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

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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 *Anaprionus* Okada, with approximately 10 species in the Eastern region, and the *Zaprionus* subgenus, 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, David 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. ghesquierei* (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 (La-
chaise and Tsacas 1983, Schmitz et al., 2007). However,
other factors, including aspects of its reproduction, adap-
tation 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 selec-
tion (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 num-
ber of sperm and their developmental stages are also factors
that can contribute to the reproductive potential of the spe-
cies.

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

Material and Methods

Origin of insects

A strain of Z. indiana 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 ± 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 measure-
ments. For such measurements, the spiral testes from Z.
indiana 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 fol-
lowing 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 per-
formed according to Cotta-Pereira et al. (1976), with mod-
ifications. The testes of adult Z. indiana and Z. sepsoides
males were removed at different developmental stages (1,
3, 5 and 8 days old) and fixed in a solution of 4% para-
formaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate
buffer, pH 7.4. After initial fixation for 4 h at 4 °C, the tes-
tes 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 bot-
tle 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 ac-
etone 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 dehydra-
tion, 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 µm sections of the testes were stained
with 1% toluidine blue and analyzed using a light micro-
scope. Ultra-thin, 70 nm sections were contrasted with ura-
nyl 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 spe-
cies, as follows (Mello and Vidal, 1980, with modifica-
tions). A solution of 0.5% periodic acid was applied to the
semi-thin, 0.5 µm 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 dig-
ital imaging software, Version 4.8 for Windows. The mate-
rial prepared using histological techniques was analyzed
and photographed using a JEOL 1011 transmission elec-
tron microscope operated at 80 kV.

Results

Testes morphology of Z. indiana and Z. sepsoides
males

The testes of Z. indiana and Z. sepsoides are yellow
in color (Figures 1A and 1C, respectively). In Z. indiana,
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).
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 spermatozoa 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 inferred 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-

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**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.
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 4 - TEM micrographs of spermatozoa (flagellum) of *Z. indiana*. A. Transverse section of the testes showing various spermatozoa bundles organized within cysts. B. Transverse section of the testes showing a bundle containing 64 spermatozoa 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.
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; Scharer *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 Azevedo-Oliveira, 1997; Tartarotti and Azeredo-Oliveira, 1999; Severi-Aguiar and Azeredo-Oliveira, 2005; Miliardi-Souza and Azeredo-Oliveira, 2008; Costa *et al.*, 2008; Peruquetti *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 spermatozoids, were ob-

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**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 spermatozoid 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. A detail 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.
erved 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 are 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 contributes 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; Gönçzy 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 spermatogenesis within a spermatic 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 observations 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+2+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; Kieler, 1970; Phillips, 1970; Dallai and Azfelius, 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 Zapronius 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 Zapronius has been extensively debated (Yassin et al., 2007, 2008, 2010, Yassin and David, 2010) and current reports agree that Zapronius 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 Zapronius 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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