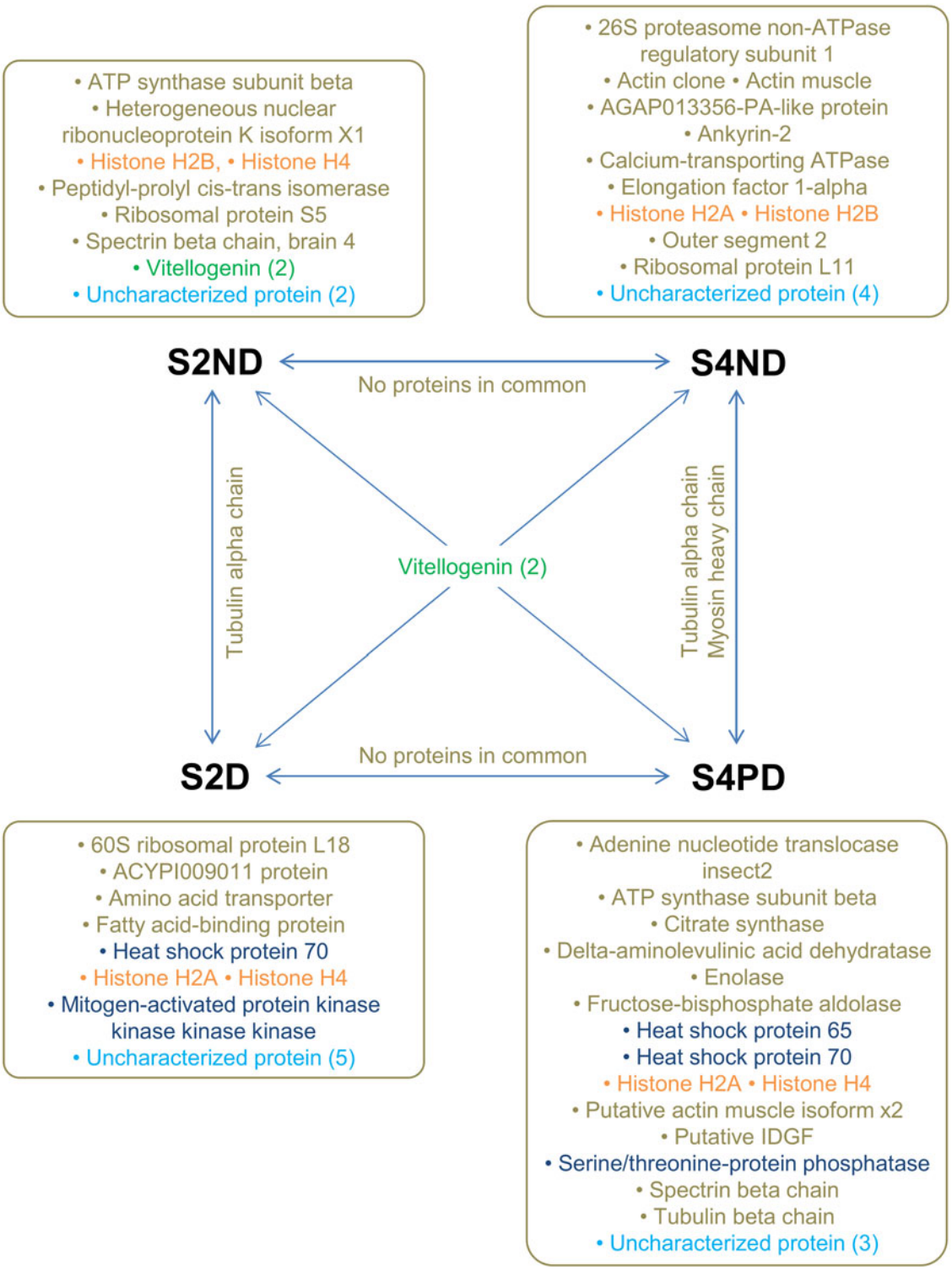

Figure 3. Schematic representation of the specific and common proteins found in non-diapausing (S2ND and S4ND), diapausing (S2D) and post-diapausing (S4PD) eggs of *M. spectabilis*. Numbers in parentheses indicate the number of proteins with the same function. Some proteins discussed in the paper are highlighted in different colors. Proteins in light blue letters indicate results with *E*-value $>1 \times 10^{-3}$. Common proteins not represented in the figure are: vitellogenin, putative hemoglobin subunit alpha and one uncharacterized protein between S2ND and S4PD; vitellogenin and putative hemoglobin subunit alpha between S4ND and S2D; vitellogenin-like among S2ND, S2D and S4PD; and one uncharacterized protein among S2ND, S4ND and S2D. No common proteins were detected among S2ND, S4ND and S4PD.

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as 221 significantly different proteins between diapausing and non-diapausing pupae of *Praon volucre* Haliday (Hymenoptera: Aphidiinae) (Colinet *et al.,* 2012), 58 differently expressed proteins between diapause and non-diapause female adults of the seven-spotted ladybird beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) (Ren *et al.,* 2016), 24 up-regulated proteins between diapause and non-diapause eggs of the migratory locust, *Locusta migratoria* L. (Orthoptera: Acrididae) (Cui *et al.,* 2019) and 3175 in non-diapause-destined and diapause-destined female adults of the cabbage beetle, *Colaphellus bowringi* Baly (Coleoptera: Chrysomelidae) (Tan *et al.,* 2017). In these studies, the number of annotated proteins were 14 (Colinet *et al.,* 2012), eight (Ren *et al.,* 2016) and 138 (Tan *et al.,* 2017). In our study, we identified 87 proteins within the eggs of non-diapausing, diapausing and post-diapausing eggs of *M. spectabilis*, a high proportion of which (68 out of 87) were annotated. Thus, the number of annotated proteins here is greater than some previous reports (Colinet *et al.,* 2012; Zhang *et al.,* 2012; Ren *et al.,* 2016). We have detected 19 uncharacterized proteins (representing from 16.7 to 38.5% of the specific proteins from each phase). It is not possible to rule out a putative role of these uncharacterized proteins in diapausing or post-diapausing eggs of *M. spectabilis* and future studies could provide more information about these uncharacterized proteins. These studies may focus on new searches in updated databanks, sequencing larger fragments of the proteins and even determining the protein structures at atomic or near-atomic resolution in order reveal their potential role in insect biology.

Histones (H2A, H2B and H4) and tubulin alpha chain were found in all embryonic developmental and diapause phases while vitellogenins were found in different phases of embryonic development (fig. 3, tables S1 and S2). Vitellogenin was also found in non-diapause and diapause eggs of the silk moth *Bombyx mori* L. (Lepidoptera: Bombycidae) when an LC-MS/MS strategy was employed (Fan *et al.,* 2013). Vitellogenin is a yolk precursor that forms vitellin, a phospholipoglycoprotein that is a major component of the egg yolk and provides nutrients for the embryo during embryogenesis (Masuda and Oliveira, 1985; Adams *et al.,* 2002). The other protein found in all developmental and diapause phases was tubulin alpha chain. That protein is a component of microtubules, which are involved in a number of functions including forming the cytoskeleton. A higher number of peptides for tubulin alpha chain was detected in post-diapausing eggs (S4PD) than in diapausing eggs (S2D) of *M. spectabilis*. Interestingly, a tubulin beta chain, which is also a component of microtubules, was detected only in S4PD eggs. This indicates that greater quantity of tubulins is necessary to assembly microtubes during post-diapause since this stage is associated with increased cellular activity in oppose to the reduced activity during diapause. Similarly, cDNA encoding *beta-tubulin* was lower during adult diapause of the house mosquito, *Culex pipiens* L. complex (Diptera: Culicidae) (Kim and Denlinger, 2009). It appears that different types of tubulins might be differently affected by diapause since a tubulin beta chain was down-regulated in diapause maintenance of *L. migratoria* and diapause preparation of *Cx. pipiens* (Hao *et al.,* 2017; Zhang *et al.*, 2019), a tubulin gamma-2 chain was increased during summer diapause of *Galeruca daurica* (Joannis) (Coleoptera: Chrysomelidae) (Ma *et al.,* 2019) and tubulin alpha chain was up-regulated in diapause preparation and maintenance of *Cx. pipiens* (Zhang *et al.*, 2019). Meanwhile, although histones were found in eggs in the same developmental phase (S2) but from different diapause phases (non-diapausing and diapausing), five histone peptides were found in S2ND and only two in S2D. This means that the number of histones found in diapausing eggs was lower (table S1). Histones are proteins that wrap the DNA in nucleosomal core particles and are important regulators of gene expression where, by bending the DNA, they obstruct transcription (Lawrence *et al.,* 2016). The three types of histones found in this study (H2A, H2B and H4) are part of the nucleosomal core particle and they can have a role in regulating transcription (Lawrence *et al.,* 2016). Histones may also have post-translational modifications (such as acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation, ADP (adenosine diphosphate) ribosylation, deamination, propionylation and butyrylation) that influence gene expression in many ways including increase DNA compaction and influence the recruitment of transcription factors (Lawrence *et al.,* 2016). The level of histones has been found to be low in other insects that undergo diapause as the initial steps of diapause in *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) (Wolschin and Gadau, 2009) and the maintenance of diapause in eggs of *L. migratoria* (Hao *et al.,* 2017). A lower level of histones is associated with reduced protein synthesis and cell replication (MacRae, 2010) and that is consistent with the observation that histones were less abundant in S2D eggs of *M. spectabilis*.

When focusing on the peptides specifically found in S2D and/or S4PD eggs of *M. spectabilis*, two heat shock proteins (Hsp65 and Hsp70), one mitogen-activated protein kinase kinase kinase kinase (MAP4K) and one serine/threonine-protein phosphatase can be highlighted. Heat shock proteins from families Hsp60 and Hsp70 are commonly found in studies of diapause in insects (King and MacRae, 2015). Hsp70 is one of the major families of heat shock proteins found in insects and this protein, along with heat shock proteins from other families as Hsp60, interact with other proteins and promote protein folding, degradation and disaggregation that influences several processes like protein synthesis, cell signaling, transcription and metabolism (King and MacRae, 2015). The Hsp70 detected in this study was found only in diapausing (S2D) and post-diapausing (S4PD) eggs of *M. spectabilis*. Other reports have found similar results as Hsp70 being more abundant during pupal diapause in *P. volucre* (Colinet *et al.,* 2012) and up-regulated during diapause in *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) and *G. daurica* (Rinehart *et al.,* 2000; Ma *et al.,* 2019). Although preventing the production of Hsp70 does not alter the entrance or the duration of diapause in *S. crassipalpis*, the lack of Hsp70 decreased the cold tolerance of diapausing pupae (Rinehart *et al.,* 2007). Thus, we hypothesized that Hsp70 may be also important for diapause egg survival of *M. spectabilis* during the dry season. On the other hand, our results showed that an Hsp65 was found exclusively in post-diapausing (S4PD) eggs of *M. spectabilis*. Heat shock proteins with different molecular weights, Hsp65 and Hsp72, have developmental and tissue-specificity in flesh fly, *S. crassipalpis*. For instance, under heat stress, third-instar larvae produced Hsp65 in the brain and integument while Hsp70 is induced from pupariation throughout the rest of development, and in 3-day-old adult males Hsp65 is observed in the terminalia and flight muscle while Hsp70 is detected in the brain and integument (Joplin and Denlinger, 1990). Thus, it is possible that Hsp65 has a role in post-diapause but not in diapause of *M. spectabilis*. In fact, an Hsp60 was shown to be upregulated in the post-diapause of *Bactrocera minax* (Enderlein) (Diptera: Tephritidae) (Dong *et al.,* 2014). Other heat shock proteins, such as the Hsp90 family, were also proposed to have a role in diapause termination and post-diapause development in *S. crassipalpis* and *Lucilia sericata* (Meigen)

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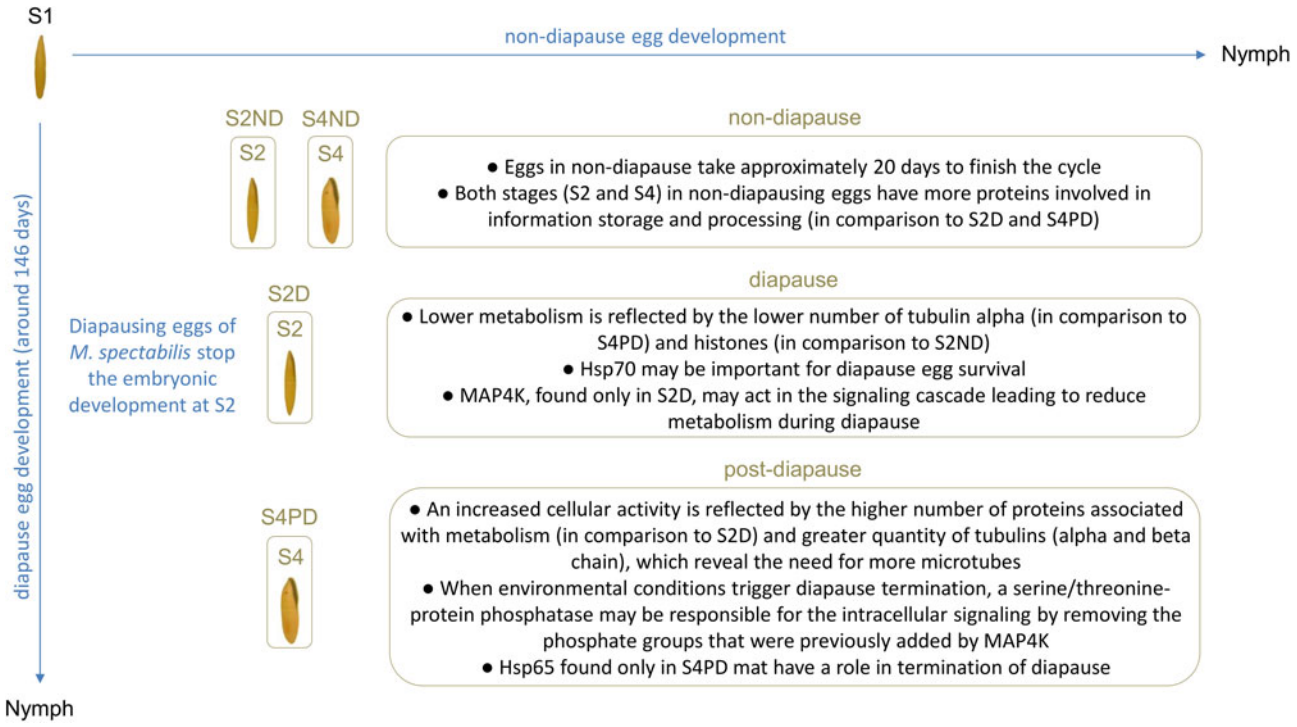

Figure 4. Model of key proteins and information associated with egg diapause regulation in *M. spectabilis*. Different phases are indicates as non-diapause (S2ND and S4ND), diapause (S2D) and post-diapause (S4PD).

(Diptera, Calliphoridae) (Rinehart and Denlinger, 2000; Tachibana *et al.*, 2005) as well as in post-diapause offspring of the crustacean *Daphnia pulex* (Kaupinis *et al.*, 2017). Clearly, more research is required to test the importance of heat shock proteins for diapause egg survival and post-diapause of *M. spectabilis*. In case Hsp70 or Hsp65 are confirmed as important for diapause or post-diapause of *M. spectabilis*, blocking their synthesis in eggs of *M. spectabilis* through the overexpression of *hsp65* or *hsp70* antisense cDNA in forage plants could be used as an alternative strategy to manage this insect in the field. In that case, it should be carefully evaluated the impact of the antisense cDNA on down-regulating these proteins in eggs of *M. spectabilis*, on reducing reproduction of spittlebugs as well as on harming non-target insects.

The other protein that can be highlighted for being found only in diapausing (S2D) eggs of *M. spectabilis* is MAP4K. MAPK is a serine/threonine-specific protein kinase that has a role in signaling cellular responses such cell proliferation and gene regulation after the cell receives a stimulus. MAPK-mediated signaling has been reported as part of the diapause regulation in coleopteran, dipteran and lepidopteran (Fujiwara *et al.*, 2006; Kidokoro *et al.*, 2006; Fujiwara and Denlinger, 2007). MAPK might also play a role in stress tolerance since it potentially has an important role in freeze avoidance by *Epiblema scudderiana* (Clemens) (Lepidoptera: Olethreutidae) (Zhang and Storey, 2017). Moreover, in a transcriptome study of diapause and non-diapause eggs in *L. migratoria*, the MAPK signaling pathway was found to represent 2.19% of the total differentially expressed genes with pathway annotation (Tu *et al.*, 2015) and MEKK3, a component of the MAPK signal cascade, was identified in the transcriptome of diapausing *S. crassipalpis* pupae (Rinehart *et al.*, 2010). Interestingly, we found a serine/threonine-protein phosphatase only in post-diapausing eggs (S4PD) of *M. spectabilis*. That phosphatase is responsible for removing the phosphate added by MAPK4 and other serine/threonine-specific protein kinases. Removing the phosphate is also a form of post-translational modification that regulates many cellular responses. The detection of a MAP4K in diapausing eggs and a serine/threonine-protein phosphatase in post-diapausing eggs let us to hypothesize a mechanism involving phosphorylation and dephosphorylation for diapause entrance, maintenance and termination in spittlebugs (fig. 4). This mechanism possibly involves the action of MAP4K, whose ability in transfer phosphate groups to serine and threonine residues may act as important signal to reduce metabolism to enter and maintain diapause. When environmental conditions trigger diapause termination, a serine/threonine-protein phosphatase may be responsible for the intracellular signaling by removing the phosphate groups. Important to note that not only MAP4K and the serine/threonine-protein phosphatase, but also the two heat shock proteins detected here, Hsp70 and Hsp65, can promote post-translational modifications of proteins and are highlighted in a model of key proteins potentially involved in the diapause regulation of *M. spectabilis* (fig. 4). As pointed out previously (Storey and Storey, 2012), post-translational modifications are important mechanisms to reorganize metabolism during diapause without using valuable energetic resources, like ATP, since the organism is passing through an energy-saving period. Our results indicate that post-translational modifications are also important for diapause regulation in spittlebugs.

In conclusion, this paper reported the first analysis of proteins from non-diapausing, diapausing and post-diapausing eggs of the spittlebug *M. spectabilis*. The methodology employed allows us to identify 87 proteins being 13 of them specific to the diapausing eggs (S2D). Although 19 uncharacterized proteins were found, we were able to identify two important targets for future research. These targets are two heat shock proteins (Hsp65 and Hsp70),

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one MAP4K enzyme and one serine/threonine-protein phosphatase that might have a role in the maintenance and regulation of diapause/post-diapause in *M. spectabilis*. Better understanding the diapause mechanism in this important insect paves the way for a deeper knowledge about its evolutionary strategies along with potentially providing new targets for control strategies.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485321000754

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Conflict of interest. The authors declare no competing interests.

References

Adams TS, Filipi PA and Yi SX (2002) Effect of age, diet, diapause and juvenile hormone on oogenesis and the amount of vitellogenin and vitellin in the two spotted stink bug, *Perillus bioculatus* (Heteroptera: Pentatomidae). *Journal of Insect Physiology* **48**, 477–486.

Alvarenga R, Auad AM, Moraes JC, Silva SEB and Rodrigues BS (2019) Tolerance to nymphs and adults of *Mahanarva spectabilis* (Hemiptera: Cercopidae) by forage plants in fertilized soils. *Pest Management Science* **75**, 2242–2250.

Assad ED, Sano EE, Masutomo R, Castro LHR and Silva FAM (1993) Veranicos na região dos cerrados brasileiros: freqüência e probalidade de ocorrência. *Pesquisa Agropecuária Brasileira* **28**, 993–1003.

Auad AM and Carvalho CA (2009) Desenvolvimento e viabilidade de ovos de *Mahanarva spectabilis* (Distant, 1909) (Hemiptera: Cercopidae) submetidos a diferentes regimes de hidratação. *Arquivos do Instituto Biológico* **76**, 711–714.

Auad AM, Simoes AD, Leite MV, Silva SEB, Santos DR and Monteiro PH (2011) Seasonal dynamics of egg diapause in *Mahanarva spectabilis* (Distant, 1909) (Hemiptera: Cercopidae) on elephant grass. *Arquivos do Instituto Biológico* **78**, 325–330.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Bryon A, Kurlovs AH, Van Leeuwen T and Clark RM (2017) A molecular-genetic understanding of diapause in spider mites: current knowledge and future directions. *Physiological Entomology* **42**, 211–224.

Colinet H, Renault D, Charoy-Guével B and Com E (2012) Metabolic and proteomic profiling of diapause in the aphid parasitoid *Praon volucre*. *PLoS ONE* **7**, e32606.

Cui DN, Tu XB, Hao K, Raza A, Chen J, McNeill M and Zhang ZH (2019) Identification of diapause-associated proteins in migratory locust, *Locusta migratoria* L. (Orthoptera: Acridoidea) by label-free quantification analysis. *Journal of Integrative Agriculture* **18**, 2579–2588.

Dong Y, Desneux N, Lei C and Niu C (2014) Transcriptome characterization analysis of *Bactrocera minax* and new insights into its pupal diapause development with gene expression analysis. *International Journal of Biological Sciences* **10**, 1051–1063.

Fan L, Lin J, Zhong Y and Liu J (2013) Shotgun proteomic analysis on the diapause and non-diapause eggs of domesticated silkworm *Bombyx mori*. *PLoS ONE* **8**, e60386.

Fujiwara Y and Denlinger DL (2007) High temperature and hexane break pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, by activating ERK/MAPK. *Journal of Insect Physiology* **53**, 1276–1282.

Fujiwara Y, Shindome C, Takeda M and Shiomi K (2006) The roles of ERK and P38 MAPK signaling cascades on embryonic diapause initiation and termination of the silkworm, *Bombyx mori*. *Insect Biochemistry and Molecular Biology* **36**, 47–53.

Hao K, Jie W, Xiong-Bing T, Whitman DW and Zhang ZH (2017) Transcriptomic and proteomic analysis of *Locusta migratoria* eggs at different embryonic stages: comparison for diapause and non-diapause regimes. *Journal of Integrative Agriculture* **16**, 1777–1788.

Joplin KH and Denlinger DL (1990) Developmental and tissue specific control of the heat shock induced 70 kDa related proteins in the flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology* **36**, 239–249.

Kaupinis A, Aitmanaitė L, Strepetkaitė D, Valius M, Lazutka JR and Arbačiauskas K (2017) Proteomic and gene expression differences between post-diapause and subitaneous offspring phenotypes in the cyclic parthenogen *Daphnia pulex*. *Hydrobiologia* **798**, 87–103.

Kidokoro K, Iwata KI, Takeda M and Fujiwara Y (2006) Involvement of ERK/MAPK in regulation of diapause intensity in the false melon beetle, *Atrachya menetriesi*. *Journal of Insect Physiology* **52**, 1189–1193.

Kim M and Denlinger DL (2009) Decrease in expression of *beta-tubulin* and microtubule abundance in flight muscles during diapause in adults of *Culex pipiens*. *Insect Molecular Biology* **18**, 295–302.

King AM and MacRae TH (2015) Insect heat shock proteins during stress and diapause. *Annual Review of Entomology* **60**, 59–75.

Koštál V (2006) Eco-physiological phases of insect diapause. *Journal of Insect Physiology* **52**, 113–127.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lawrence M, Daujat S and Schneider R (2016) Lateral thinking: how histone modifications regulate gene expression. *Trends in Genetics* **32**, 42–56.

Ma HY, Zhou XR, Tan Y and Pang BP (2019) Proteomic analysis of adult *Galeruca daurica* (Coleoptera: Chrysomelidae) at different stages during summer diapause. *Comparative Biochemistry and Physiology – Part D* **29**, 351–357.

MacRae TH (2010) Gene expression, metabolic regulation and stress tolerance during diapause. *Cellular and Molecular Life Sciences* **67**, 2405–2424.

Masuda H and Oliveira PL (1985) Characterization of vitellin and vitellogenin from *Rhodnius prolixus*: identification of phosphorylated compounds in the molecule. *Insect Biochemistry* **15**, 543–550.

Peck DC (2002) Distribución y reconocimiento del salivazo de los pastos (Homoptera: Cercopidae) en la Costa Caribe de Colombia. *Pasturas Tropicales* **24**, 4–15.

Pereira JF, Cunha GRD and Moresco ER (2019) Improved drought tolerance in wheat is required to unlock the production potential of the Brazilian Cerrado. *Crop Breeding and Applied Biotechnology* **19**, 217–225.

Ragland GJ, Armbruster PA and Meuti ME (2019) Evolutionary and functional genetics of insect diapause: a call for greater integration. *Current Opinion in Insect Science* **36**, 74–81.

Ren XY, Zhang LS, Han YH, An T, Liu Y, Li YY and Chen HY (2016) Proteomic research on diapause-related proteins in the female ladybird, *Coccinella septempunctata* L. *Bulletin of Entomological Research* **106**, 168–174.

Rinehart JP and Denlinger DL (2000) Heat-shock protein 90 is down-regulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. *Insect Molecular Biology* **9**, 641–645.

Rinehart JP, Yocum GD and Denlinger DL (2000) Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochemistry and Molecular Biology* **30**, 515–521.

Rinehart JP, Li A, Yocum GD, Robich RM, Hayward SA and Denlinger DL (2007) Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proceedings of the National Academy of Sciences* **104**, 11130–11137.

Rinehart JP, Robich RM and Denlinger DL (2010) Isolation of diapause-regulated genes from the flesh fly, *Sarcophaga crassipalpis* by suppressive subtractive hybridization. *Journal of Insect Physiology* **56**, 603–609.

Shevchenko A, Tomas H, Havlis J, Olsen JV and Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols* **1**, 2856–2860.

Silveira Neto S, Nakano O, Barbin D and Vila Nova NA (1976) Diapausa. In *Manual de Ecologia de Insetos*. Ed. Agron. Ceres Ltda. Piracicaba, pp. 70–75.

Storey KB and Storey JM (2012) Insect cold hardiness: metabolic, gene, and protein adaptation. *Canadian Journal of Zoology* **90**, 456–475.

Tachibana SI, Numata H and Goto SG (2005) Gene expression of heat-shock proteins (*Hsp*23, *Hsp*70 and *Hsp*90) during and after larval diapause in the blow fly *Lucilia sericata*. *Journal of Insect Physiology* **51**, 641–647.

Tan QQ, Liu W, Zhu F, Lei CL, Hahn DA and Wang XP (2017) Describing the diapause-preparatory proteome of the beetle *Colaphellus bowringi* and identifying candidates affecting lipid accumulation using isobaric tags for mass spectrometry-based proteome quantification (iTRAQ). *Frontiers in Physiology* **8**, 251.

Tu X, Wang J, Hao K, Whitman DW, Fan Y, Cao G and Zhang Z (2015) Transcriptomic and proteomic analysis of pre-diapause and non-diapause eggs of migratory locust, *Locusta migratoria* L. (Orthoptera: Acridoidea). *Scientific Reports* **5**, 11402.

Valério JR (2009) *Cigarrinhas das pastagens*. Campo Grande: Embrapa Gado de Corte, Documentos 179, 51 p.

Wolschin F and Gadau J (2009) Deciphering proteomic signatures of early diapause in *Nasonia*. *PLoS ONE* **4**, e6394.

Zhang J and Storey KB (2017) Insect cold hardiness: the role of mitogen-activated protein kinase and Akt signalling in freeze avoiding larvae of the goldenrod gall moth, *Epiblema scudderiana*. *Insect Molecular Biology* **26**, 181–189.

Zhang Q, Lu YX and Xu WH (2012) Integrated proteomic and metabolomic analysis of larval brain associated with diapause induction and preparation in the cotton bollworm, *Helicoverpa armigera*. *Journal of Proteome Research* **11**, 1042–1053.

Zhang C, Wei D, Shi G, Huang X, Cheng P, Liu G, Guo X, Liu L, Wang H, Miao F and Gong M (2019) Understanding the regulation of overwintering diapause molecular mechanisms in *Culex pipiens* pallens through comparative proteomics. *Scientific Reports* **9**, 6485.

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