



## Spermatogenesis of *Zaprionus indianus* and *Zaprionus sepsoides* (Diptera, Drosophilidae): Cytochemical, structural and ultrastructural characterization

Letícia do Nascimento Andrade de Almeida Rego, Rosana Silistino-Souza,  
Maria Tercília Vilela de Azeredo-Oliveira and Lilian Madi-Ravazzi

*Departamento de Biologia, Instituto de Biociências, Letras e Ciências Exatas,  
Universidade Estadual Paulista “Julio Mesquita Filho”, São José do Rio Preto, SP, Brazil.*

### Abstract

*Zaprionus indianus* is a drosophilid native to the Afrotropical region that has colonized South America and exhibits a wide geographical distribution. In contrast, *Z. sepsoides* is restricted to certain African regions. The two species differ in the size of their testes, which are larger in *Z. indianus* than in *Z. sepsoides*. To better understand the biology and the degree of differentiation of these species, the current study evaluated spermatogenesis in males of different ages by conventional staining techniques and ultrastructural analysis. Spermatogenesis and the ultrastructure of spermatozoa were similar in the two species, and the diploid number was confirmed to be  $2n = 12$ . A greater number of spermatozoa were observed in young *Z. indianus* (1-3 days old) compared to *Z. sepsoides* males, which showed a higher frequency of cells at the early stages of spermatogenesis. The head of the sperm was strongly marked by silver staining, lacto-acetic orcein and the Feulgen reaction; the P.A.S. reaction revealed glycogen granules in the testes of both species. Both species presented similar arrangement of microtubules (9+9+2), two mitochondrial derivatives of different size and 64 spermatozoa per bundle. Such similarity within the genus *Zaprionus* with other species of *Drosophila*, indicates that these structures are conserved in the family Drosophilidae. The differences observed the number and frequency of sperm cells in the early stages of spermatogenesis, between the young males of *Z. indianus* and *Z. sepsoides*, are features that may interfere with reproductive success and be related to the invasive potential of *Z. indianus*.

*Keywords:* *Zaprionus indianus*, *Zaprionus sepsoides*, spermatogenesis, cytochemistry, ultrastructure, meiosis.

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### Introduction

The genus *Zaprionus* is divided into two biogeographically separate subgenera: the subgenus *Anaprius* Okada, with approximately 10 species in the Eastern region, and the *Zaprionus* subgenera, with approximately 50 species in the Afrotropical region (Okada and Carson, 1983; Yassin *et al.*, 2008). Phylogenetically, *Zaprionus* is very close to the genus *Drosophila* (Yassin *et al.*, 2007, 2008, 2010; Yassin and David, 2010).

The species *Zaprionus indianus* (Gupta, 1970) originated in Africa was first identified in Brazil in 1999 and is considered an invasive species that has infested fig plantations (Vilela, 1999; Tidon *et al.*, 2003). After 1999, *Z. indianus* has been detected in different regions of Brazil (Toni *et al.*, 2001, Castro and Valente, 2001; Santos *et al.*, 2003; Kato *et al.*, 2004; Mattos-Machado *et al.*, 2005, Da-

vid *et al.*, 2006), Uruguay (Goñi *et al.*, 2001) and, in 2005, Florida (USA) (Van der Linde *et al.*, 2006) and Argentina (Soto *et al.*, 2006).

Studies examining the invasive potential of *Z. indianus*, included its life cycle (Amoudi *et al.*, 1991; Stein *et al.*, 2003), larval competition (Amoudi *et al.*, 1993a) and aspects of fitness (Amoudi *et al.*, 1993b; Setta and Carareto, 2005). Allozyme studies have indicated that plasticity in the distribution of the allelic frequency at the *Est-3* locus may have also contributed to the success of this species in spreading across the Americas (Galego and Carareto, 2007, 2010). However, few studies have examined the biology and reproduction of *Z. indianus* (Araripe *et al.*, 2004) or other *Zaprionus* species (Yassin *et al.*, 2007; Yassin and David, 2010; Yassin *et al.*, 2010).

Of the 49 species of the genus *Zaprionus*, only three have become invasive: *Z. indianus*, *Z. tuberculatus* and *Z. ghesquieri* (Chassagnard and Kraaijeveld, 1991). *Z. indianus* is the most widespread species of the genus and the ecologically most diverse of the Afrotropical drosophilid fauna (Yassin *et al.*, 2007, 2010; Yassin and David, 2010). The general habits of this species may be a major

factor in its successful occupancy of four continents (Lachaise and Tsacas 1983, Schmitz *et al.*, 2007). However, other factors, including aspects of its reproduction, adaptation and ecology, may also be involved in the invasion process.

Male characteristics that influence the competitive ability to mate and fertilization success can also interfere with the fitness of the species. For example, sperm size is a male trait that is predicted to be under strong sexual selection (Hosken *et al.*, 2003, Snook, 2005; Scharer *et al.*, 2008), even though the selective advantage of different sizes of sperm remains largely unclear (García-González and Simmons, 2007; Amitin and Pitnick, 2007). The number of sperm and their developmental stages are also factors that can contribute to the reproductive potential of the species.

The present study aims to expand on previous studies on the biology of *Z. indianus* and the characteristics that possibly influence its adaptation to various environments, as reflected by its successful invasion of different continents. More specifically, this paper analyzes spermatogenesis and sperm ultrastructure of a geographical strain of *Z. indianus* and of *Z. sepsoides* (Duda, 1939). *Z. sepsoides* is restricted to certain regions of East Africa and differs from *Z. indianus* in testes size and sperm. Furthermore, it is not considered an invasive species.

## Material and Methods

### Origin of insects

A strain of *Z. indianus* from Ubatuba, SP, Brazil, and a strain of *Z. sepsoides* originating from the Congo region of Africa were used in the present work. These strains were maintained at  $\pm 25$  °C in standard culture medium: banana and yeast (*Saccharomyces cerevisiae*).

### Measurements of testes

Pairs of testes were dissected from 12 eight-day-old adult males of each species for obtaining linear measurements. For such measurements, the spiral testes from *Z. indianus* were first uncoiled.

### Preparation of slides and cytochemical techniques

In this analysis, the testes were removed from males of different ages (1-8 days of life) from both species. The testes were stained using lacto-acetic orcein following De Vaio *et al.* (1985) and impregnated with silver nitrate following Howell and Black (1980), with modifications. After the usual preparation of the slides, the procedure for the Feulgen reaction was followed according to Mello and Vidal (1980), with modifications, for both species.

The structural and ultrastructural analyses were performed according to Cotta-Pereira *et al.* (1976), with modifications. The testes of adult *Z. indianus* and *Z. sepsoides* males were removed at different developmental stages (1,

3, 5 and 8 days old) and fixed in a solution of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4. After initial fixation for 4 h at 4 °C, the testes were washed three times in 0.1 M phosphate buffer, pH 7.4, for 15 min each. Next, the material was post-fixed in a 1% osmium tetroxide solution for 2 h in a dark, sealed bottle in the refrigerator. After this procedure, the material was washed three times in distilled water for 5 min each and then dehydrated in a series of increasingly concentrated acetone solutions (30%, 50%, 70% and 90% acetone), with 15 min per solution, followed by dehydration in 95% and 100% acetone three times for 15 min each. After dehydration, the material was embedded in a 1:1 mixture of resin (Araldite) and 100% acetone for infiltration overnight at room temperature. The next day, the material was removed from the resin-acetone solution, placed in resin and placed in a 37 °C oven for 2 h. The testes were then removed from the bottles containing the pure resin, and the embedding process was initiated in the mold, which was placed in a 60 °C oven for 72 h for polymerization.

Semi-thin, 0.5  $\mu$ m sections of the testes were stained with 1% toluidine blue and analyzed using a light microscope. Ultra-thin, 70 nm sections were contrasted with uranyl acetate and lead citrate and analyzed by transmission electron microscopy.

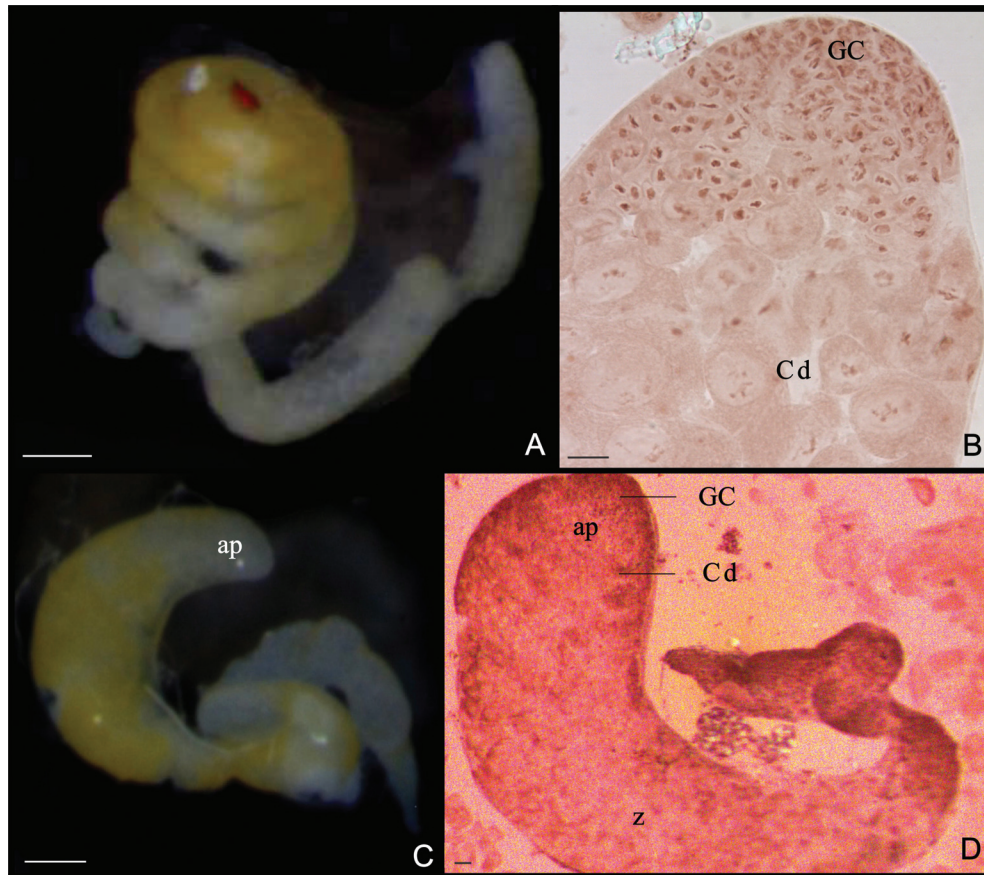
For periodic acid-Schiff (P.A.S.) staining, the slides were first prepared for structural analysis for the two species, as follows (Mello and Vidal, 1980, with modifications). A solution of 0.5% periodic acid was applied to the semi-thin, 0.5  $\mu$ m sections, which were then placed in a 60 °C oven for 14 min. Next, the sections were washed in distilled running water for 5 min. A Schiff reagent was added to slides, which were protected from light and were placed in a 60 °C oven for 45 min. Following the washing of each sample in 3 distilled water baths for 5 min each; the slides were air-dried and mounted with nail polish.

The material prepared by means of conventional cytochemical and staining techniques was analyzed using a light microscope (Olympus BX40) and Axiovision LE digital imaging software, Version 4.8 for Windows. The material prepared using histological techniques was analyzed and photographed using a JEOL 1011 transmission electron microscope operated at 80 kV.

## Results

### Testes morphology of *Z. indianus* and *Z. sepsoides* males

The testes of *Z. indianus* and *Z. sepsoides* are yellow in color (Figures 1A and 1C, respectively). In *Z. indianus*, the testes consist of two coiled tubes of approximately 5 mm in length each (Figure 1A), whereas in *Z. sepsoides*, the testes are two kidney-shaped tubes of approximately 2 mm in length each (Figure 1C).



**Figure 1** - Testes and cells of *Zaprionus indianus* (A-B) and *Zaprionus sepsoides* (C-D). A and C. Testes seen under a stereo microscope. B and D. Squash staining with lacto-aceto orcein showing the germinal center (GC) in the apex of the testes (ap), followed by cells in division (Cd) and spermatozooids (z) present in the central region of the testis. Scale bars: A and C (0.5 mm); B and D (10  $\mu$ m).

### Lacto-acetic orcein staining and Feulgen reaction

In the apical part of the testes, a large number of dividing cells was observed. During prophase I, the nuclei of the primary spermatocytes in the diplotene and diakinesis stages exhibited chromosomes that were arranged in a circular ring and were associated with each other. The sex chromosomes were separate from this ring, as indicated by arrows in Figures 2A and 2J.

The metaphase stage of meiosis I revealed six pairs of bivalent chromosomes ( $2n = 12$ ) in the nucleus in a circular arrangement (Figures 2B-C, 2E-F and 2K and 2L).

At the end of telophase I, the formation of two nuclei was observed (Figures 2D, 2G and 2M). During spermiogenesis, the spermatids become elongated (Figures 2H and 2N) and organized into long bundles in *Z. indianus* (Figure 2I) and short bundles in *Z. sepsoides* (Figure 2O). In *Z. sepsoides*, these bundles stained strongly and revealed a concentration of genetic material at their tips, corresponding to the nuclei of spermatozooids during individualization (Figure 2O). This strong staining indicated the presence of aldehyde groups, based on the color produced by the reactive Schiff stain.

Mainly primary spermatocytes were observed in the apical part of the testes of *Z. indianus* and *Z. sepsoides*, whereas in other parts of the testes, secondary spermatocytes, spermatids and spermatozoa were noted (Figures 1B and 1D).

### Impregnation by AgNOR

Cells in various stages of spermatogenesis were observed by impregnation with AgNOR. For both species, spermatocytes in the early stages of prophase I were observed, with nucleolar corpuscles scattered throughout the cell nucleus (Figures 2P and 2S). Spermatids at later stages of elongation showed no impregnation with AgNOR along their entire length (Figures 2Q and 2T), whereas spermatozoa arranged into long bundles and strongly stained with AgNOR in their extremities were frequently observed in *Z. indianus* (Figure 2R). In contrast, in *Z. sepsoides*, sperm bundles did not exhibit this staining pattern (Figure 2U).

### Structural and ultrastructural analysis of the testes and spermatogenesis of *Z. indianus* and *Z. sepsoides*

The testes of *Z. sepsoides* and *Z. indianus* were analyzed at different stages of development (1, 3, 5 and 8 days



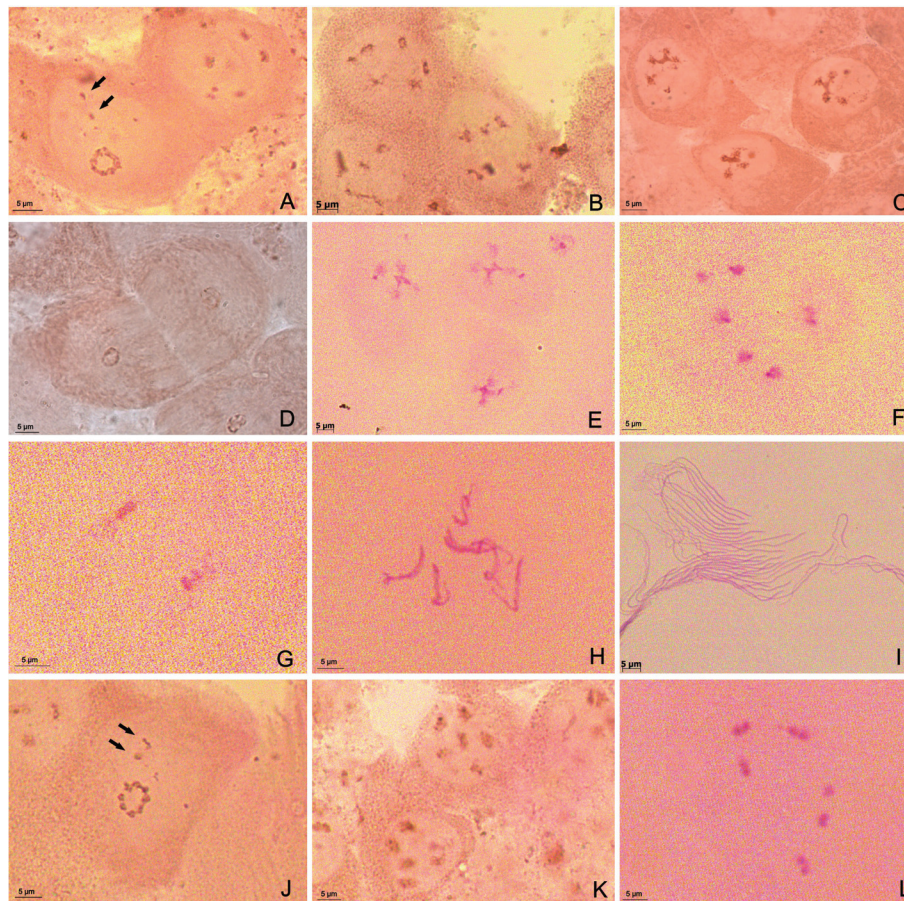
old). In *Z. sepsoides*, the dividing cells (spermatocytes) and elongating cells (spermatids) were more easily detected in young males (1-3 days old), whereas in older ones spermatozooids were noted more frequently. In contrast, in *Z. indianus*, a greater abundance of sperm bundles was observed in young males (1-3 days old), with only few cells in the early stages of spermatogenesis.

In the testes of both species, diverse and unique cellular structures were found. The germinal center was the location of a considerable number of germline stem cells, which were identified at the apex of the testis in both *Zaprionus* species. The testicular content could be divided into two parts: germline stem cells at the apex of the testis and the remainder of the testes filled with cells corresponding to other stages of spermatogenesis. The testicle is coated by a layer of cells and more internally by an envelope (Figures 1B, 1D, 3A, 3B, 4A, 4C and 5A). Strongly stained granules were also observed, particularly in the testes of *Z. sepsoides*. These granules may contain glycogen as in-

ferred from the results of the P.A.S. reaction on these testes (Figure 3B).

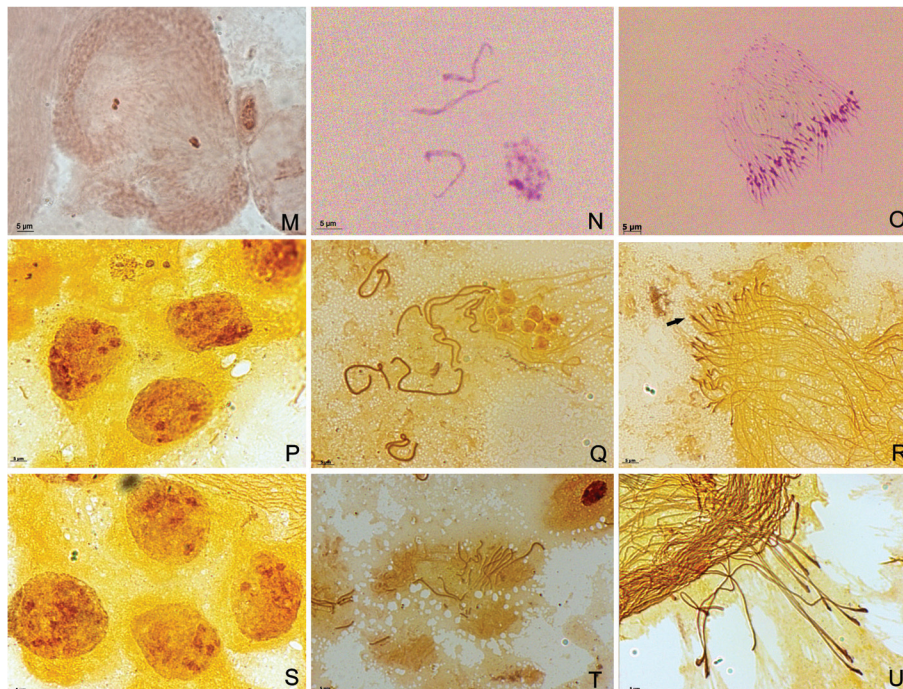
The successive stages of spermatogenesis were not visible in the testes of *Z. indianus*, perhaps due to their spiral morphology and size, which hampered their proper preparation as ultra-thin sections. Alternatively, the peak of the initial stages may have occurred earlier in development, for instance in the pupal stage. In *Z. sepsoides*, some stages of spermatogenesis were observed, with dividing cells occupying the entire apical portion of the testis together with the presence of many granules. Additionally, in regions such as the testicular apex, many circular and elongated spermatids were observed, and in other regions, bundles of spermatozoa were distributed in all directions (Figures 3A-B).

The differentiation of the spermatids in both species within the cysts is synchronous (Figures 4A-B and 5A-B). Another observation at ultrastructural level was the consis-



**Figure 2** - Histological and cytochemical staining results. *Zaprionus indianus* cells stained using lacto-aceto orcein (A-D) and subjected to the Feulgen reaction (E-I). *Zaprionus sepsoides* cells stained using lacto-aceto orcein (J, K, M) and subjected to the Feulgen reaction (L, N, O). Testicular cells of *Zaprionus indianus* (P-R) and *Zaprionus sepsoides* (S-U) after silver impregnation. **A**. Prophase I (diplotene / diakinesis phase), showing the chromosomes arranged in a circular ring and the sex chromosomes (arrows) as separate from this ring. **B, C, E, F**. Metaphase I of meiosis showing the six pairs of bivalent chromosomes. **D, G**. Telophase I showing the genetic material in the center of each nucleus. **H**. Elongated spermatids **I**. Spermatozooids organized into long bundles. **J**. Prophase I (diplotene / diakinesis phase), showing the chromosomes arranged in a circular ring and the sex chromosomes (arrows) as separate entities from this ring. **K, L**. Metaphase I of meiosis showing the six pairs of bivalent chromosomes. Scale bars: 5 µm.





**Figure 2 (cont.)** - M. Telophase I showing the genetic material in the center of each nucleus. N. Elongated spermatids. O. Spermatozoid bundle presenting strong staining and a high concentration of chromosomal material at the tip of the bundles, this corresponding to the spermatozoid nuclei. (P-U). Testicular cells of *Zaprionus indianus* after silver impregnation. P. Spermatocytes in the initial state of prophase I, showing nucleolar bodies spread throughout the entire nucleus of each cell. Q. Spermatids in a more advanced stage of elongation not presenting specific impregnation along their entire length; R. Spermatozoids organized into long bundles with extremities strongly stained by AgNOR (arrow). (S-U) Testicular cells of *Zaprionus sepsoides* after silver impregnation. S. Spermatocytes in prophase I showing the nucleolar bodies of the cells revealed through silver impregnation; T. Spermatids in a more advanced stage of elongation not presenting specific impregnation along their entire length; U. Spermatozoids not showing strong AgNOR staining in one of their extremities. Scale bars: 5  $\mu$ m.

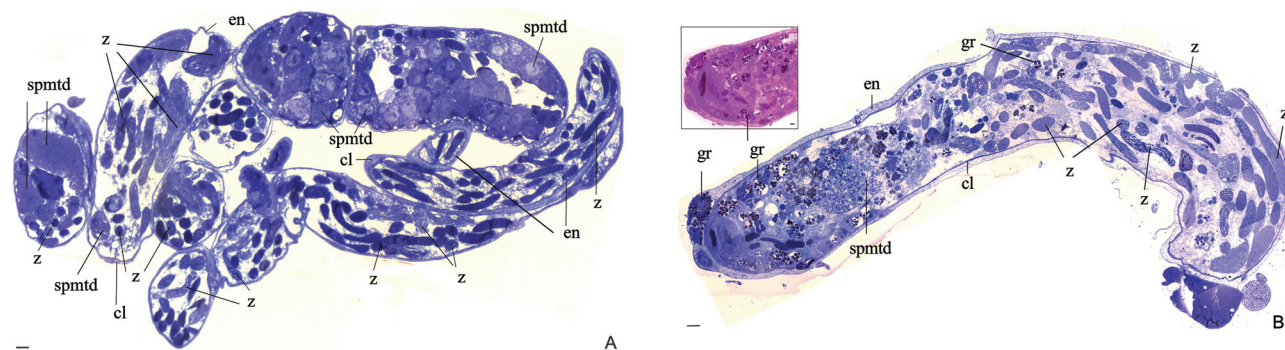
tent number of 64 spermatozoids per bundle in both *Z. indianus* (Figures 4A-B) and *Z. sepsoides* (Figures 5A-B).

The ultrastructures of spermatozoids in both *Z. indianus* and *Z. sepsoides* revealed that the axonemes of both species possess a 9+9+2 configuration, consisting of one pair of central microtubules, nine double peripheral microtubules formed by fibril A and fibril B, surrounded by nine additional accessory microtubules, plus nine spokes (Figures 4G-H and 5D-E, respectively). Additionally, proximal to the axoneme, two mitochondrial derivatives of

different size were present (Figures 4B, 4D-G, and 5B-D) in both species. The larger mitochondrial derivative contained distinct paracrystalline material, particularly in *Z. indianus* (Figures 4D-E).

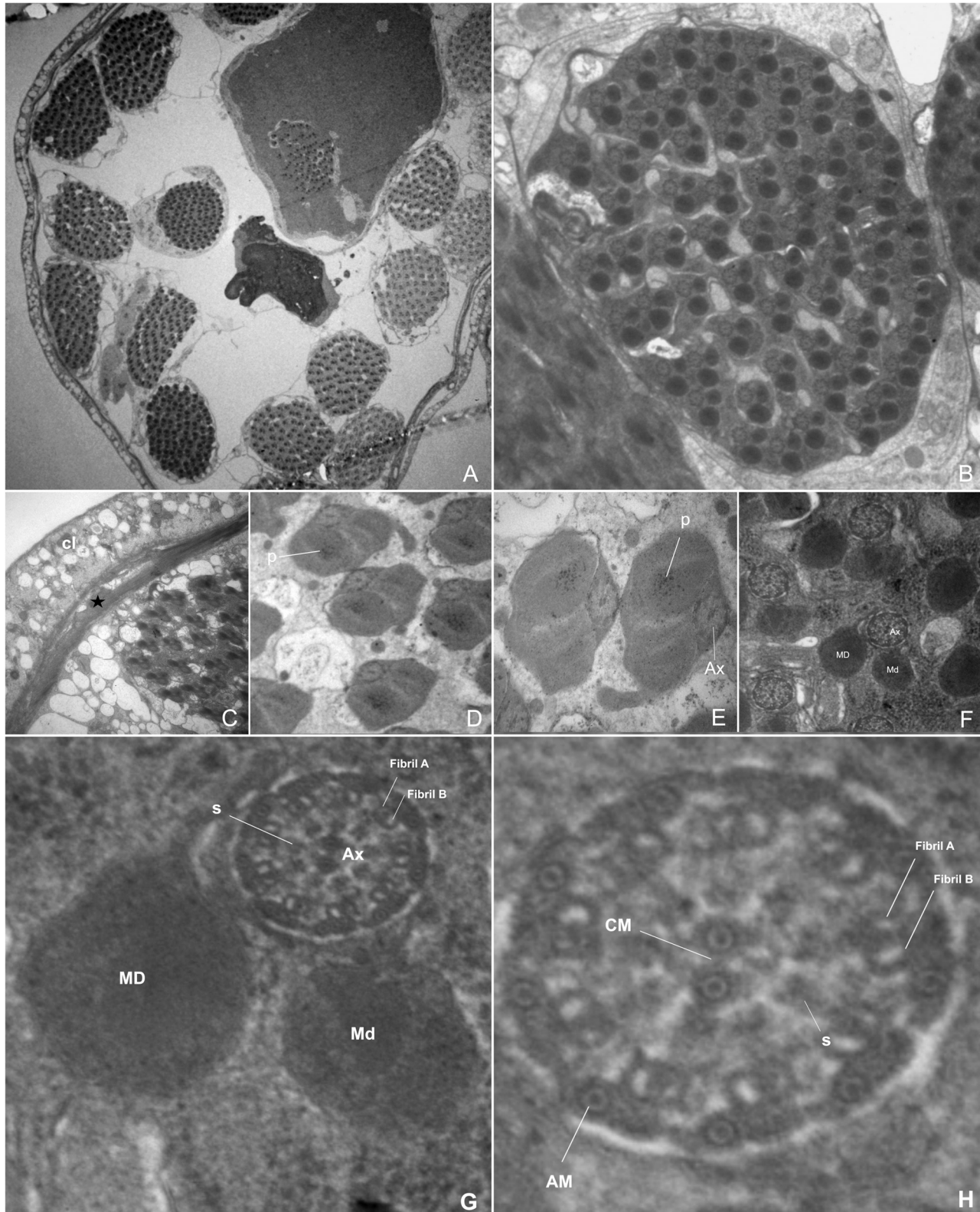
## Discussion

The rapid dispersion of *Z. indianus* across different continents, and especially throughout Brazil, where this species is considered a pest on fig plantations (Stein *et al.*,

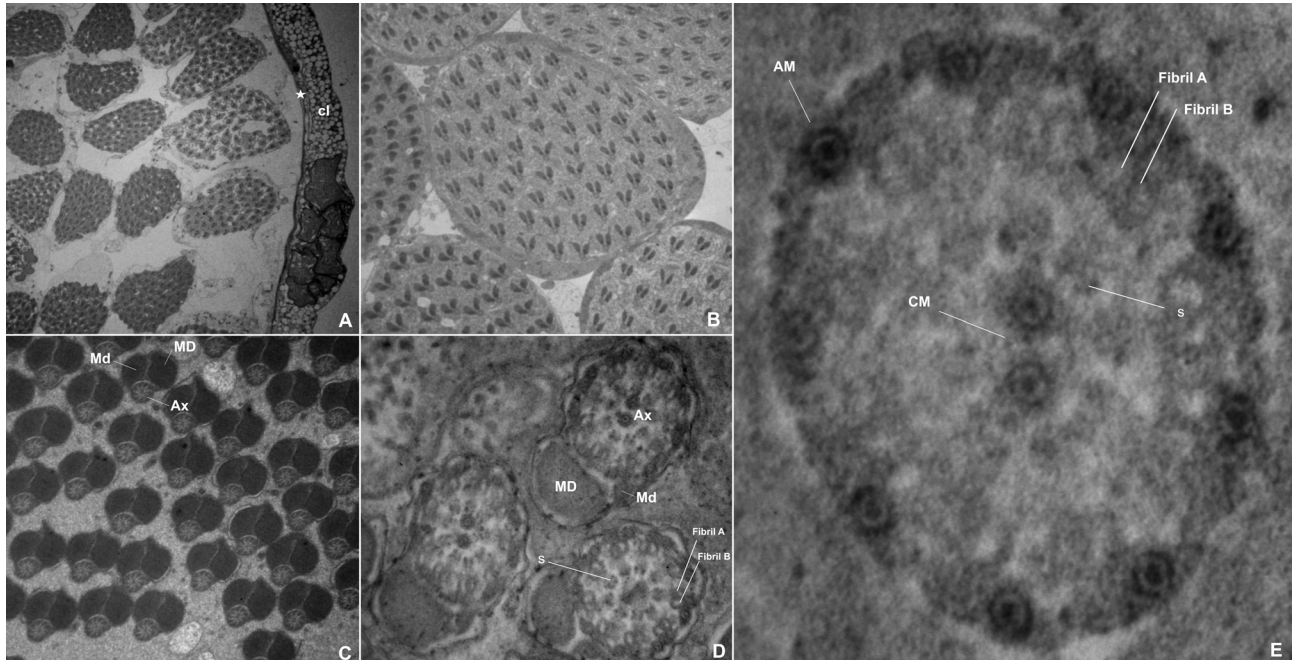


**Figure 3** - Semi-thin histological sections of *Zaprionus indianus* (A) and *Zaprionus sepsoides* (B), showing spermatids (spmt), followed by spermatozoid bundles arranged transversely and longitudinally (z). Other structures are also present in the testes, such as the coating layer (cl), the envelope (en) and glycogen granules (gr) revealed by P.A.S. reaction (note in detail of Figure B). Note these histological sections were 0.5 mm in length and stained with 1% toluidine blue. Scale bars: 10  $\mu$ m.





**Figure 4** - TEM micrographs of spermatozoids (flagellum) of *Z. indianus*. **A.** Transverse section of the testes showing various spermatozoid bundles organized within cysts. **B.** Transverse section of the testes showing a bundle containing 64 spermatozoids in detail. **C.** Transverse section of the testes showing the coating layer (cl) and envelope (star). **D-G.** Transverse sections of a spermatozoid bundle, showing the tail region with axonemes (Ax) and mitochondrial derivatives of different size: larger mitochondrial derivatives (MD) in which the accumulation of paracrystalline material (p) is visible and smaller mitochondrial derivatives (Md). **H.** details of a flagellum showing the 9+9+2 arrangement: one pair of central microtubules (CM), nine double peripheral microtubules (formed by fibril A and fibril B), surrounded by nine additional accessory microtubules (AM) and nine spokes (s). Scale: Figure A: 2000x; Figure B: 10000x; Figure C: 8000x; Figures D, E, F: 67000x; Figure G: 84000x; Figure H: 100000x.



**Figure 5** - TEM micrographs of spermatozoids (flagellum) of *Z. sepsoides*. **A**. Transverse section of the testes showing various spermatozoid bundles organized within cysts. **B**. Transverse section of the testes showing a bundle containing 64 spermatozoids in detail. **C**, **D**. Transverse section of a spermatozoid bundle, showing the tail region with axonemes (Ax) and mitochondrial derivatives of different size: larger mitochondrial derivatives (MD) and smaller mitochondrial derivatives (Md) are visible, beside a coating layer (cl) and an envelope (star). **E**. details of a flagellum, showing the 9+9+2 arrangement: one pair of central microtubules (CM), nine double peripheral microtubules (note fibril A and fibril B), surrounded by nine additional accessory microtubules (AM) and nine spokes (s). Note the accumulation of electron-dense material in the nine accessory microtubules and in the two central microtubules. Scale: Figure A: 2000x; Figure B: 4000x; Figure C: 20000x; Figures D: 67000x; Figure E: 100000x.

2003), has spurred a growing number of studies on its biology and history of invasion. A similar reason motivated the present study on the spermatogenesis of this species in comparison to *Z. sepsoides*, as the two species differ in their ecological characteristics and testicular morphology. We showed that the testes of *Z. indianus* are formed by two coiled tubes of approximately 5 mm in length, whereas the testes of *Z. sepsoides* consist of two tubes of kidney shaped appearance of approximately 2 mm each. These measurements confirm those of Yassin and David (2010) obtained on testes of different species of *Zaprionus*. The *inermis* species can be classified into two categories: those with small testes, ranging from 1.0 to 2.0 mm in length (*Z. sepsoides*, *Z. inermis*, *Z. cercus*, *Z. kolodkinae* and *Z. tsacasi*), and those species with large testes, varying from 5.2 to 5.4 mm in length (*Z. indianus*, *Z. africanus* and *Z. taronus*).

Araripe *et al.* (2004) showed that the anatomy and reproductive physiology of *Z. indianus* differs from *D. melanogaster*. For example, *Z. indianus* sperm are longer (5 mm) than the sperm of *D. melanogaster* (1.8 mm). Joly and Bressac (1994) measured the size of the sperm and testes of several drosophilid species and found that sperm size is approximately 6.7 mm in *Z. indianus* and 0.78 mm in *Z. sepsoides* and that the testicular sizes are 6.53 mm and 1.42 mm, respectively. These measurements correspond to the testes measurements performed in this study.

The sperm bundles in *Z. sepsoides* were shorter than in *Z. indianus*, reflecting its smaller size and thus smaller testes. As frequently reported in the literature, testes are as long as the sperm they produce (Lindsley and Tokuyasu, 1980; Pitnick and Markow, 1994; Snook, 2005; Schärer *et al.*, 2008).

The diploid number of *Z. indianus* and *Z. sepsoides* was confirmed to be  $2n = 12$  chromosomes. In this study, the AgNOR technique could not distinguish the nucleolar organizer regions (NORs) in the meiotic chromosomes. In prophase stage nuclei and in sperm, a NOR was intense protein synthesis in the anterior region, corresponding to the spermatozoid heads. Although this marking has been observed in studies of other organisms (Tavares and Azeredo-Oliveira, 1997; Tartarotti and Azeredo-Oliveira, 1999; Severi-Aguiar and Azeredo-Oliveira, 2005; Morielle-Souza and Azeredo-Oliveira, 2008; Costa *et al.*, 2008; Perquetti *et al.*, 2008, 2010), few studies have examined AgNOR markings in the genus *Zaprionus*. Gupta and Kumar (1987) studied the association of polytene chromosomes with the nucleolus in four distinct Indian populations of *Zaprionus indianus* and observed that different chromosomes, such as X chromosome and microchromosomes, exhibit NOR activity.

In young *Z. sepsoides* males (1-3 days old), cells in the early stages of spermatogenesis, such as spermatocytes I and II, spermatids and a few spermatozooids, were ob-



served more frequently than in young *Z. indianus* males. In contrast, the young *Z. indianus* males presented larger numbers of spermatids and spermatozoa and few cells at the initial stage of spermatogenesis. This finding suggests a difference in sperm maturation dynamics between the two species. In the genus *Drosophila*, spermatocytes enter in the first meiotic division in the pupal stage (Cooper, 1950), and the testes of most species of *Drosophila* are not fully developed at hatching and, with certain variability, continue to mature in the adults (Pitnick and Miller, 2000). The results of the present work indicate that a similar process occurs in *Z. indianus*, because one-day-old males already exhibited significant quantities of sperm bundles, whereas in *Z. sepsoides* such quantities were only found in older males (8 days old).

These results contradict those of Pitnick and Miller (2000), who showed that in *D. hydei*, apparently selected for larger testes, there is an increase in development time (egg to adult) and time of maturation of spermatozoa post-hatching, and this may contribute to a delay in reproduction of this population. In *Z. indianus*, which has larger testes and sperm than *Z. sepsoides*, this does not occur, because young males already present considerable amounts of mature sperm (Madi-Ravazzi, L.; David J., “personal communication”)

The fertility of *Z. indianus* is greater than that of *Z. sepsoides*, at least in laboratory experiments (Madi-Ravazzi, L.; David J., “personal communication”), indicating a difference in the fitness of these species. If the size differences and maturation of sperm from these species favor greater reproductive potential, this phenomenon should be investigated in detail.

The organized distribution of spermatogenic cells observed in *Z. indianus* and *Z. sepsoides* is similar to that of other Drosophilidae (Cooper, 1950; Bairati, 1968; Meyer and Henning, 1974; Hardy *et al.*, 1979; Lindsley and Tokuyasu, 1980; Fuller, 1993; Joly and Bressac, 1994; Gönczy and Dinardo, 1996; Li and Xie, 2005; Scharer *et al.*, 2008; Mojica *et al.*, 2000; Barreau *et al.*, 2008; Cheng and Mruk, 2010).

Another relevant observation was the constant number of 64 sperm per bundle in both *Z. indianus* and *Z. sepsoides*. The mechanism underlying the formation of these bundles is similar to what occurs in *Drosophila* and other insects. In these species, a bundle of sperm is produced by a series of mitotic divisions of the spermatogonia within a spermatid cyst (Cooper, 1950; Hardy *et al.*, 1979; Lindsley and Tokuyasu, 1980; Fuller, 1993; Fabrizio *et al.*, 1998; Mojica *et al.*, 2000).

Although the mitotic divisions within the cysts are synchronous in most insects, in some species of *Drosophila* the divisions can also be asynchronous and the number of sperm per bundle may vary from 32, as in *D. hydei*, to 128, as in *D. pseudoobscura* (Mojica *et al.*, 2000). Similar ob-

servations were made in bees (Cruz-Landim, 2001) and Coleoptera (Name *et al.*, 2007).

According to Virkii (1969), basal orders of insects have more sperm per bundle than the more derived orders, and the more specialized groups tend to exhibit the lowest number of sperm per bundle. Name *et al.* (2007) demonstrated, for example, that certain scarab beetles have 128-512 sperm per bundle and *Sitophilus zeamais* and *S. oryzae* approximately 260 sperm per bundle. Fewer sperm per bundle indicates a reduced production of spermatozoa, which may correspond to a fitness that limits genetic variability (Mojica *et al.*, 2000). Although *Z. indianus* and *Z. sepsoides* have the same number of sperm per bundle (64), there may be different numbers of cysts in these species, which is indicated by a greater abundance of sperm bundles in *Z. indianus* than in *Z. sepsoides*.

The present study confirmed the organizational pattern of the axoneme of insects following the 9+9+2 scheme, which is an arrangement of an internal 9+2 microtubules surrounded by nine additional accessory microtubule. Studies of the structural organization of the axoneme in *Drosophila* have been performed by various researchers (Perotti, 1969; Kiefer, 1970; Phillips, 1970; Dallai and Afzelius, 1991; Dallai *et al.*, 1993; Fabrizio *et al.*, 1998; Mojica *et al.*, 2000), who all confirmed the conservation of this structure in different species of *Drosophila* and the family Drosophilidae. Two mitochondrial derivatives of different size are also present in both *Zaprionus* species. In insects, these organelles have been studied ultrastructurally using a phylogenetic approach and this morphology of mitochondrial derivatives is observed in highly evolved species, being a apomorphic character (Phillips, 1970; Mojica *et al.*, 2000). Some functions of mitochondrial derivatives may be related to the process of storing and releasing energy to power the mobility of the flagellum (Phillips, 1970; Lindsley and Tokuyasu, 1980).

Phylogenetic relationships of the genus *Zaprionus* has been extensively debated (Yassin *et al.*, 2007, 2008, 2010; Yassin and David, 2010) and current reports agree that *Zaprionus* is more closely related to *Drosophila* than to the *Sophopora* subgenera, to which the *melanogaster* group belongs (Russo *et al.*, 1995; Kwiatowski and Ayala, 1999; Da Lage *et al.*, 2007; Commar *et al.*, 2012). The ultrastructural morphology of the mitochondrial derivatives present in *Zaprionus* is very similar to those found in *D. melanogaster*. However, based on the above-cited studies, such similarity is possibly a homoplasy and not informative regarding the phylogenetic relationships of these taxa.

The findings presented here indicate differences in sperm maturation between the species analyzed. These differences may favor the reproductive success and fitness of *Z. indianus* and thus also relate to the invasive potential of the species.



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