Research Article

Spermatogenesis and Nucleolar Activity in *Triatoma klugi* (Triatomine, Heteroptera)

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Abstract

Triatoma klugi is a Chagas disease vector in the Rio Grande do Sul State. Triatominae chromosomes are holocentric and sex chromosomes segregation is post-reductional. In this paper we describe the karyotype of male *T. klugi* and a meiotic analysis including the nucleolar behavior during spermatogenesis. Testis cells were analyzed after lacto-acetic orcein and silver nitrate staining. Two autosomes and the heterochromosomes presented nucleolar activity (Ag-NORs) during diplotene-diakinesis. The analysis of metaphase I and II revealed a karyotype with 2n = 20+XY. In metaphase I a prominent nucleolar mass was observed in the cell periphery and small silver grains were detected in metaphase II. During anaphase, the chromosomes segregated in parallel and a typical holocentric late migration behavior was observed. The restoration of the nucleolus was an important feature in this phase. During telophase nucleolar masses persisted and in early spermiogenesis the spermatids presented a small peripheral mass until elongation. The present study is a contribution to the study of chromatin behavior and nucleolar persistence in meiosis.

Introduction

The hematophagous insects belonging to the Triatominae subfamily are best known as triatomines. These arthropods are vectors of the Chagas disease etiological agent, the protozoan *Trypanosoma cruzi (T. cruzi)*. The disease is endemic in South America with pronounced relevance in heart illnesses. It is considered that *T. cruzi* infects about 11 million people in endemic regions (Dias *et al.*, 2002). Chagas disease represents the third most common parasitic affection, after malaria and schistosomiasis (Guzmán-Bracho, 2001).

The Triatominae subfamily includes 137 insect species (Galvão *et al.*, 2003) divided into six tribes: *Alberproseniini, Bolboderini, Cavernicolini, Linshcosteini, Rhodniini, and Triatomini*. Eighteen triatomine genera have been described: *Alberprosenia, Belminus, Bolbodera, Microtriatoma, Parabelminus, Cavernicolai, Linshcosteus, Psammolestes, Rhodnius, Dipetalogaster, Eratyrus, Hermanlentia, Meccus, Mepraia, Nesotriatoma, Paratriatoma, Panstrongylus,* and *Triatoma* (Galvão *et al.*, 2003).

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Triatomines are homogeneous in relation to their hematophagous behavior and all species are potential vectors of Chagas disease (Tartarotti *et al.*, 2004). *Triatoma klugi* (*T. klugi*) is an important vector in sylvan environments and is distributed throughout the state of Rio Grande do Sul. This species is part of the "oliverai complex", which also includes *T. matogrossensis*, *T. williami*, *T. guazu* and *T. jurbergi* (Carcavallo *et al.*, 2001). *Triatoma klugi* specimens susceptible to different lineages of *Trypanosoma cruzi* showed an infection similar to that observed in *Triatoma infestans (T. infestans)* (Joukoski *et al.*, 2000). Under laboratory conditions this species presented an infection rate for the *T. cruzi* lineages 60% higher than previously observed (Emmanuelle-Machado *et al.*, 2002).

Cytogenetic studies revealed that the typical number of chromosomes in Triatominae is 2n = 22, with 20 autosomes and two sex chromosomes (XX, XY) (Ueshima, 1979). Most species have 20 autosomes, except for *Triatoma rubrofasciata* (22A), *Panstrongylus megistus* and *T. nitida* (18A) (Dujardin *et al.*, 2000). Fifty species have had their karyotypes described and 30 of them were shown to possess 2n = 20A+XY (Panzera *et al.*, 1998; Morielle and Azeredo-Oliveira, 2004). The variation in the number of chromosomes was mainly due to the presence of different sex determining systems. The subfamily displayed a frag-

mentation of the males X chromosome and the following systems have been observed: XY, X_1X_2Y and $X_1X_2X_3Y$. These three systems have been observed in the genus *Triatoma* (Panzera *et al.*, 1998). A fourth sex determination mechanism, $X_1X_2Y_1Y_2$, has been recently described in *Mepraia spinolai* (Frías and Atria, 1998).

Triatomines have holokinetic chromosomes, which are characterized by the absence of centromere in the primary chromosome constriction (Pérez *et al.*, 2000). These insects possess a peculiar behavior during meiosis, in which the first meiotic division is reductional for autosomes and equational for the sex chromosomes (González-García *et al.*, 1996).

Several authors have studied the nucleolus organizer regions (NORs) in Heteroptera (Camacho *et al.*, 1985; Fossey and Liebenberg, 1995; Cattani and Papeschi, 2004). In the subfamily Triatominae the nucleolus persists during meiosis (Tartarotti and Azeredo-Oliveira, 1999b; Morielle and Azeredo-Oliveira, 2004). Some studies performed in insects indicated that silver nitrate stained structures could represent kinetochore or centromere material (Suja *et al.*, 1991; Rufas and Gosálvez, 1982). Ultrastructural analysis of *Tricholepidion* (Insecta) spermiogenesis evidenced a lump of diffuse granular material, the related to the centriole in early spermatids (Dallai *et al.*, 2001). This structure resembles the nucleolar bodies present in triatomine spermatids. *Triatoma klugi* has been recently described by Carcavallo *et al.* (2001) and it is morphologically related to *T. oliveirai*, justifying its inclusion in the oliveirai complex (Emmanuelle-Machado *et al.*, 2002). The purpose of this paper was to describe the karyotype of male *Triatoma klugi* and to analyze the chromosomes meiotic behavior and nucleolar cycle during spermatogenesis.

Materials and Methods

Specimens of *Triatoma klugi* (subfamily Triatominae, family Reduviidae, order Heteroptera; Carcavallo *et al.*, 2001) were supplied by the Araraquara Special Health Service (SESA), part of the Epidemiology Department of the Public Health Faculty of University of São Paulo (USP).

Triatoma klugi is found in gaps between rocks and is distributed throughout the district of Nova Petrópolis, in the state of Rio Grande do Sul. These insects are predominantly black; with a central yellow spot on each connexivum segment (Carcavallo *et al.*, 2001).

We analyzed testes from 20 young adults. After the seminiferous tubules were squashed, part of the material was stained with lacto-acetic orcein (De Vaio *et al.* 1985) and part was submitted to silver nitrate staining (Howell and Black, 1980). Measurements of the sex chromosomes were performed with the Image Tool version 3 for Windows (UTHSCSA, 1995/2002) in ten metaphase I cells. The photomicrographs were taken in Zeiss-Jenaval photomicroscope.

Results

Karyotype and meiosis of *Triatoma klugi*

Triatoma klugi males (Figure 1a) presented a karyotype with 2n = 22, consisting of 20 autosomes and a XY pair (Figure 1b). The Y chromosome (2,21 ± 0,12 µm) was larger than the X (1,85 ± 0,16 µm). The polyploid nuclei of nutritious cells from the seminiferous tubules walls presented on average five positive heteropycnotic corpuscles (Figure 2a). In spermatogonial interphase cells, six positive heteropycnotic corpuscles were observed (Figure 2b). In the diffuse stage of prophase I, the nuclei had one or two positive heteropycnotic corpuscles, probably formed by sex chromosomes (Figure 2c-d), and were more compacted during the diffuse phase (Figure 2 e-f). During diplotene-diakinesis autosome bivalents presented chiasmata and the sex chromosomes were highly condensed (Figure 2g). At metaphase I the ten autosome bivalents and the sex chromosomes were arranged on the metaphase plate (Figure 3a) and usually disposed in a ring configuration (Figure 3b). In metaphase II, ten autosomes and one sex chromosome were present (Figure 3c-d). During anaphase II, late migrating chromosomes were seen (Figure 3e-f), which were sometimes also observed later at telophase II (Figure 3g). Positive heteropycnotic corpuscles were observed in early spermatids, but not in (Figure 3h) cells at more advanced differentiation stages (Figure 3i).

Silver nitrate staining

After silver nitrate staining the polyploid nuclei of nutritious cells from the seminiferous tubules walls presented a central nucleolus (Figure 4a). Chromosomes joined by chromatin filaments were observed at spermatogonial metaphases (Figure 4b), while a central/lateral nucleolus and small nucleolar bodies could be seen in spermatocytes (Figure 4c). Dispersed nucleolar masses were present at the beginning of the diffuse stage (Figure 4d) and during diplotene-diakinesis the sex chromosomes sometimes presented an active NOR (Figures 4e-g). At these same stages NORs were detected on two autosome bivalents (Figure 4g) or, alternatively, a prominent NOR was visible in one autosome bivalent (Figure 5a); in both situa-
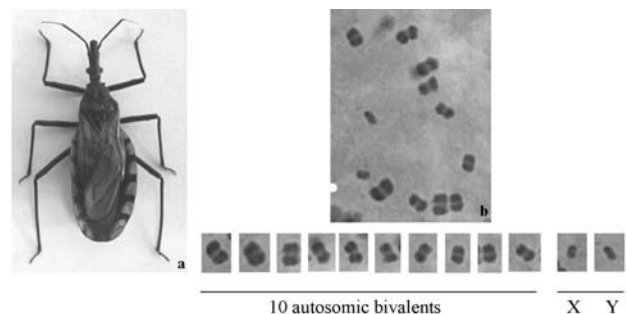
Figure 1 - a) *Triatoma klugi male*; b) Metaphase I and karyotype with ten bivalent autosomes and two sex chromosomes (XY).
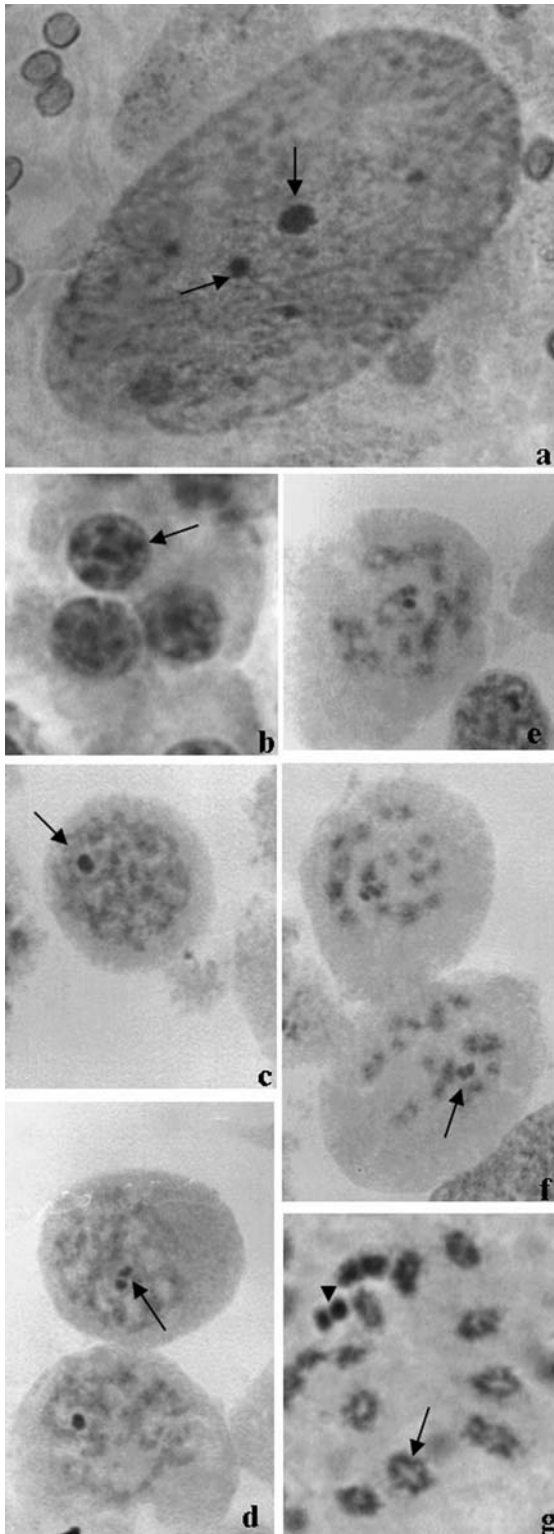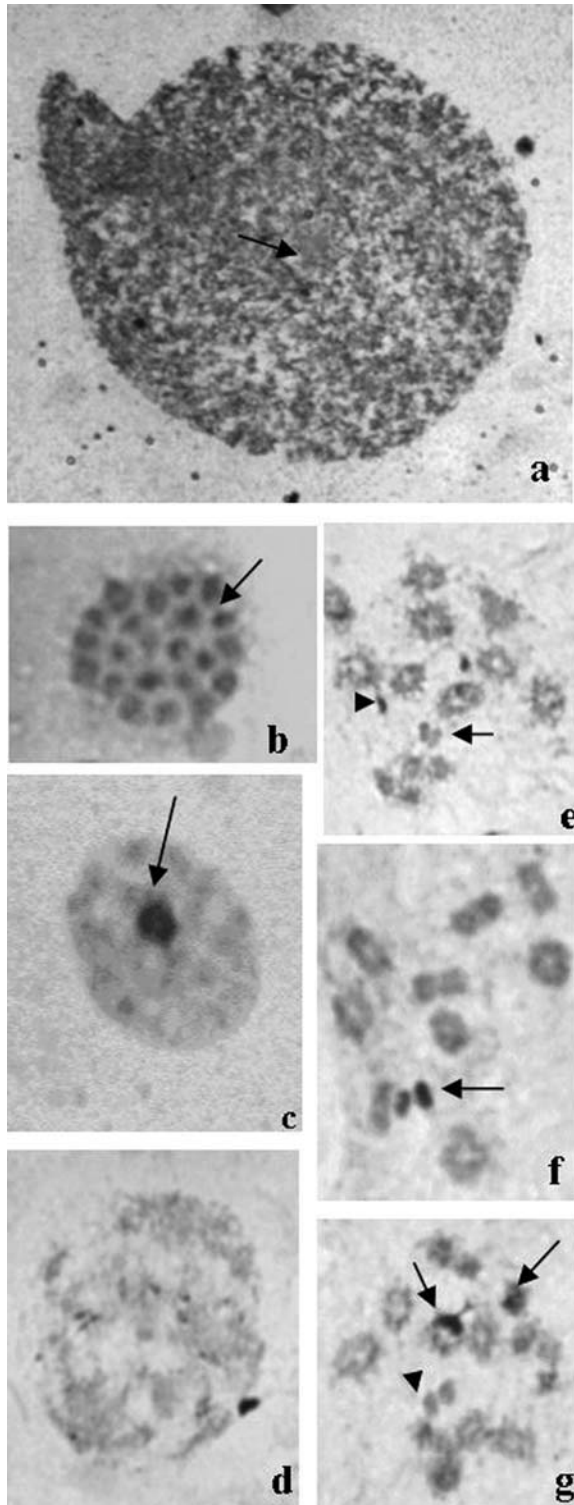
Figure 2 - Testicular tubules of *Triatoma klugi* after lacto-acetic orcein staining. (a) Polyploid nuclei with on average five heteropycnotic corpuscles, arrows indicate corpuscles; (b) Spermatogonial interphase cells with six pycnotic corpuscles, the arrow indicates the corpuscles; (c-d) Nuclei at the beginning of prophase I (diffuse stage) presenting one or two condensed corpuscles formed by the sex chromosomes, arrows indicate corpuscles; (e-f) Nuclei in early prophase I, sexual corpuscle is indicated by arrow; (g) Nucleus in diplotene-diakinesis, arrow indicates bivalent autosomes and the arrowhead points to the pseudobivalent sex chromosomes.
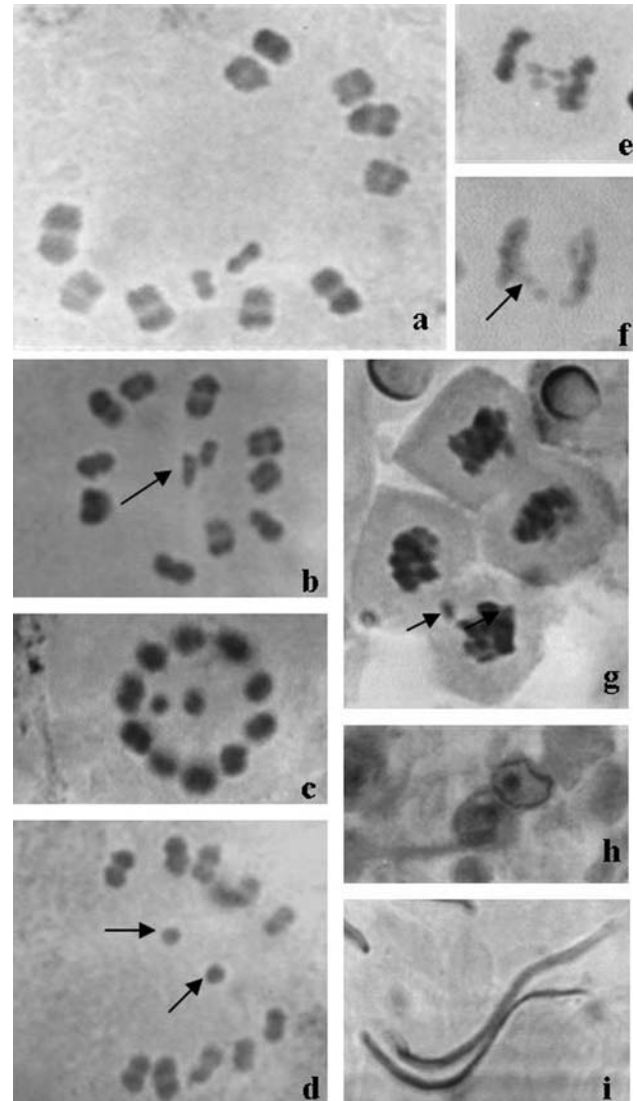
Figure 3 - Testicular tubules of *Triatoma klugi* after lacto-acetic orcein staining: (a-b) Metaphase I with ten bivalent autosomes and two sex chromosomes indicated by the arrow; (c-d) Metaphase II with equational division of the sex chromosomes shown by arrows. Note that in (c) the chromosomes are arranged in a ring; (e-f) Anaphase with late chromosomes migration, the arrow indicates parallel chromosome migration; (g) Telophase II with four gametic cells, the arrow indicates late migrating chromosomes; (h) Spermatids with heteropycnotic corpuscles in early spermiogenesis, (i) Sperm cells at the advanced differentiation stage.

tions the sex chromosomes presented inactive NORs (Figure 4g and 5a). A large peripheral nucleolar mass was observed during metaphase I (Figure 5b) and small silver stained corpuscles were common during metaphase II (Figure 5c). In this phase, at least one autosome presented an active NOR (Figure 5d). At anaphase, the chromosomes segregated to the poles in a parallel configuration and were late migrating, as it is typical for holokinetic chromosomes (Figure 5e). In this stage a prominent nucleolar mass reappeared (Figure 5f) and at telophase nucleolar remnants could be detected (Figure 5g). Early spermatids also had a small peripheral nucleolar mass (Figure 5h) and during the

Figure 5 - Silver stained testis tubules of *Triatoma klugi*: (a) Diplotene-diakinesis, the arrow indicates a silver stained autosome bivalent; (b) Metaphase I; the arrow indicates a large remaining nucleolar mass; (c) Metaphase II, the arrows show dispersed nucleolar masses; (d) Metaphase II with one silver stained autosome shown by arrow; (e) Anaphase I, observe the parallel migration of chromosomes; (f) Anaphase I, note the parallel chromosome disposition, the arrow indicates a prominent nucleolar mass; (g) Telophase I, note the nucleolar masses in both daughter cells; (h) Spermatids in early spermiogenesis with peripheric nucleolar mass shown by arrow; (i) Spermatids in the elongation stage with nucleolus shown by arrow.

observed in *Rhodnius domesticus* (Morielle and Azeredo-Oliveira, 2004), *Panstrongylus megistus* and *P. herreri* (Tartarotti and Azeredo-Oliveira, 1999a).

Late chromosome migration was observed during meiotic anaphase in *T. klugi*, a phenomenon also described in *Panstrongylus herreri* (Tartarotti and Azeredo-Oliveira, 1999a), *T. brasiliensis, T. delpontei, T. lecticularia, T. rubrovaria* and *T. sordida* (Tavares and Azeredo-Oliveira, 1997). Late migrating chromosomes can appear either spontaneously or as a consequence of radiation in somatic or germinative cells (Hughes-Schrader and Schrader, 1961).

In *T. klugi* an interaction between microtubules and chromosome ends was specifically seen at anaphase I (Figure 1f-g). The kinetic activity was located at one extremity of the chromatids because the migration is parallel to the spindle axis. This phenomenon was also detected in *T. infestans* suggesting that the interaction of microtubules during meiosis possesses at least two steps: first, an initial holocentric interaction which determines the stabilization of chromosomes; and second, a restricted interaction towards the end that involves segregation (Pérez *et al.*, 2000).

The restriction of kinetic activity to chromosome ends is a general characteristic of holocentric chromosomes. It has been suggested that these characteristics can facilitate the meiotic processes, such as crossing over, chiasmata terminalization and bivalent orientation on the metaphase plate (Pimpinelli and Goday, 1989).

In *T. klugi* the chromosomes behave as bivalents in metaphase I even after chiasmata terminalization. In Heteroptera bivalents usually display a single chiasma in any chromosome region and its presence persists beyond metaphase I (Pérez *et al.*, 1997). The sex chromosomes of *T. klugi* were univalent and pseudobivalent at metaphase I and were predominantly univalent at metaphase II. The sex chromosomes appeared as univalents at metaphase I of the *Antiteuchus* species of heteropterans, while in metaphase II they were pseudobivalents (Lanzone and Souza, 2006). Nevertheless, a pseudobivalent XY was observed in both the first and second meiotic divisions in the Heteroptera *Graphosoma italicum* (González-García *et al.* 1996).

Silver staining revealed impregnated bodies in the polyploid nuclei of *T. klugi*. Nucleolar bodies have also been observed in testis polyploid nuclei of *Panstrongylus megistus* and *P. Herreri* (Tartarotti and Azeredo-Oliveira, 1999b). The increase in nucleolar bodies in polyploid nuclei may be related to increased rRNA synthesis (Tavares and Azeredo-Oliveira, 1997).

During meiotic prophase and metaphases I and II of *T. klugi*, an alternation of ribosomal genes activity between autosomes and sex chromosomes was observed. The same phenomenon has already been described *P. megistus* and *P. herreri* (Tartarotti and Azeredo-Oliveira, 1999b) and indicates that not all ribosomal genes are necessarily active. The presence of NORs in heterochromosomes is a common

phenomenon in insects and several *Drosophila* species, for instance, have ribosomal genes located on their sex chromosomes (Bicudo, 1984).

Dispersed nucleolar bodies were observed at metaphase II in *T. klugi* and a large nucleolar mass was also observed during metaphase I, anaphase I and telophase I. This confirms the occurrence of "nucleolar material persistence" as suggested by Tartarotti and Azeredo-Oliveira (1999b) and provides evidence that the nucleolus does not completely disappear during meiosis in triatomines, but is reorganized from pre-existing nucleolar elements.

Nevertheless, some authors suggest that silver nitrate impregnation may also reveal other chromosome structures. Silver staining of *Schistocerva gregaria* (Orthoptera) early spermatids apparently revealed kinetochore or centromere material, while silver stained structures in elongated spermatids were attributed to a particular kind of condensed chromatin. Another study performed during meiosis in Orthopera led to the conclusion that silver staining reveals three distinct chromosome structures: (i) active nucleolar organizer regions (ii) kinetochores and (iii) cores that run along each chromatid except at their distal ends (Suja *et al.*, 1991).

In *T. klugi*, silver staining observed in early spermatids disappears during elongation. Post-meiotic NORs reactivation has already been observed in mammalian cells submitted to silver staining (Hofgartner *et al.*, 1979). It is possible that the conservation of post-meiotic NORs reactivation in vertebrates and invertebrates indicates the need for post-meiotic RNA synthesis related to differentiation. Alternatively, the silver stained structures seen in spermatids may represent he centriole adjunt observed in ultrastructural studies of spermiogenesis. An electron microscope analysis of *Tricholepidion* (insecta) showed that, after meiosis is completed, the spermatid has only a single centriole located close to the nucleus. Also connected to the centriole is a lump of diffuse granular material, named the centriole adjunt. The terms "centriole adjunt" or "postnuclear body" have been given to aggregates of granular masses, which initially accumulate around the centriole and that are believed to differentiate later into various sperm components (Dallai *et al.*, 2001).

This study presents the description of the karyotype of male *Triatoma klugi* and contributes to a better understanding of chromatin and nucleolar behavior during meiosis.

Acknowledgments

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