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Spermatheca of Four Species of Ants of the Tribe Attini (Hymenoptera: Myrmicinae). Morphological Specialization

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Abstract: The function of the insects spermatheca is to store the spermatozoa from the time of copulation until the fertilization of the eggs. This organ consists of a sac, usually associated to glands. The ants *Atta sexdens rubropilosa*, *Acromyrmex landolti*, *Acromyrmex balzani* and *Acromyrmex landolti balzani* are species considered pests and cause serious economical damages to several crops. Queens' spermatheca of these four species were analyzed through techniques of Scanning Electron Microscopy (SEM), histology and histochemistry. It was verified that these structures had the morphology of a sac consisting of two lobes, which fusing at the median region. In *A. s. rubropilosa* and *A. l. balzani*, groups of cells with secretory characteristics form the spermathecal gland, but were absent in *A. landolti* and *A. balzani*. In these latter species the secretory cells are arranged like a single epithelium forming an internal coat inside the spermatheca. The results obtained in the present work shows that the spermatheca of these species of ants have morphological differences. In this sense, the spermatheca of *A. landolti* presents an internal secretory epithelium of columnar morphology throughout its extension, thus suggesting that all the cells would produce the secretion. The spermatheca of the one *A. balzani* presented an internal secretory epithelium with columnar and cubical portions, where a greater secretion likely occurs at the columnar one. *A. l. balzani* presents internal secretory epithelium and external spermathecal gland connected by a duct, while *A. s. rubropilosa* presents only the spermathecal gland.

Key words: Spermatheca, histology, histochemistry, spermathecal gland, attini

INTRODUCTION

Leaf-cutting ants are the main agricultural pests of the neotropical region and their control can be performed through the use of chemical substances of low specificity that accumulate in the environment.

These ants are represented by some species of the genera *Atta* and *Acromyrmex*, which are the most derived of the tribe Attini and whose colonizing success of agricultural crops is attributed to their great voracity and capability to proliferate.

Ant queens live unusually long for insects, which generally have short lives. They store viable spermatozoa for a decade or more and produce workers and reproductives over such long periods, that almost certainly has contributed to the propensity for ants to evolve diverse life histories that been so successful in a wide range of environments. After mating, which occurs in a few minutes during the mating flight, a queen is capable of storing viable spermatozoa for dozens of years; these spermatozoa will be gradually used to fertilize the eggs and thus originate all the individuals of the colony.

The ability to store spermatozoa releases from the risk and energy expenditure involved in finding subsequent mates (Thornhill and Alcock, 1983). Maintenance of spermatozoa viability can be a critical aspect of female reproduction in insects, yet the mechanisms mediating this phenomenon are poorly understood.

The maintenance of these spermatozoa for such a long time requires the presence of a specialized organ in the queens, known as spermatheca which presents a particular organization for each phylogenetic group that has been studied.

The spermatheca is an organ of ectodermic origin and it is internally coated with cuticle. It basically consists of a storage sac, often accompanied by associated glands, known as spermathecal glands, which produce secretions that allow the maintenance of a microenvironment of appropriate pH, ideal ionic constitution and nutrients, in addition to other factors necessary for the survival of the spermatozoa. In

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some insects these glands might be absent and the internal coating epithelium performs the secretory role (Davey, 1985).

Generally, the spermatheca consists of a single sac, but two have been observed in *Blaps* (Coleoptera) and *Phlebotomus* (Diptera) and three occur in the most derived flies (Chapman, 1998). In ants in general only one spermatheca has been described.

The basic functions of the spermathecal secretions have been poorly studied, however, some observations suggest that their main function would be to maintain the viability of the spermatozoa during the time of mating until the fertilization of the eggs (Berry, 1985). The spermatozoa of insects become active inside the spermatheca and may remain there for several months or up to several years.

In view of the all the facts described above and given the economical importance of these insects, the present work had the object of carrying out a study of the spermatheca of queen ants of *Atta sexdens rubropilosa*, *Acromyrmex landolti*, *Acromyrmex balzani* and *Acromyrmex landolti balzani* through the use of the techniques of Scanning Electron Microscopy (SEM), histology and histochemistry. The specific aim of this study is to show a morphological diversity between the spermatheca in ants of the tribe Attini.

MATERIALS AND METHODS

Mated queens of ants of the sp. *A. landolti*, *A. balzani* and *Acromyrmex landolti balzani* were collected from nests in the campus of the UNESP in Rio Claro, SP-Brazil and queen ants of *Atta sexdens rubropilosa* were collected in the region of Botucatu, SP-Brazil. The equipments used are available in laboratories of Histology of the Biology Department, Biosciences Institute, UNESP, campus in Rio Claro, SP-Brazil.

Scanning Electron Microscopy (SEM): The spermatheca were removed from the *A. s. rubropilosa* queens, fixed en Karnovsky for 24 h and dehydrated in a graded 70-100% ethanol and acetone series. The material was critical point dried, sputtered with gold and examined under a Phillips SEM 505.

Histology: The spermatheca from queens of all species were removed, fixed in paraformaldehyde, dehydrated a graded 70-95% alcohol series and embedded in JB-4 resin at 4°C in the dark. Then they were placed in plastic molds at 4°C in order to delay premature polymerization. The plastic molds containing the material were filled with JB-4 resin and polymerization was completed at room temperature. The sections were placed on clean glass slides and air dried. Then they were stained with hematoxylin-eosin, just applied in *Atta sexdens rubropilosa*.

Histochemistry: Individuals were cold anesthetized and dissected in saline solution. The spermatheca were fixed in paraformaldehyde. Dehydration was performed in a standard alcohol series (70, 80, 90 and 95%) at 15 min intervals. The material was embedded in resin and sectioned to a 3 μm thickness. The sections were arrayed on glass slides and stained with PAS/Alcian Blue (for the detection of polyssaccharides) for all four studied species, Bromophenol Blue (for protein detection) just applied in the sp. *Acromyrmex landolti balzani* and Sudan Black B (for lipids detection) just applied in *Atta sexdens rubropilosa*.

RESULTS

Morphology: The morphological studies with spermatheca of *Atta sexdens rubropilosa*, *Acromyrmex landolti*, *Acromyrmex balzani* and *Acromyrmex landolti balzani* revealed that in the four species this structure was located at the beginning of the common oviduct. In all four species, the spermatheca appeared as a bi-lobed sac that fuses partially at the median region and thus present a common lumen (Fig. 1A) and an ample reservoir for the storage and maintenance of the spermatozoa.

Figure 1 B illustrates the morphology of the spermatheca of queen ants of *Atta sexdens rubropilosa*, which is the same observed in the other three species. In the sp. *A.s. rubropilosa* and *A.l. balzani* the spermatheca was divided into two distinct regions: a) an external secretory portion composed by rounded or elongated cellular groups (resembling acini), denominated spermathecal glands and b) a reservoir (Fig. 1B). In *A. s. rubropilosa* the spermathecal gland was only located at the region of fusion of the two spermathecal lobes, while in *A.l. balzani* this gland coated the whole upper portion of the spermatheca. In the sp. *A. landolti* and *A. balzani* there was no subdivision of the spermatheca into two portions, given that the secretory portion was only found coating the reservoir internally.

Histology and histochemistry: In order to obtain a better understanding and visualization the histological and histochemical results were summarized in Table 1.

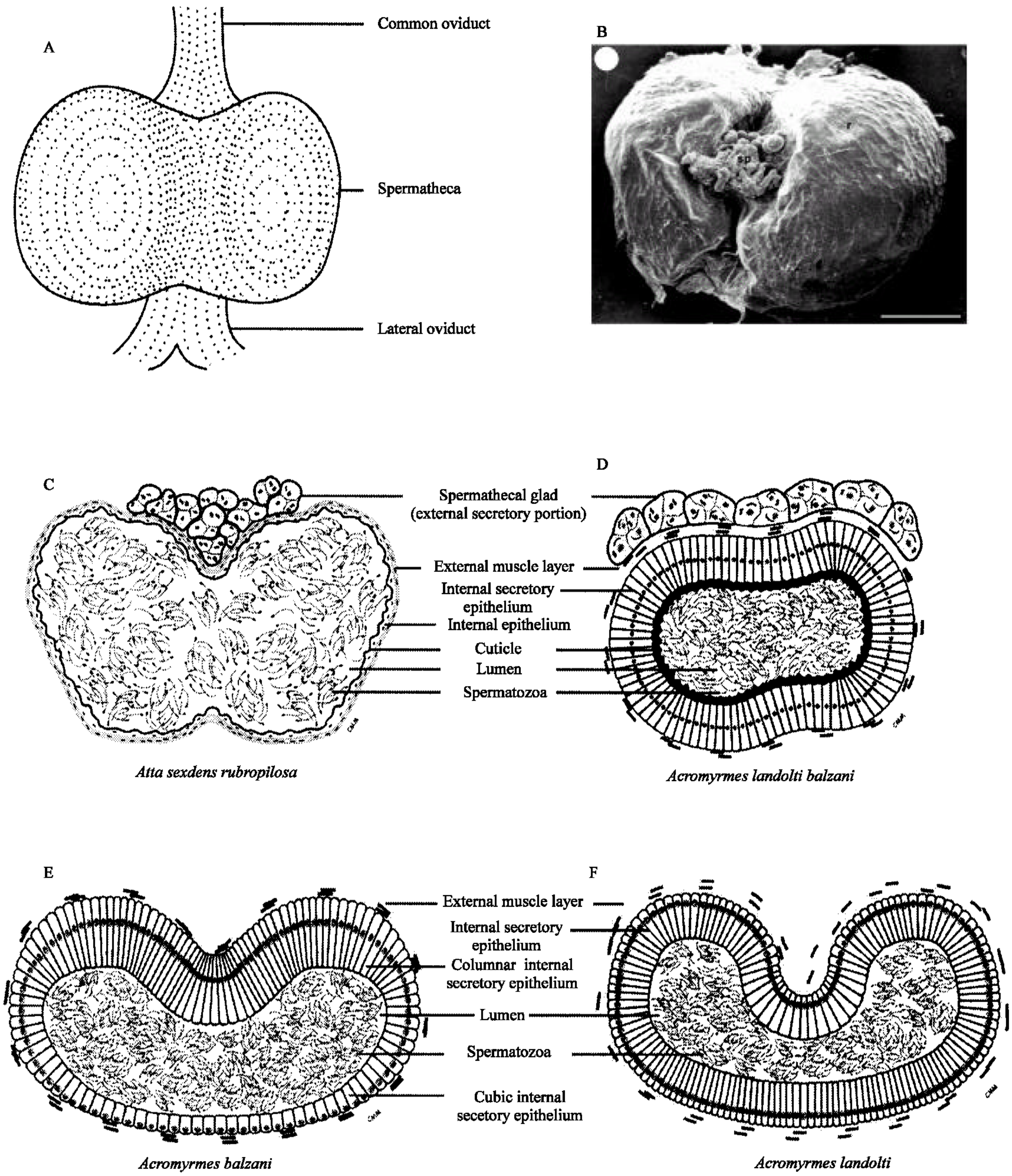

Fig. 1: A. Schematic diagram showing the bi-lobed sac morphology of the spermatheca of the ants *Atta sexdens rubropilosa*, *Acromyrmex landolti*, *Acromyrmex balzani* and *Acromyrmex landolti balzani*. B. SEM of the spermatheca of *A.s. rubropilosa*, r = reservoir, sp. = secretory portion, Scale bar = 1 µm. C. D. E. F. Schematic diagram of the spermatheca obtained through the histological sections

Table 1: Histology and histochemistry of the spermatheca of queens of the ants *Atta sexdens rubropilosa*, *Acromyrmex landolti*, *Acromyrmex balzani* and *Acromyrmex landolti balzani*

Species structure	*Atta sexdens rubropilosa*	*Acromyrmex landolti*	*Acromyrmex balzani*	*Acromyrmex landolti balzani*
Spermathecal gland	-Present in the form of rounded or elongated cellular groups forming acini (Fig. 1B, C; 2A) -Rounded or polygonal secretory cells located at the point of fusion of the two spermathecal lobes (Fig. 1C; 2A) -Rounded nuclei (Fig. 1C; 2A) -Sudan Black B +++ (Fig. 2D)	-Absent	-Absent	-Present in the form of polygonal secretory cells forming acini (Fig. 1D; 2F, G) -Coats the whole upper portion of the spermatheca (Figs. 1D; 2F, G) -PAS/Alcian Blue + (Fig. 2F) -Bromophenol Blue +++ (Fig. 2G)
Portion of the canaliculi near to the the cell nucleus found in the spermathecal gland	-Showing infoldings around the lumen and a thin cuticle coating it internally (Fig. 2A)	-Absent	-Absent	-Showing infoldings around the lumen and a thin cuticle coating it internally (Fig. 2F) -PAS/Alcian Blue + (Fig. 2F)
Portion of the canaliculi near to the reservoir found in the spermathecal gland	-No infoldings in the lumen -The cuticle is thicker than the one found in the canaliculi (Fig. 2A)	-Absent	-Absent	-Not observed
Internal secretory epithelium	-Absent	-Simple epithelium of columnar cells (Fig. 1F; 2I) -Rounded nuclei found at the basal region (Fig. 1F; 2I) -PAS/Alcian Blue + (Fig. 2I)		-Simple epithelium of columnar cells (Fig. 1D; 2F, G, H) -Spherical nuclei located at the median-basal portion of the cells (Fig. 1D; 2F, G, H) -PAS/Alcian Blue + (Fig. 2F) -Bromophenol Blue +++ (Fig. 2G, H)
Reservoir wall	-Thin -Composed by an epithelium varying from scamous to cubical (Fig. 1C; 2B) -PAS/Alcian Blue +++ (Fig. 2C) -Sudan Black B +++ (Fig. 2E)	-Thin -Composed by an internal columnar secretory epithelium (Fig. 1F; 2I) -PAS/Alcian Blue + (Fig. 2I)	-Thin -Composed by an internal secretory epithelium varying from cubical to columnar (Fig. 1E; 2J, K, L) -PAS/Alcian Blue + (Fig. 2J, K, L)	-Thin -Composed by an internal columnar secretory epithelium (Fig. 1D; 2F, G) -PAS/Alcian Blue + (Fig. 2F) -Bromophenol Blue +++ (Fig. 2G, H)
Cuticle found internally coating the spermatheca	-Thick throughout most of its extension -PAS/Alcian Blue +++ (Fig. 1C; 2B, C) (Fig. 2C) -Sudan Black B ++ (Fig. 2E)	-Absent	-Absent	-Thick throughout its extension (Fig. 1D; 2F, G) -Bromophenol Blue +++ (Fig. 2G)
Musculature found externally surrounding the spermatheca	-Few layers (Fig. 1C; 2B, E) -PAS/Alcian Blue +++ (Fig. 2C) -Sudan Black B ++ (Fig. 2E)	-Numerous layers that thicken at he point of fusion between the two lobes (Fig. 1F: 21) -PAS/Alcian Blue +++ (Fig. 2I)	-Numerous layers (Fig. 1E; 2J, K, L) -PAS/Alcian Blue +++ (Fig. 2J, K, L)	-Few layers (Fig. 1D; 2F, G, H) -PAS/Alcian Blue +++ (Fig. 2F) -Bromophenol Blue +++ (Fig.2G, H)
Morphology of the spermatozoa	-Elongated without a distinction between the head and tail -PAS/Alcian Blue ++ -Sudan Black B + (Fig. 2E)	-Elongated without a distinction between the head and tail (Fig. 2I) -PAS/Alcian Blue ++ (Fig. 2I)	-Elongated without a distinction bet- -ween the head and tail (Fig. 2J, K, L) -PAS/Alcian Blue ++ (Fig. 2J, K, L)	-Elongated without a distinction between the head and tail (Fig. 2G) -Bromophenol Blue +++ (Fig. 2G)

+Weak positive reaction, ++Medium positive reaction, +++Strong positive reaction, -Negative reaction

DISCUSSION

The spermatheca of ants in general has been considered important for the adaptive success of these species, given that it is responsible for the reception and storage of the spermatozoa from the moment of mating until the fertilization of the eggs, thus being the source for the production of new individuals.

The morphology of spermatheca is generally described as a sac, into which a few secretory glands may open. These associated glands are known as spermathecal glands.

In the species of ants of the genera *Atta* and *Acromyrmex* analyzed in the present study, the spermatheca shows the same sac morphology described for insects in general; however, in these species there were two lobes that fused partially at the median region of the structure.

It was possible to verify that, in the spermatheca of these species, the regions of probable secretory function were distributed in two different manners: a) as cells grouped together forming structures similar to acini and located externally in relation to the spermatheca and b) in the form of a secretory epithelium coating the spermatheca internally. Depending on the species, was observed that the secretory cells might be distributed in three different manners: a) at the external portion likely acini, b) at the internal portion in the shape of an epithelium that coats the reservoir, or c) at both the internal and external portions.

The external secretory portion was found in *A.s. rubropilosa* and *A.l. balzani* located precisely at the point of fusion between the two spermathecal lobes of the reservoir and it was denominated spermathecal gland. Nevertheless, in the spermatheca of queens *A. balzani* and *A. landolti* we could verify the absence of cells with secretory characteristics located externally in relation to the spermatheca, in other words, these species lacked the spermathecal glands.

In insects in general, the external secretory portion of the spermatheca, both at the level of light microscopy and electron microscopy, has been described as constituted by cells presenting intra and extracytoplasmic canaliculi (Gupta and Smith, 1969; Davey, 1985).

In the species *A.s. rubropilosa* and *A.l. balzani*, which presented the spermathecal gland, the presence of these canaliculi was also noted and they have the function of collecting the secretion produced by the secretory cells and transport it into the reservoir's lumen. In these two species we suggest that the secretion produced by the spermathecal gland might undergo some sort of modification through the path it travels, from the site where it was produced intracellularly, going through the canaliculi and finally reaching the reservoir. This suggestion is based on the results of the histochemical tests, which revealed that the final secretion released does not present the exact constitution of when it was produced. In the spermatheca of queens of *A.s. rubropilosa* the two portions of the canaliculi were observed. In the intracellular portion the canaliculi showed numerous infoldings around the lumen, as well as a thin internal cuticular coat. In the extracellular portion, no infoldings were observed in the surface of the lumen and the cuticle that forms the internal coat appeared thicker.

The occurrence of a modification in the composition of the secretion has already been described in some insects by other authors, who reported that the secretion produced by the secretory cells is generally more dense at the beginning of the extracytoplasmic canaliculi and becomes more fluid as it travels through them, thus facilitating its passage into the spermathecal lumen.

The spermatheca of the queens of the three species of the genus *Acromyrmex* appeared as organs of considerable size, showing thick walls due to the presence of an internal secretory epithelium. However, in the species *A.s. rubropilosa*, the reservoir wall appeared extremely thin due to the absence of the secretory epithelium composed by cells of columnar morphology. These cells are probably responsible for the production of the cuticle and may also play a role in the passage of material.

The presence of an internal secretory epithelium in the spermatheca of the queens of all three species of ants of the genus *Acromyrmex* suggests that the physiology for the maintenance of the spermatozoa in viable conditions in order to use them at the time of fertilization of the eggs in these species must be different than the physiology observed in *A.s. rubropilosa*, in which only an external secretory portion was observed.

In *A.l. balzani* in addition to the spermathecal gland, the internal secretory epithelium appeared composed by columnar cells. In the spermatheca of queens of *A. balzani* and *A. landolti*, which did not present the spermathecal gland, the secretory cells forming a single internal epithelium. In *A. landolti*, this epithelium was constituted by columnar cells too. In the spermatheca of queens of *A. balzani* we observed a differentiation in the cellular morphology of the internal secretory epithelium, with the upper 2/3 of the spermatheca approximately being composed by columnar cells, while the remaining lower 1/3 of the spermatheca approximately was composed by cubical cells. This variation in the hight of the secretory cells must depend on their degree of functional activity. It could be inferred that the greatest activity occurs in the columnar region of the epithelium probably due to the fact that, previously in the evolutionary scale, the spermathecal gland would have been located externally to this precise region.

According to Wheeler and Krutzsch (1994) the apical margin of the columnar epithelium of the spermatheca of ants *Crematogaster opuntiae* displays dense arrays of microvilli and numerous-bounded pinocytotic vesicles. We also observed that the apical region of the epithelial cells of the secretory epithelium of *A. balzani* and *A. landolti* is differentiated. Due to the fact that the portion responsible for the production of the secretion is located internally and coats the whole reservoir, the secretion produced by the cells would be released directly inside the reservoir through a process of exocytosis, in which vesicles from the apical cytoplasm of the cells to forward into the spermathecal lumen. Consequently, there would be no participation of external cells to the spermatheca in the production of secretion to replace the spermathecal fluid.

Muscle layers were observed externally surrounding the spermatheca of all four species of ants described in the present study. The musculature appeared relatively thin, with the exception of the sp. *A. balzani*, in which was verified the presence of several layers and in *A. landolti*, in which these layers thicken particularly at the region of the point of fusion between the two spermathecal lobes. The musculature by contracting all the structure has the function of aiding the movement of the spermatozoa inside the spermatheca and also in their expulsion at the time of egg fertilization.

From the histochemical point of view, the data presented in this research revealed that the external secretory portion of the spermatheca of *A.s. rubropilosa*, the internal secretory epithelium and the external secretory portion in *A.l. balzani* and the internal secretory epithelium in *A. landolti* and *A. balzani*, reacted weakly to the test for the detection of polysaccharides, thus indicating low levels of production of these elements in the spermatheca of the four species. The polysaccharides observed in the spermatheca of *A.s. rubropilosa*, *A. landolti* and *A.l. balzani* correspond to neutral polysaccharides, since they did not react to the Alcian Blue showing the absence of acid polysaccharides. However, the presence of acid ones was observed in the spermatheca of *A.l. balzani*.

In the spermatheca of *A.s. rubropilosa* was observed a large amount of polysaccharides in the cytoplasm of the cells reservoir. Therefore, since the cuticle is a structure that presents large amounts of polysaccharides, these elements might have the cells of the epithelium of the reservoir as source.

The low level of polysaccharides found in the spermatheca of ants of the genera *Atta* and *Acromyrmex* might suggest that these compounds would be forming complexes with other elements, such as glycoproteins, which would in turn have the function of maintaining the spermatozoa by providing them with an energy source while they remain stored, but mainly by supplying the energy necessary for their metabolism at the time of fertilization.

Atta sexdens rubropilosa spermatheca had a low level of lipids, thus suggesting that they might also be produced with the purpose of establishing complexes that would act in the maintenance of the spermatozoa stored.

The protein detection revealed that in *A.l. balzani* the internal secretory epithelium, the cuticle that coats the reservoir internally, the musculature that surrounds the whole structure externally and the secretion stored inside the spermathecal lumen are positive to this element.

The studies concerning the specific composition of the secretion produced by the secretory cells of insect's spermatheca, or of the secretion contained inside the lumen shown that it is a product of specialized glandular cells found in this structure, has a glycoproteic nature (Clements and Potter, 1967; Happ and Happ, 1970; Bhatinagar and Musgrave, 1971; Grodner and Steffens, 1978; Ahmed and Gillot, 1982 a, b; Gardner, 2004).

The spermatozoa of most insects are described as elongated and filamentous structures (Bacceti, 1972; Dallai and Afzelius, 1993; Lino-Neto and Dolder, 2002), an observation that was corroborated in the ants of the genera *Atta* and *Acromyrmex* analyzed in the present study. The histochemical studies showed that the spermatozoa contain polysaccharides and proteins in their basic constitution, with lipids having been observed only in *A.s. rubropilosa*. The spermathecal fluid stored inside the lumen of the spermatheca had proteins as its basic component and has the function of maintaining the spermatozoa alive.

The analysis of the external morphology of the spermatheca of ants of the genera *Atta* and *Acromyrmex*, showed many more similarities than differences when examined by the naked eye. Thus, histological studies were needed in order to corroborate such similarity or verify that, although the spermatheca are externally similar, they present variations in the arrangement of cells and tissues.

According to the morphological data up to now obtained for the spermatheca of *A.s. rubropilosa*, *A. balzani*, *A. landolti* and *A.l. balzani*, it was possible to propose a specialization of the structure (Fig. 1 C, D, E, F), since the spermatheca of *A.s. rubropilosa* only presents an external secretory portion; *A.l. balzani*, presenting the internal secretory epithelium and the external secretory portion and the spermatheca of *A. balzani* and *A. landolti* only present an internal secretory epithelium. However, *A. landolti* presents a higher degree of differentiation than *A. balzani* since its secretory epithelium was differentiated into two portions (columnar and cubical), thus suggesting that the greater production of secretion would occur in the columnar portion of the epithelium. In *A. landolti* there was no such differentiation of the epithelium presenting only columnar cells throughout, thus suggesting that the secretion is produced uniformly.

In *A. balzani* and *A. landolti*, the fact that the secretory epithelium is exclusively located in the internal region of the spermatheca suggests that, in these two species, the spermatheca itself would be self-sufficient to produce all the elements that compose the secretion necessary to maintain the spermatozoa viable. A lesser or none dependence on the supply of exogenous elements that contribute for an adequate microenvironment for the spermatozoa until the moment of fertilization of the eggs would render greater chances of survival to the species, even at times of unfavorable environmental conditions such as low temperatures, high pluviosity, scarce food sources, etc.

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