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Anatomy of the Spermatophore in Triatomines (Hemiptera, Reduviidae, Triatominae) and Its Applications to the Study of Chagas Disease Vector Biology

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Abstract. The present study focused on spermatophore structure, transfer, and subsequent destination inside bloodfeeding females of the species *Triatoma infestans* and *Rhodnius neglectus*. The morphology of the spermatophore differed between the species studied, such that in *T. infestans*, the shape was ovaloid, whereas in *R. neglectus*, the shape resembled a rod. Structures' spine-like cuticulars distributed across the inner surface of the vagina of both species were observed; however, the role of these cuticulars is unknown in Triatominae. In both species, there was an opening in the spermatophore exactly where the common oviduct is connected, thereby making it possible to confirm that the process of spermatozoid migration takes place through this opening. The results obtained show that the spermatophores of *T. infestans* and *R. neglectus* differ in size, shape, and structure. Therefore, they can be used as taxonomic markers and may provide information regarding physiology and evolution.

INTRODUCTION

During courtship and mating, males of many species of insects provide females with nutritional contributions that can include captured prey, male body parts, and substances of the male accessory glands.[1]

The male accessory glands of insects secrete a viscous substance that surrounds the spermatozoids and solidifies on them to form the spermatophore. This spermatophore is then transferred to the female during copulation, thereby ensuring that the spermatozoids are transported.[2] The female reproductive system of insects, in general, comprises a pair of ovaries and two lateral oviduct openings in a common oviduct that continues until the chamber genital or bursa copulatrix.[3]

Although this chamber has been referred to as the bursa copulatrix,[3,4] we have adopted the term vagina in describing this posterior chamber, which was already described in *Rhodnius*.[5,6]

The spermathecae are blind-ended tubes coming off of the common oviduct that receive and store the sperm until the eggs are fertilized. The development of a more spacious spermatheca and an expanded vagina can be adapted to receive nutrients secreted and also allow a prolonged stock sperm.[7]

The females of many species digest, eat, or remove the spermatophore after it has been transferred. Thus, it has been proposed that the spermatophore size remains large as a form of parental investment. In this manner, females that receive large spermatophores subsequently present long refractory periods.[8]

Although few studies have reported on the morphology of spermatophores in insects, it has been proven that they differ in size, shape, and structure between species and that these differences may be reliable as species-specific markers.[2] Study on diversity (size and shape) of ejected spermatophores in Reduviidae in Southern India showed that there was variation between species.[9]

In this light, the present study focused on spermatophore structure, transfer, and subsequent destination inside bloodfeeding females of the species *Triatoma infestans* and *Rhodnius neglectus*. In this way, it may contribute to the basic knowledge of Triatominae through identifying new parameters for studies on systematics and reproduction, because this structure is essential for spermatozoids to be transferred and the species to be maintained.

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MATERIALS AND METHODS

The insects used were obtained from colonies maintained in the insectary of the Laboratory of Parasitic Diseases, Instituto Oswaldo Cruz, FIOCRUZ/RJ.

To monitor copulation, 10 couples of *T. infestans* and *R. neglectus* were separated out and placed on Petri dishes that were 15 cm in diameter and lined with filter paper. Immediately after copulation, the male was removed from the dish and isolated in a receptacle. Ten minutes later, dissection of the female was started using physiological solution (0.7% NaCl + 0.3% KCl) to avoid desiccation of the internal structures. The copulatory pouch and spermatophore were removed and the following analyses were made.

Spermatophore and cuticular structure morphology. Five females triatomine bugs were transversely cut in the sixth abdominal segment, washed, dehydrated in an alcohol series, and oven-dried at 50°C.[10] In the other 10 females, after mating, the vaginas containing the spermatophore were removed, fixed in 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer, pH 7.2, for 1 hour, and post-fixed in 1% osmium tetroxide in the same buffer for 1 hour at room temperature in the dark. The samples were washed with sodium cacodylate buffer, dehydrated in a graded acetone series of up to 100%, and submitted to the critical point drying method using super dry CO_2.

Ventral sides of the external genitalia and vagina were placed on metallic supports and coated with a thin layer of gold, and the analysis was performed by the Scanning Electron Microscope JEOL 6390LV of the Electron Microscopy Platform, Instituto Oswaldo Cruz, FIOCRUZ.

Morphometry. The spermatophores were photographed using a digital camera (Sony DSC S730), which was coupled manually to a Leica stereoscopic microscope at a magnification of 20×. The images were analyzed, and the measurements were made using the Image-Pro 4.5 software.

Statistical analyses were conducted with the aid of the Bioestat software, version 5. The morphometric data relating to the size and width of the spermatophores of the two species studied were compared using a Student *t* test with a confidence interval of 95% ($P = 0.05$).

Histology. The spermatophores inside the copulatory pouch were fixed in 2.5% glutaraldehyde and 0.1 M cacodylate buffer (pH 7.2) and then dehydrated in an ascending ethanol series (7.5%, 15%, 30%, 50%, 70%, 90%, and 100%). Subsequently, the samples were embedded in Leica histological resin. The material was sectioned using a microtome in slices of thickness of 4 μm, and these sections were stained using hematoxylin and eosin. Finally, the sections were analyzed and photographed under a Zeiss Primo Star microscope that was coupled to a Zeiss AxioCam ICc 1 camera.

RESULTS

Copulatory behavior. No differences in copulatory behavior were observed between the two species. However, there was a difference in the time taken to perform copulation. *T. infestans* took 5–10 minutes on average, which could be extended to 20 minutes, whereas *R. neglectus* took 30–50 minutes, which could be extended to 62 minutes.

Morphology of the spermatophore and cuticular structures. The spermatophore in *T. infestans* is ovaloid, the secretion is completely translucent, and the spermatozoid mass is located in the anterior portion in relation to the female's body (Figure 1). However, in *R. neglectus*, the spermatophore resembles a rod, and the secretion has a translucent appearance in almost all the structures, except for an opaque portion in the posterior region. In this species, the spermatozoid mass is also located in the posterior region (Figure 2).

By means of scanning electron microscopy, an opening could be seen above the spermatozoid mass in both species exactly where the common oviduct is connected. In *T. infestans*, it was in the anterior portion in relation to the female's body (Figure 3), whereas in *R. neglectus*, it was in the posterior portion in relation to the female's body (Figure 4).

The vagina inner face of both species has cuticular structures similar to spines. In *T. infestans*, single spines are predominant, and all may be seen at the same region (Figure 5). *R. neglectus* ranges from two to five spines close to each other in one region (Figure 6A), and in other regions, there is only

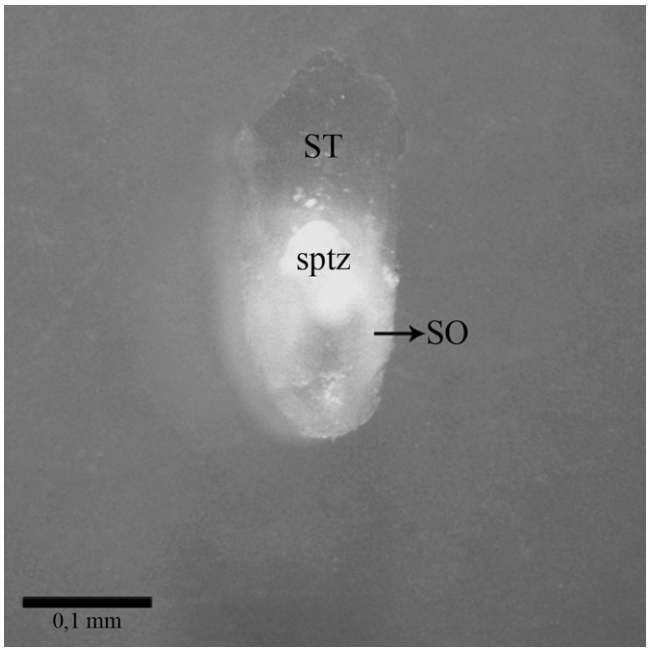

FIGURE 2. Spermatophore removed from the vagina of *R. neglectus*. SO = opaque secretion; sptz = spermatozoid mass; ST = translucent secretion.

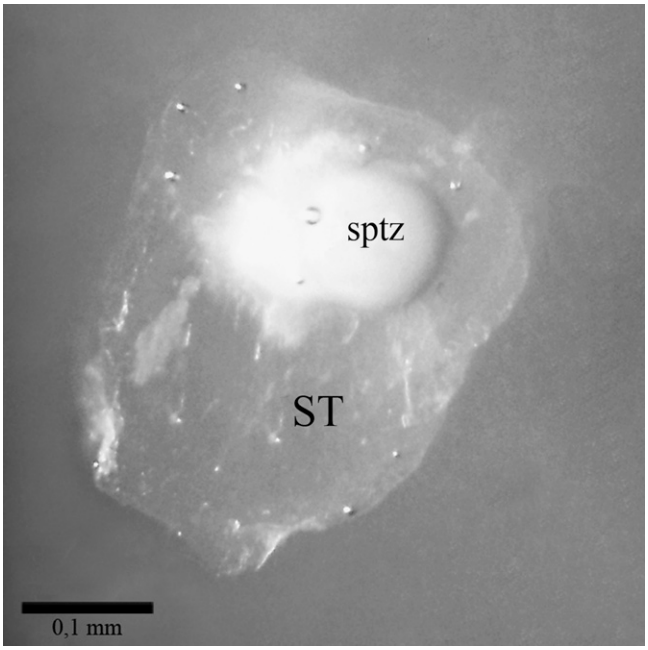

FIGURE 1. Spermatophore removed from the vagina of *T. infestans*. sptz = spermatozoid mass; ST = translucent secretion.

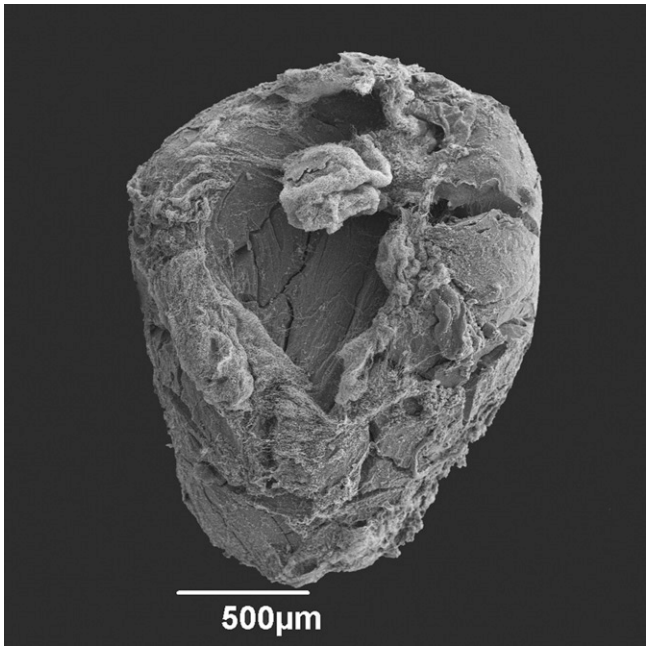

FIGURE 3. Electron micrograph of the spermatophore of *T. infestans*.

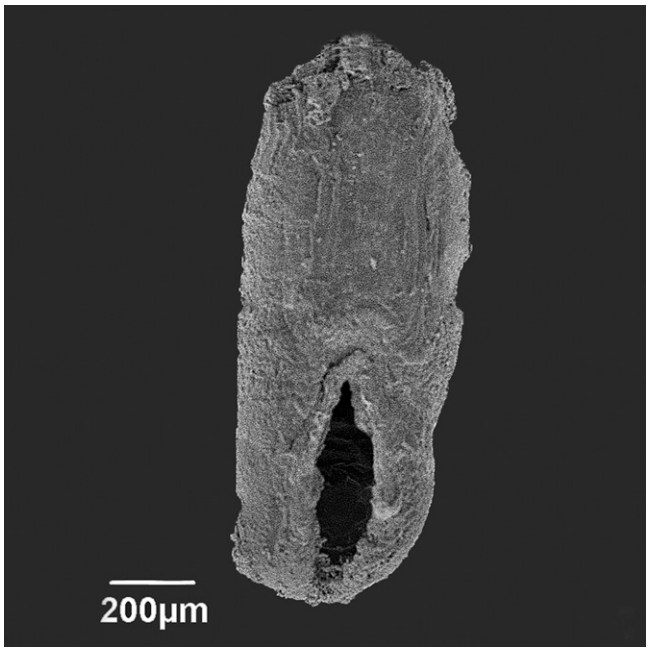

FIGURE 4. Electron micrograph of the spermatophore of *R. neglectus*.

a single spine (Figure 6B). The external genitalia of both species have spines similar to the spines observed inside the vagina. However, the spines of the genitalia are shorter than the spines in the vagina (Figure 7).

Morphometry. In the morphometric analysis, 11 spermatophores of *T. infestans* and 9 spermatophores of *R. neglectus* were photographed. The measures showed that the differences in width were significant ($P = 0.0011$). The spermatophore of *T. infestans* was broader (2.49 ± 0.25 mm) than the spermatophore of *R. neglectus* (1.94 ± 0.43 mm). The differences in length were not significant ($P = 0.035$): *T. infestans* (4.22 ± 0.8 mm) versus *R. neglectus* (3.68 ± 0.42 mm) (Table 1).

Histology. On the histological sections from the spermatophore inside the vagina of *T. infestans* and *R. neglectus*, translucent secretions and a spermatozoid mass forming a compact structure, a thick cuticle surrounding the entire spermatophore, epithelium, and muscle fiber cells could be seen (Figure 8).

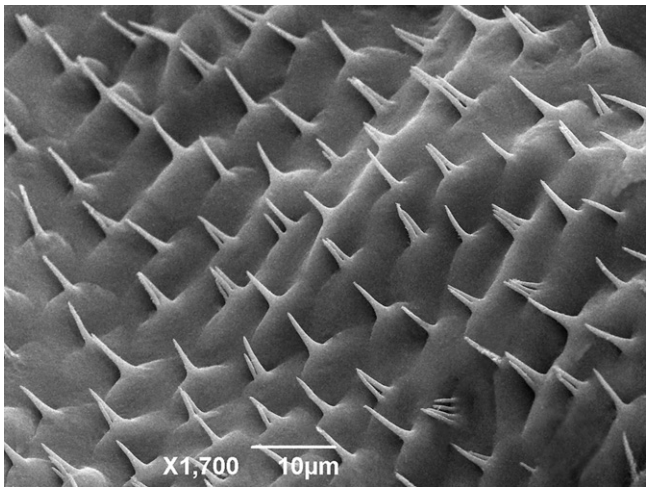

FIGURE 5. Electron micrograph of the spines in the vagina of *T. infestans*.

The simple epithelium in *T. infestans* presented with low cells and rounded nuclei (Figure 8), whereas in *R. neglectus*, it presented high cells and elongated nuclei (Figure 9) seated on a thick cuticle. In both species, the circular and longitudinal muscle fiber cells had a columnar appearance with decentralized nuclei.

Ten minutes after copulation, the spermatozoids were observed coming out of the spermatophore and crossing the epithelium (Figure 10). In the region where this process was taking place, no surrounding cuticle was seen (Figure 10).

DISCUSSION

Although reproductive behavior has been studied in a number of species of Triatominae,[11–14] there is little information about the production, transfer, and ejection of the spermatophore.

The copulation behavior of triatomines has been described similarly in all studies conducted on them. However, the time taken for copulation varies between the species. It has been reported to be 10–16 minutes among *Triatoma*,[11,12,14] which corroborates the time taken by *T. infestans* in the present study. In the species *Rhodnius*, no specific times have been reported, but times of 30–50 minutes were found for *R. neglectus* in the present study.

Courtship, named dance of love, has been described in studies on the copulation behavior of triatomines, and it is important for the male's reproductive success.[11,15] In *T. infestans* and *R. neglectus*, this behavior was little observed, probably because the species studied were obtained from colonies maintained in a laboratory. This finding suggests that this process may be related to the species' evolutionary biology, such that species with a high degree of adaptation to closed environments may display sexual behavior that is less stereotyped.[16]

In insects, the secretions from the male accessory glands are related to protection, storage, and activation of sperm and behavioral changes in females, such as reduction of attractiveness, fecundity, ovulation, and egg production.[17] In Triatominae, the male accessory glands consist of four pairs of lobules that are morphologically similar. However, in *Triatoma*, all of them present translucent secretions[18]; in *Rhodnius*, three pairs present translucent secretions, and one pair presents opaque secretions.[19] In *R. prolixus*, the spermatophore is formed within the vagina, with the sperm and opaque gland material entering the vagina first followed by the relatively large amount of secretion from the transparent gland before the intromittent organ is pulled out.[19]

In the present study, the spermatophores extracted from the vagina of the female are similar to other spermatophores described for other insect species.[7,20,21] In *T. infestans*, the secretions from the spermatophore are completely translucent, whereas in *R. neglectus*, the posterior region is opaque, thus indicating that it is part of the opaque gland secretion described in *R. prolixus*.[19]

The spermatophore of *R. prolixus* varies in size considerably according to the quantity of secretion produced by the male accessory glands and the insect nutritional status.[20] Among males that were fed, larger volumes of glandular secretion were observed, and a large spermatophore was produced. However, males that were not fed did not produce a spermatophore. Thus, it was reported that fasting reduced the

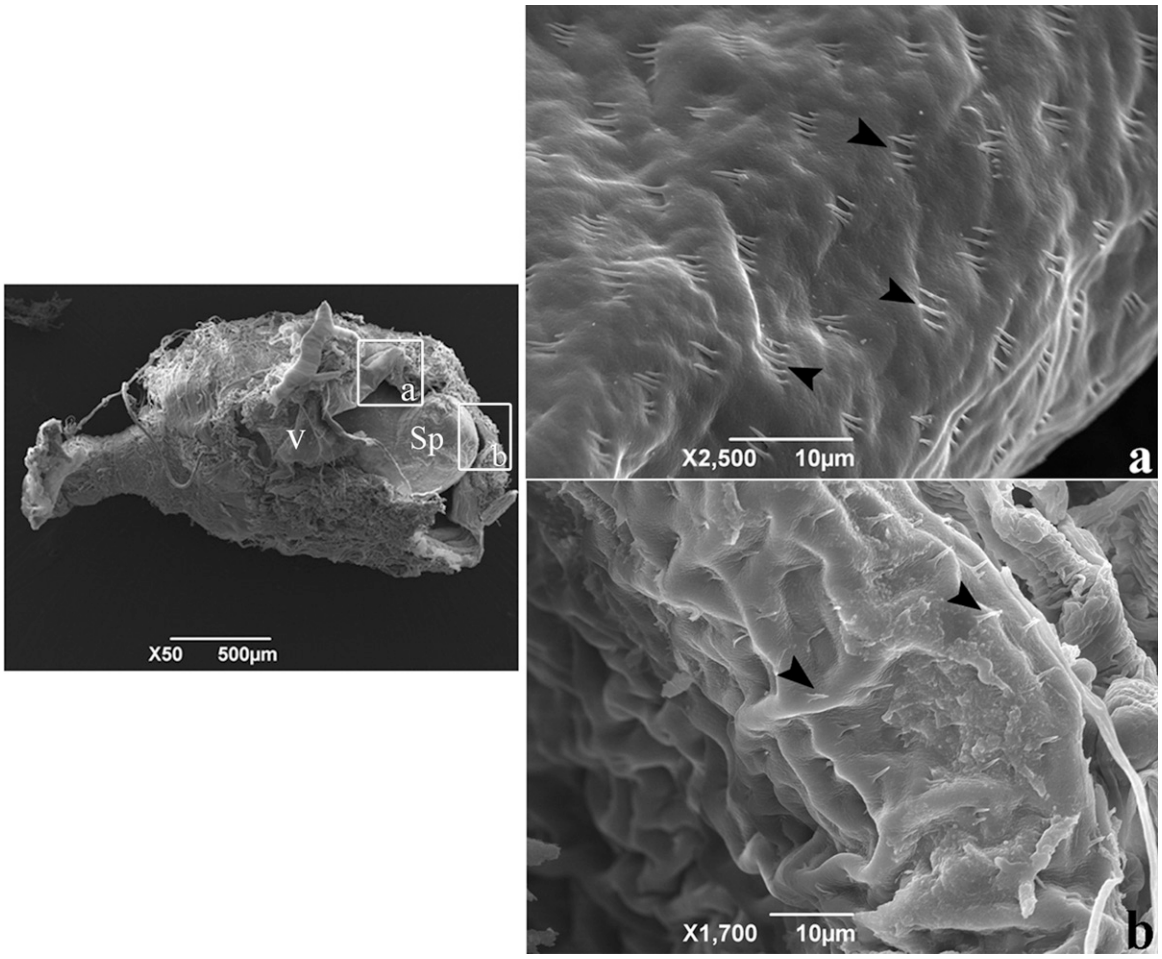

FIGURE 6. Electron micrograph of the vagina of *R. neglectus*. (**A**) Region with two to five spines (arrowheads). (**B**) Region with a single spine (arrowheads). Sp = spermatophore; V = vagina.

quantity of secretion from the male accessory glands, and consequently, copulation did not take place, even if the seminal vesicle was full of spermatozoids. The first copulation in *T. infestans* is associated with the need for another blood meal to complete the glandular activity (i.e., in this species, the males only have sufficient secretion to form the spermatophore with another blood meal).[22]

The definitive form for the spermatophore in *R. prolixus* is given by the male's aedeagus before the transfer to the female's vagina.[19] Differing from this process, the spermatophores of *T. infestans* and *R. neglectus* are transferred to the vagina as a viscous or mucous substance, and they solidify in the vagina.

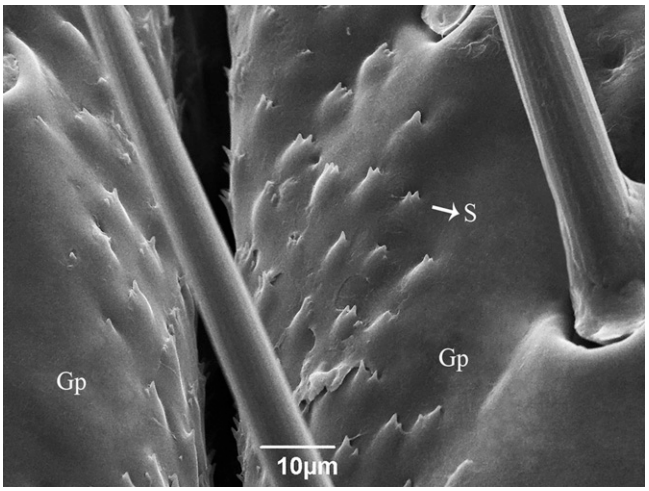

FIGURE 7. Electron micrograph of the genitalia of *T. infestans*. Gp = gonapophyses; S = spines.

TABLE 1

Measurements of the lengths and widths of the spermatophores of *T. infestans* and *R. neglectus* at a magnification of 20× under stereoscopic microscope

	T. infestans		*R. neglectus*	
Specimen	Length (mm)	Width (mm)	Length (mm)	Width (mm)
1	3.01	2.60	4.44	2.61
2	3.78	2.76	4.39	1.80
3	3.79	2.66	5.10	2.43
4	3.65	2.85	3.93	1.63
5	3.15	2.24	5.07	1.90
6	4.10	2.37	4.32	1.66
7	4.46	2.82	2.67	1.67
8	3.99	2.13	3.31	1.35
9	3.51	2.30	4.73	2.37
10	3.63	2.35	–	–
11	3.44	2.28	–	–
Mean	3.68	2.49	4.22	1.94
SD	0.42	0.25	0.8	0.43

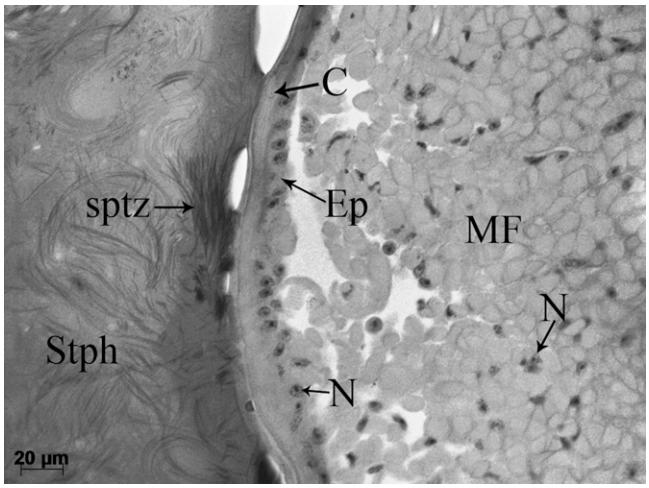

FIGURE 8. Histological section of the spermatophore of *T. infestans* inside the vagina. C = cuticle; Ep = epithelium; MF = muscle fibers; N = nuclei; sptz = spermatozoids; Stph = spermatophore.

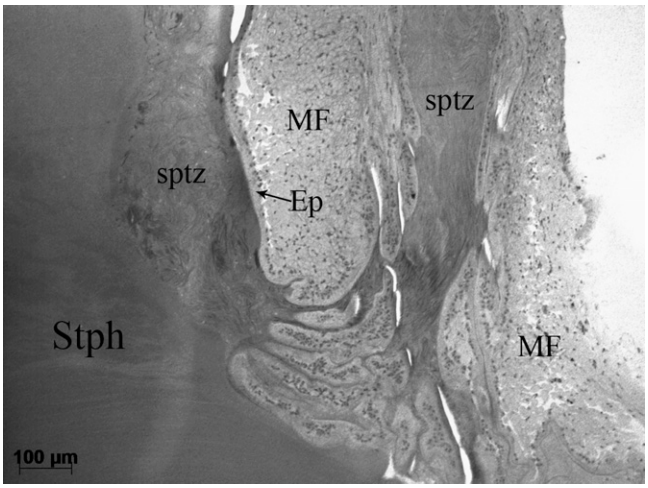

FIGURE 10. Histological section of spermatophore of *T. infestans* inside the vagina showing spermatozoid migration. Ep = epithelium; MF = muscle fibers; sptz = spermatozoids; Stph = spermatophore.

Thus, the lumen of the vagina is responsible for giving the definitive format to the spermatophore, such as in Coleoptera.[7]

In the present study, it was observed that, when the insects spent a long time fasting, copulation did not take place in either of the species studied given that the main element of spermatophore composition involves secretions from the accessory glands.[7,23] We took the view that this structure represented a substantial investment of energy by the male and was a trophic source when food was scarce, which could have an influence on reproduction.

In the histological sections through the spermatophore inside the vagina, it could be seen that there was a thick cuticle that originated from invagination of the exocuticle during the embryonic development of the female. Comparison between the vaginal epitheliums of virgin females and females in different physiological states in the presence or absence of a spermatophore may clarify whether this structural morphology is standard or whether it is related to distension of the vagina, which occurs with epithelial cells of the intestine in some insects. The latter are long and change their morphological pattern after the insect has fed.[24]

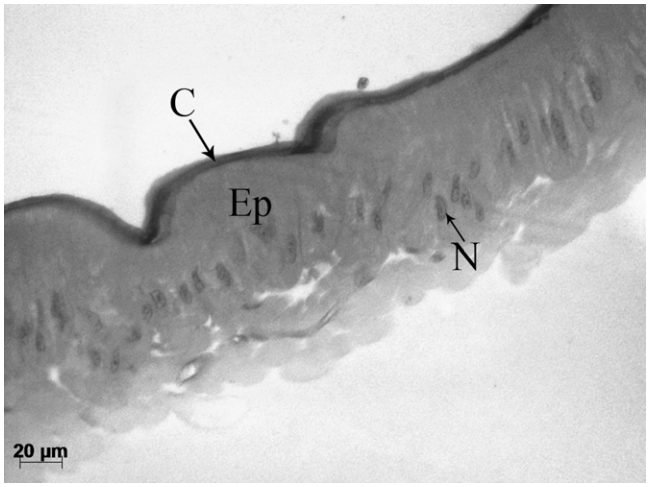

FIGURE 9. Histological section of the vaginal epithelium of *R. neglectus*. C = cuticle; Ep = epithelium; N = nucleus.

Visceral muscles in insects do not have a connection to the body wall, such as in skeletal muscles. They form mainly around the intestine and the ducts of the reproductive system. In the species studied, the circular and longitudinal muscle fibers that cover the surface of the vagina are responsible for vaginal contraction and help in spermatozoid migration to the spermathecae and in oviposition.[5]

The means by which spermatozoids migrate from the spermatophore to the spermatheca diverge between different insects. In *Danaus plexippus* (Lepidoptera), the copulatory pouch has a region with rows of chitinous spines and another region covered with bristles. The presence of the spermatophore inside the copulatory pouch causes muscle contractions that promote contact between the spines and the spermatophore wall. These contractions result in release of spermatozoids, and then, the spermatophore wall is digested by proteolytic enzymes from the copulatory pouch.[25] In triatomines, the presence of an opening in the anterior region of the spermatophore that is subjected to pressure from contractions of the common oviduct opens up to release spermatozoids to the spermathecae.[20] In the present study, it was observed that the cuticle surrounded the spermatophore, but in the region where the spermatozoids came out of the spermatophore, no cuticle was seen to exist. By means of optical microscopy and electron microscopy, an opening in the spermatophore of both species could be seen exactly where the common oviduct is connected, through which bundles of spermatozoids migrate. This finding suggests that the region without cuticle is the location of the opening where the common oviduct is connected.

By scanning electron microscopy, the present work shows the presence of cuticular structures similar to spines that has not been described before in this group of insects within the vagina of the *T. infestans* and *R. neglectus*. The presence of spines between within the vagina is caused by an invagination of the outer cuticle, because these spines were also observed between gonapophyses of the external genitalia.

In insects with a distinct bursa copulatrix separate from the vagina (for example, the monarch butterfly), this chamber serves to hold and break up the spermatophore, thus releasing the spermatozoa.[25] In reduviids, there is no need to break the

spermatophore, because it is not encased in an outer shell (unpublished observations). It is unlikely that these spines serve any physical purpose to either break apart a spermatophore that does not need to be broken or move spermatozoa that are being moved by rhythmic muscular contractions.[5]

Because significant differences in the length and width of the spermatophores of *T. infestans* and *R. neglectus* were observed, these parameters can be considered to be genus-level markers for Triatominae. They may also be considered to be markers at the species level given that they are different species.

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