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INSIGHTS INTO THE PROTEOME OF THE SPERMATHECA OF THE LEAF-CUTTING ANT *ATTA SEXDENS RUBROPILOSA* (HYMENOPTERA: FORMICIDAE)

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The genus *Atta* (Hymenoptera: Formicidae) consists of leaf-cutting ants, which are distributed from the southern United States to central Argentina, but do not occur in Chile (Mariconi 1970). These ants live in eusocial nests with large, populous colonies and cultivate a fungus on fresh plant parts, particularly leaves. The cutting of plant parts by *Atta* spp. often causes enormous economic losses in agriculture and forestry (Della Lucia et al. 1993; Hölldobler & Wilson 1990).

Atta spp. have great reproductive potentials, with a typical *Atta sexdens rubropilosa* (Forel, 1908) nest having 5 to 8 million workers, *Atta laevigata* (Fr. Smith, 1858) having 3.5 million (Riley et al. 1974) and *Atta colombica* (Guérin, 1845) having 1 to 2.5 million (Fowler et al. 1986). This high reproductive capacity allows a queens to store the sperm in her saculiform ectodermic spermatheca throughout her reproductive life (Klenk et al. 2004).

The storage time of the sperm varies between insect species, but in the queens of Hymenopterans, it can be stored for years (Klenk et al. 2004). The continued viability of the gametes depends on the products secreted by the spermathecal epithelium and glands (Den Boer et al. 2009). The identification of proteins expressed in the spermatheca might contribute to the advancement of knowledge on the roles of proteins in prolonged sperm storage (Zareie et al. 2013).

To gain a greater understanding of the reproductive biology of this ant species, as well as to lay a foundation for the possible application of proteomic studies in insect population control, we evaluated the profile of the differentially expressed proteins in the spermathecae of the virgin and fertilized *A. sexdens rubropilosa* queens. We collected queens of *A. sexdens rubropilosa* in the field in Viçosa, Minas Gerais, Brazil. A total of 30 spermathecae were dissected and stored at -20 °C with the protease inhibitor (phenylmethylsulfonyl fluoride and benzamidine 1.0 mM).

The proteins from the spermathecae were extracted with 1.0 mL of Tris-HCl buffer (40 mM; pH 7.5) supplemented with 1.0 mM PMSF. The homogenate was centrifuged at 20,000 g for 15 min at 4 °C and the supernatant precipitated with trichloroacetic acid (TCA/acetone). The precipitate was washed and re-suspended in 100 µL of solubilization buffer (7 M urea, 2 M thiourea, 2% CHAPS, DTT 0.3%). The protein concentration was determined by the Bradford method (Bradford 1976). Two-dimensional electrophoresis was performed on 7 cm strips with linear immobilized pH gradient (pH 3-10). The strips were rehydrated with 137.5 µL of a solution containing 150 µg of protein, 40 mM DTT, 2% ampholyte (IPG Buffer pH3-10) and Destreak rehydration solution (GE Healthcare). The two-dimensional gels obtained were analyzed with the appropriate software, the spots with differential expression (ANOVA; $P < 0.05$) were selected, excised and the proteins digested with a solution of 0.5 ng trypsin per sample (Trypsin Gold-PROMEGA). The tryptic peptides were analyzed by mass spectrometry (MS) MALDITOF/TOF model Ultraflex III (Bruker Daltonics®). The spectra of the spots were identified by the Peptide Mass Fingerprinting (PMF) method with the MASCOT software (http://www.matrixscience.com) in the NCBI and Swiss-Prot databases.

The two-dimensional protein profiles obtained of the spermathecae, showed 22 spots with differential expression (Fig. 1). Nine of the 22 samples submitted to MS showed homology to proteins of several species of *Drosophila* by PMF. Six others were categorized as hypothetical proteins, they did not present homology with the proteins of known function. The seven remaining spectra showed no protein sufficient for identification under PMF. The proteins identified were separated into 4 functional categories, i.e., (i) energy and metabolism, (ii) cell cycle, (iii) cell processes biosynthesis and modification of proteins and (iv) structure and structural organization (Table

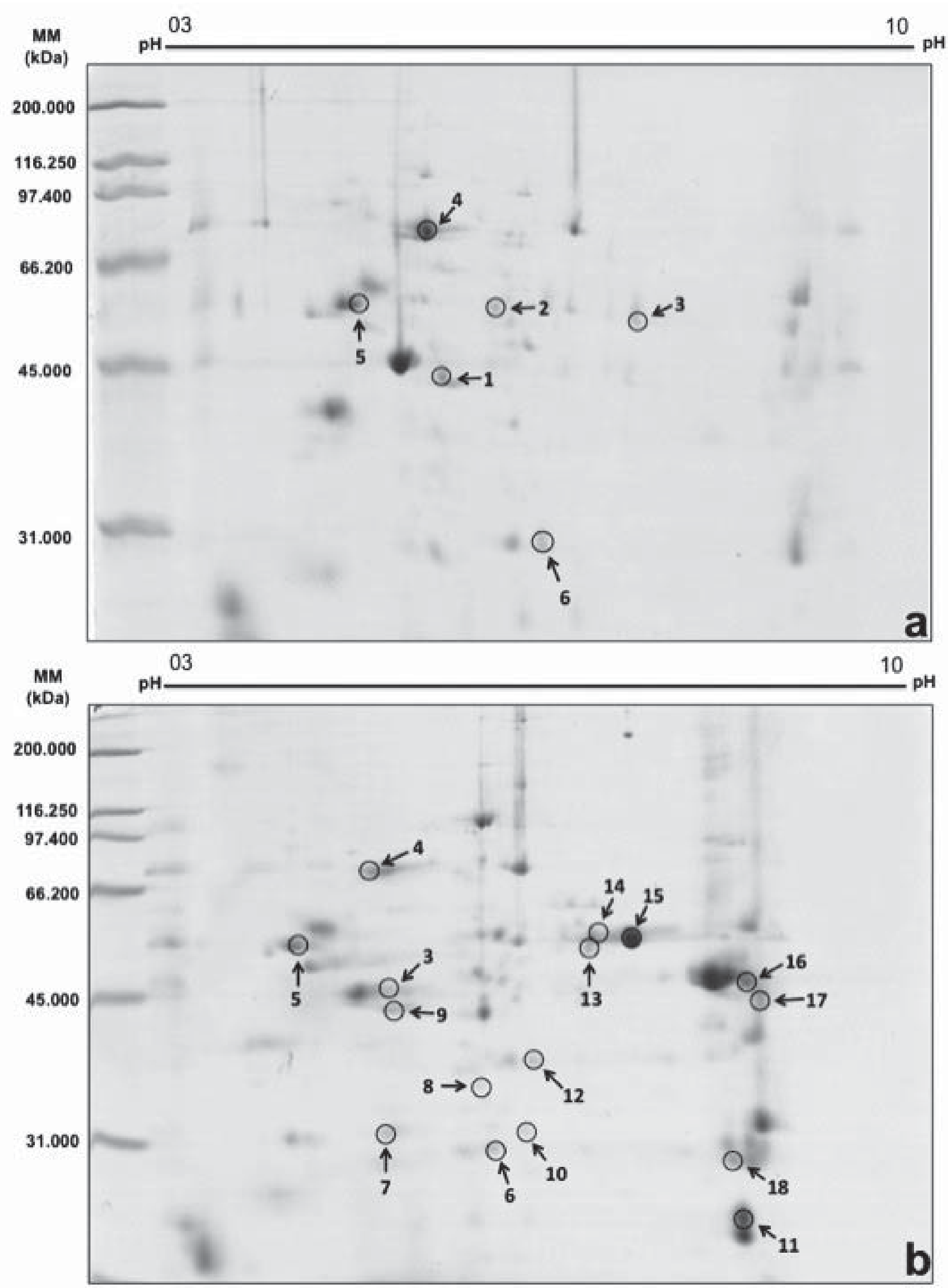

Fig. 1. Two-DE profiles of the spermatheca of virgin (a) and fertilized (b) queens of *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). Arrows indicate differentially expressed spots. The isoeletric focalization was run on 7cm strips on pH 3-10, and the 2-DE was run on SDS-PAGE 10%. MM - molecular weight (Broad Range - BioRad). Gels were stained by Coomassie Brilhant Blue.

TABLE 1. PROTEINS PREDICTED BY PEPTIDE MASS FINGERPRINT (PMF) IN THE SPERMATHECAE OF VIRGIN AND INSEMINATED *ATTA SEXDENS RUBROPILOSA* QUEENS.

					Mascot-search results (Peptide Mass Fingerprinting - PMF)						
Spot ID	Identified protein	iP/MW (kDa) Theorical [a]	Fold Changed Unmated	Fold Changed Mated	iP/MW (kDa) observed[b]	Score	Sequence coverage (%)	Macthing peptides	Protein name/ organism	Access number	Database
Energy and metabolism proteins											
1	Cytochrome P450	8,14/58.565			9.12/58.451	45/50	24%	11	Cytochrome P450 (*Dme*)	C6A14-DROME	SwissProt/0,5
2	Vitellogenin	9,61/47.002			7.74/49.744	14/50	7%	2	Vitellogenin-2 (*Dme*)	VIT2 -DROME	SwissProt/0,1
3	Lipase 3	5,67/43.542	1.90		5.36/45.214	40/50	25%	6	Lipase 3 (*Dme*)	LIP3-DROME	SwissProt/0,5
4	Yolk protein 1	7,22/39.875			6.02/31.013	33/66	20%	4	Yolk protein 1 (*Dma*)	gi:1490463	NedCBI/0,1
Structure and structural organization											
5	Actin - 5C	5,93/48.433			5.30/42.196	54/50	30%	8	Actin-5C (*Dme)*	ACT1-DROME	SwissProt/0,5
Cell cycle related proteins											
6	Replication factor C subunit 2	9,48/46.258			7.62/37.549	44/50	34%	11	Replication factor C subunit 2 (*Dme*)	RFC2-DROME	SwissProt/0,5
7	Cdc6	5,50/78.446	6.05		9.27/73.900	49/66	20%	13	Cdc6 (*Dmau)*	gi:113197051	NCBI/0,5
Biosynthesis and modification proteins											
8	Elongation factor 1alpha 2	7,78/58.745			9.07/51.030	45/50	24%	10	Elongation factor 1alpha 2 (*Dme*)	EF1A2-DROME	SwissProt/0,5
9	NEDD8 (activating enzyme E1 -catalytic subunit	7,65/53.560		1.77	5.26/50.855	35/50	10%	5	NEDD8-activating enzyme E1 catalytic subunit (*Dme)*	UBA3-DROME	SwissProt/0,5
Hypothetical proteins - uncharacterized proteins											
10	GI13461	9,40/26.069			9.88/22.686	47/66	29%	6	GI13461 (*Dmo*)	gi:195127850	NCBI/0,5
11	GL23714	4,73/56.106	2.37		6.13/89443	50/66	13%	10	GL23714 (*Dpe*)	gi:195143705	NCBI/0,5
12	GI24073	5,81/32.640			5.94/24.081	46/66	29%	5	GI24073 (*Dmo*)	gi:95117162	NCBI/0,5
13	GM23230	6,54/38.553			8,90/34.358	45/66	17%	8	GM23230 (*Dse)*	GM23230 gi|194865752	NCBI/0,5
14	GG14359	6,68/29.686	1.26		8.69/19.672	58/66	34%	7	GG14359 (*Der*)	gi:194865752	NCBI/0,5
15	GL23619	7,16/33.227			5.60/45.970	47/66	11%	6	GL23619 (*Dpe*)	gi:195144050	NCBI/0,5

[A]Isoeletric Point (iP)

[B]Molecular Weight (MW) Theorical Observed *Drosophila melanogaster* (DME) *Drosophila macrothrix* (DMA), *Drosophila mauritiana* (DMAU), *Drosophila mojavensis* (DMO), *Drosophila persimilis* (Dpe), *Drosophila sechellia* (Dse) and *Drosophila erecta* (Der).

1), and some proteins with no known functions, which were referred to as hypothetical proteins, i.e., GI13461, GM23230, GG14359, GI24073, GL23714 and GL23619 (Table 1).

The identification of differentially expressed proteins in the spermathecae of virgin and fertilized *A. sexdens rubropilosa* queens represents an important step towards understanding the molecular regulation of sperm storage by the queens of the ants (Baer et al. 2009a; Zareie et al. 2013). The presence of proteins associated with the metabolic pathways/products (i.e., yolk protein-1 (YP-1), vitellogenin, cytochrome P450 and lipase-3) in the spermathecae of fertilized queens could be an indication of their participation in energy metabolism that may be required to produce energy as needed in this organ after fertilization. Also, the differential expression of lipase in virgin queens may be related to lipid metabolism, prior to reproduction. Although, lipase-3 had not been previously reported in the spermathecae of ants, the activity of this enzyme in the reproductive tract of other insects is known. For instance, *Phlebotomus papatasi* (Loew, 1845) (Diptera: Psychodidae) females synthetize lipase, which was recognized as the main component involved in the accessory glands secretion process in the reproductive tract (Rosetto et al. 2003).

The YP-1 in the spermathecae of the fertilized *A. sexdens rubropilosa* queens may have come from the male during copulation as shown for yolk protein-2 (supposedly related to the maturation process of the sperm) in the seminal fluid of *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) males (Bebas et al. 2008). In spite of this, the role of YPs in the insect spermatheca is still unknown.

Vitellogenin is differentially expressed in the spermatheca of the fertilized *A. sexdens* rubropilosa queen, and this has also been reported in the spermathecae of virgin and inseminated *Apis mellifera* (Linnaeus, 1758) (Hymenoptera: Apidae) queens (Baer et al. 2009a). The vitellogenin in the spermatheca of fertilized *A. sexdens rubropilosa* queens may have an antioxidant role (Koeniger 1986), whereby the spermatheca is protected from reactive oxygen species (ROS);. This antioxidant function probably increases sperm longevity by reducing the levels of ROS (Collins et al. 2004).

Protein elongation factor 1-alpha (EF1-α), which is differentially expressed in the spermatheca of fertilized queens, is important in promoting protein biosynthesis in eukaryotic cells as well as in regulating apoptosis (Andersen et al. 2003). The increased expression of EF1-α in *D. melanogaster* (Meigen, 1830), suggests that this protein could be a factor in the promotion of longevity (Wang et al. 2004). Thus, the presence of EF1-α in the spermatheca of the fertilized queens could relate to homeostasis and consequently contribute to increased longevity of the gametes within the spermatheca.

The cytochrome P450 proteins are involved in the detoxification of xenobiotics, and in the development of insects (Li et al. 2007). Therefor, the expression of P450 in the spermatheca of fertilized *A. sexdens rubropilosa* queens could be in response to the detoxification of toxic substances in the spermatheca, especially when it is filled with the male's seminal fluid.

Proteins Cdc6 and RFC2 are related to the cell cycle and DNA replication, but when expressed in the spermathecae of virgin and fertilized queens, their roles remain unknown in *A. sexdens rubropilosa*. However, protein Cdc6 is important in cell cycle progression, the initiation of replication and of processes that control the passage of the cell through the later stages of the cell cycle (Crevel et al. 2005). DNA alterations may occur depending on age (Mullaarte et al. 1990) and, therefore, the expression of the cell cycle proteins may be linked to the prevention of DNA damage of the spermatozoids and the spermathecal cells.

NEDD8-E1 is a ubiquitin-like protein with 81 amino acids and a sequence linked to ubiquitin that is 60% identical and 80% similar (Kumar et al. 1993). This protein is highly conserved in eukaryotes and is expressed in most or in all the tissues of these organisms (Carrabino et al. 2004). A ubiquitin-like protein has been reported in the human seminal plasma (Lippert et al. 1993) and in the seminal fluid of *Cimex lectularius* (Latreille, 1802) (Hemiptera: Cimicidae) (Reinhardt et al. 2009). The presence of this male protein in the spermathecae of the fertilized *A. sexdens rubropilosa* queens demonstrates a possible transfer from the male seminal fluid to the female spermatheca during copulation.

The protein Actin 5C, which has the highest expression in the spermathecae of the fertilized *A. sexdens rubropilosa* queens, and which has been documented to be present in the seminal fluid of *A. mellifera* males (Baer et al. 2009a, 2009b), was also identified in the spermatheca of the virgin queens. The presence of the Actin was expected since it is constitutively expressed in eukaryotic cells (Eriji 2002). Its function may be related mainly to the transport of nutrients from the lumen into the hemocoel, secretion, endocytosis and exocytosis, and to increasing the resistance of the spermathecal epithelium filled with sperm (Gobin et al. 2006; Ortiz & Camargo-Mathias 2006).

In conclusion, we found that the proteome of the spermatheca of *A. sexdens rubropilosa* provides substantial information on proteins in this organ. Fifteen of the 22 differentially expressed proteins were identified. This also highlights the possible roles of these proteins in prolonged semen storage through the likely increase in metabolic activities of the spermatheca after copulation.

Summary

Fifteen of the 22 differentially expressed proteins in the spermathecae of virgin and inseminated females of the leaf cutting ant *Atta sexdens rubropilosa* were tentatively identified. The profile of expressed proteins of the spermatheca differed significantly between virgin and fertilized females. Data from this study should contribute to the elucidation of the roles of these various proteins in prolonged storage and maintenance of viable spermatozoa within the female.

Key Words: *Atta sexdens*, leaf-cutting ants, proteomics, spermatheca

Resumo

No presente estudo foram identificadas proteínas expressas na espermateca de rainhas virgens e fertilizadas da formiga cortadeira *Atta sexdens rubropilosa*. O perfil das proteínas expressas nas espermatecas é diferente em fêmeas virgens e fertilizadas. Os dados aqui apresentados contribuem para o entendimento do papel das proteínas produzidas pelas espermatecas para o armazenamento e manutenção da viabilidade dos espermatozoides pelas rainhas.

Palabras Chave: *Atta sexdens,* formigas cortadeiras, proteômica, espermateca

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