

Cytochemical study of *Rhodnius neglectus* and *Rhodnius prolixus* salivary gland cells (Hemiptera, Triatominae)

Ana C. B. M. Anhô, Ana P. M. Lima-Oliveira & Maria T. V. Azeredo-Oliveira

Departamento de Biologia, Instituto de Biociências, Letras e Ciências Exatas – Universidade Estadual Paulista “Júlio de Mesquita Filho” – IBILCE/UNESP, São José do Rio Preto, SP, Brazil.
(Corresponding author: anacbanhe@yahoo.com.br)

ABSTRACT. Triatomines are hematophagous bugs of medical interest in South and Central America, where they may act as invertebrate hosts of the hemoflagellate protozoa *Trypanosoma cruzi* (the causative of Chagas’ disease) and *Trypanosoma rangeli* (Tejera, 1920). Triatomines of *Rhodnius* genus have salivary gland formed by two close and independent units: the principal and the accessory. This gland secretes saliva that abounds in substances that facilitate and permit feeding. Despite this importance, there are few reports on its cytochemistry. In purpose of amplifying this understanding, in this work it was investigated the nuclear structures (chromatin and nucleolar corpuscles) of salivary gland cells of *Rhodnius neglectus* (Lent, 1954) and *Rhodnius prolixus* (Stål, 1859). The salivary glands were removed from adult insects, fixed and submitted to different cytochemical methods: lacto-acetic orcein, silver ion impregnation, Feulgen reaction, Toluidine Blue, Variant method of critical electrolyte concentration and C-banding. The results evidenced predominance of binucleated cells, with bulky and polyploid nucleus, decondensed chromatin and a large nucleolar area. In addition, cytoplasmic metachromasy and a clear association between nucleolar and heterochromatic corpuscles were observed. Such characteristics were associated with intense synthesis activity to produce saliva. Besides, the heterochromatic corpuscles observed with C Banding permitted the differentiation of sexes and species.

KEYWORDS. Gland, morphology, chromatin, nucleolus, nucleus.

RESUMO. Estudo citoquímico de células da glândula salivar de *Rhodnius neglectus* e *Rhodnius prolixus* (Hemiptera, Triatominae) Os triatomíneos são insetos hematófagos que apresentam interesse médico nas América do Sul e Central por serem hospedeiros invertebrados dos protozoários hemoflagelados *Trypanosoma cruzi* (agente etiológico da doença de Chagas) e *Trypanosoma rangeli* (Tejera, 1920). Os triatomíneos do gênero *Rhodnius* possuem glândula salivar formada por duas unidades próximas e independentes: a principal e a acessória. Esta glândula secreta uma saliva que contém substâncias que facilitam e permitem a hematofagia. Entretanto, apesar da sua importância, poucos estudos realizaram sua análise citoquímica. No propósito de ampliar esse entendimento, neste trabalho foram investigadas as estruturas nucleares (cromatina e corpúsculos nucleolares) das células da glândula salivar de *Rhodnius neglectus* (Lent, 1954) e *Rhodnius prolixus* (Stål, 1859). As glândulas foram removidas de insetos adultos, fixadas e submetidas a diferentes métodos citoquímicos: Orceína Lacto-Acética, Impregnação por Íons de Prata, Reação de Feulgen, Azul de Toluidina, Variante da Concentração Eletrolítica Crítica e Bandamento C. Os resultados evidenciaram predomínio de células binucleadas, com núcleos volumosos e poliplóides, cromatina descondensada e uma grande área nucleolar. Além disso, foi observada metacromasia citoplasmática e uma clara associação entre os corpúsculos nucleolares e heterocromáticos. Tais características foram associadas às atividades de intensa síntese proteica para a produção de saliva. Além disso, os corpúsculos heterocromáticos observados no Bandamento C permitiram a diferenciação dos sexos e das espécies.

PALAVRAS-CHAVE. Glândula, morfologia, cromatina, nucléolo, núcleo.

Triatomines are vector of *Trypanosoma cruzi* (Chagas, 1909), the etiological agent of Chagas disease. In Latin America, it has been estimated that there are 15–17 million people infected and 90–100 million are exposed to infection (COURA & BORGES-PEREIRA, 2010). *Rhodnius* genus is also vector of *Trypanosoma rangeli* (Tejera, 1920), a protozoon transmitted by the bite and apparently harmless to humans. It is pathogenic to the insect vector (HECKER *et al.*, 1990).

Triatomines are hematophagous insects throughout the lifecycle. During the blood feeding (probing and engorgement phases), saliva is released (SOARES *et al.*, 2006). It is enriched with molecules that antagonize host’s hemostatic, inflammatory and immunological responses, facilitating feeding (RIBEIRO & FRANCISCHETTI, 2003). This saliva, in association with cryptic behavior, nocturnal habits of triatomines and mechanical adaptations of mouthparts, allows few perceivable bites thus enabling a successful blood meal.

Saliva is produced by a salivary gland formed by

two close and independent units: the larger is reddish and elongated (principal unit) and the smaller is round and translucent (accessory unit) (BAPTIST, 1941; REIS *et al.*, 2003). Ultrastructural analysis showed abundant endoplasmic reticulum, mitochondria and microvilli in the apical plasma membrane domain, thus enlarging the membrane area available for saliva secretion (MEIRELLES *et al.*, 2003; REIS *et al.*, 2003). In addition, an intense acid phosphatase activity was found in nucleolus, suggesting the activity of this enzyme during rRNA molecule transcription (ANHÊ *et al.*, 2007).

Recently, ROCHA *et al.* (2010) developed a primary culture of *Rhodnius prolixus* (Stål, 1859) salivary gland cells. Three types of cells were identified, mostly binucleated. In addition, some secretion vesicles were observed budding from duct cells, suggesting a possible secretory role of this structure.

Despite its importance, the cytochemical characteristics of this gland are little known. So, the purpose of this work is to describe nuclear structures (chromatin

and nucleolus) of salivary gland cells of two species of *Rhodnius*: *Rhodnius neglectus* (Lent, 1954) and *Rhodnius prolixus*. Besides, some aspects that allow comparing and differentiating sexes and species are described.

MATERIAL AND METHODS

Salivary glands of *R. neglectus* and *R. prolixus* of both sexes were used in this study. They were from Frutal (state of Minas Gerais, Brazil) (*R. neglectus*) and Colombia (*R. prolixus*) and were obtained from the Insectary of the Araraquara Special Health Service, organ of the São Paulo University (Araraquara, state of São Paulo, Brazil). Forty newly-emerged adults (twenty males and twenty females) of each species were analyzed. They fed on mice and were dissected seven days after feeding.

For cytochemical characterization, triatomines were dissected in Demerec solution. Salivary glands were fixed in acetic acid (45%) and lactic acid (50%), squashed and submitted to the following cytochemical methods: lacto-acetic orcein (DE VAIO *et al.*, 1985 - with modifications), silver ion impregnation (HOWELL & BLACK, 1980 - with modifications), Feulgen reaction (MELLO & VIDAL, 1980 - with modifications), Toluidine Blue, Variant method of critical electrolyte concentration (CEC) (MELLO *et al.*, 1993 - with modifications) and C-banding (SUMNER, 1972 - with modifications). The preparations were examined with a photomicroscope and photographed, after which the negatives were developed and printed in specialized laboratories.

RESULTS

The techniques described nuclear structures of salivary gland cells of both sexes of *R. neglectus* (Figs 1-12) and *R. prolixus* (Figs 13-24). Bulky and polyploid nuclei were observed with a predominance of binucleated cells.

Orcein (Figs 1, 7, 13, 19), Feulgen reaction (Figs 3, 9, 15, 21), Variant of CEC (Figs 5, 11, 17, 23) and C-banding (Figs 6, 12, 18, 24), techniques specific to chromatin, highly stained euchromatin as a thin granulation dispersed in the nucleus (decondensed chromatin). A unique heterochromatic corpuscle was observed just on males (Figs 1, 3, 4, 6, 13, 15, 16, 18). However, the technique of C Banding showed, in *R. prolixus*, a lot of heterochromatic corpuscles in both sexes (Figs 18, 24). Light halos were also observed in association with heterochromatin (characteristic of nucleolar corpuscles). These halos were highly stained by silver ion impregnation, specific for nucleolar corpuscles. They were small, circular and numerous: about 50 corpuscles in both sexes of *R. prolixus*, while in *R. neglectus* were observed about 60 in females and 30 in males.

Variant of CEC showed blue nucleolar corpuscles surrounded by green chromatinic halos (Figs 5, 11, 17, 23). A solution of Toluidine Blue (without magnesium chloride - MgCl₂) was used for the control technique, in which cells

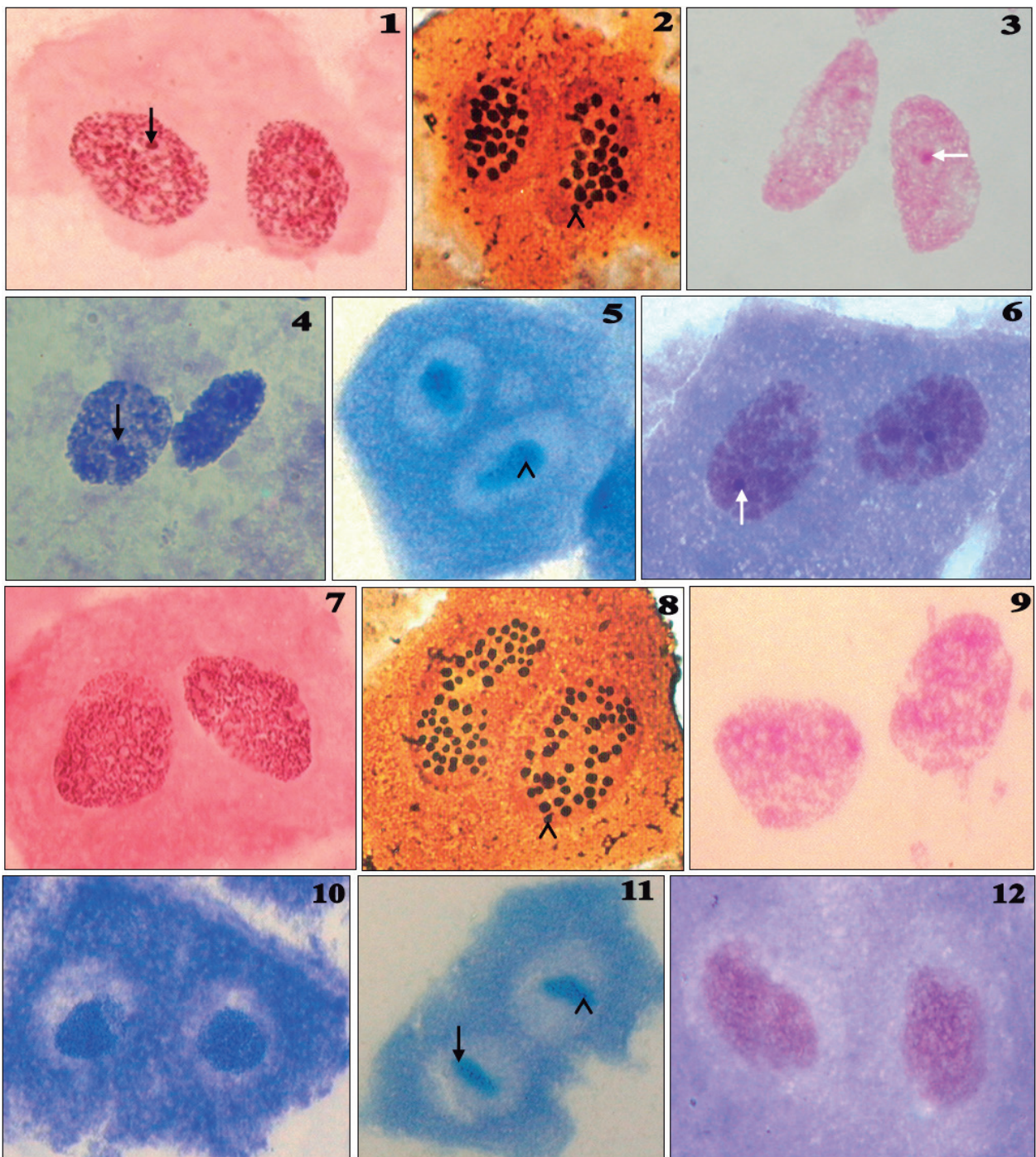
showed a metachromatic blue in all components (nuclear and cytoplasmic ones) (Figs 4, 10, 16, 22). Cytoplasm was mainly stained by the techniques of silver ion impregnation (Figs 2, 6, 14, 20), Toluidine Blue (Figs 4, 10, 16, 22) and Variant of CEC (Figs 5, 11, 17, 23).

DISCUSSION

Cytochemical techniques evidenced some characteristics of nuclei (chromatin and nucleolus) of salivary gland cells of *R. neglectus* and *R. prolixus* (both sexes). They showed a predominance of two bulky and polyploid nuclei in each cell, as observed in salivary gland of *Triatoma infestans* (Klug, 1834) and *Panstrongylus megistus* (Burmeister, 1835) (ANHÊ & AZEREDO-OLIVEIRA, 2008). According to BARTH (1954), this increasing of nuclear mass may provide the intense metabolism, essential to accelerate and regulate cellular regeneration after saliva secretion. Polyploidy is a common phenomenon in many insect tissues, as midgut, epidermis, fat body, Malpighian tubules, trachea and ovary follicle (EDGAR & ORR-WEAVER, 2001). Binucleated cells are described for salivary gland of other Hemiptera, as *Cimex hemipterus* (Fabricius, 1803) (SERRÃO *et al.*, 2008), *Rhynocoris marginatus* (Fabricius, 1794) (KUMAR & SAHAYARAJ, 2012), *Supputius cincticeps* (Stal, 1860) (DE CASTRO *et al.*, 2013) and *Podisus nigrispinus* (Dallas, 1851) (MARTÍNEZ *et al.*, 2014).

Orcein (for proteins-DNA complexed), Feulgen reaction (specific to DNA) and C-Banding (for constitutive heterochromatin), which are specific for chromatin staining, showed a predominance of decondensed chromatin, as already described for salivary glands of other Hemiptera (LACOMBE, 1999; ANHÊ & AZEREDO-OLIVEIRA, 2008; SERRÃO *et al.*, 2008; KUMAR & SAHAYARAJ, 2012; DE CASTRO *et al.*, 2013; MARTÍNEZ *et al.*, 2014). These characteristics indicated increasing nuclear mass in relation to the cytoplasm and increasing metabolic activity (LI *et al.*, 2007), necessary to produce saliva.

In addition, techniques permitted the differentiation of sexes, since just on males a unique heterochromatic corpuscle was observed. PANZERA *et al.* (1992) proposed a way of naming the sex chromosomes according to their C-heterochromatin: the Y is the heterochromatic sex chromosome. Thus, the heterochromatic corpuscle observed just on males, probably, corresponds to the Y chromosome and, so, justify its absence in females. Similar results were found in other species of triatomines. In *T. infestans*, a heterochromatic body called chromocenter (MELLO, 1971, 1975) is characteristic of males (ANHÊ & AZEREDO-OLIVEIRA, 2008) and corresponds to the packed three largest autosomes and the sex chromosome (SOLARI, 1979; PANZERA *et al.*, 1992; PEREZ *et al.*, 1997). In *P. megistus*, just males presented a heterochromatic corpuscle (ANHÊ & AZEREDO-OLIVEIRA, 2008), which is characteristic of Y chromosome and can also be present in X1 and X2 chromosomes (TARTAROTTI & AZEREDO-OLIVEIRA, 1999). In addition, C-Banding permitted the differentiation of the

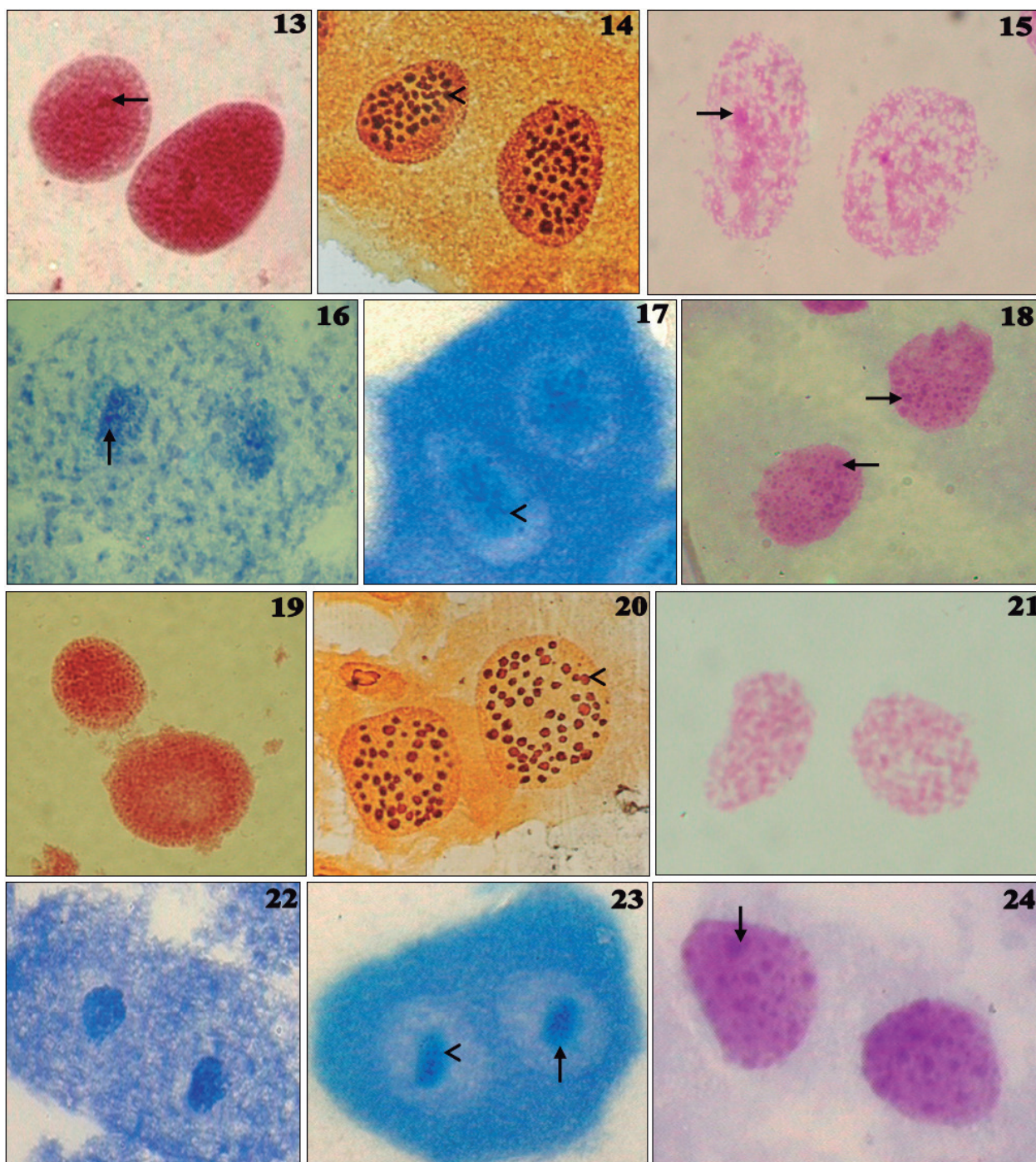


Figs 1-12, Polyloid nuclei of salivary gland of males (1-6) and females (7-12) of *Rhodnius neglectus* (Lent, 1954), stained by the following cytochemical methods: 1, 7, lacto-acetic orcein; 2, 8, silver ion impregnation; 3, 9, Feulgen reaction; 4, 10, Toluidine Blue; 5, 11, Variant method of critical electrolyte concentration; 6, 12, C-banding. Arrows indicate heterochromatic corpuscles and arrowhead the nucleolar ones. Notice the presence of heterochromatic corpuscle on males (1, 3, 4, 6). Magnifications: 1344x.

species of *Rhodnius* genus, since just *R. prolixus* showed a lot of heterochromatic corpuscles.

For nucleolar staining, silver ion impregnation was used. This technique stains proteins present in nucleolar bodies during interphase, as nucleolin (C23) and numatrin (B23) (OCHS *et al.*, 1983). The technique evidenced, in both species, numerous and small nucleolar corpuscles representing a large nucleolar area. Since fully active

nucleoli are large (NANYA & BICUDO, 1995; CARMO-FONSECA *et al.*, 2000), it can be suggested that this large nucleolar area was related with high cellular metabolism. Large nucleolar area was described for salivary glands of other Hemiptera (LACOMBE, 1999; ANHÊ & AZEREDO-OLIVEIRA, 2008; SERRÃO *et al.*, 2008; KUMAR & SAHAYARAJ, 2012; DE CASTRO *et al.*, 2013; MARTÍNEZ *et al.*, 2014). A previous study showed acid phosphatase activity in salivary gland cells of



Figs 13-24, Polyloid nuclei of salivary gland of males (13-18) and females (19-24) of *Rhodnius prolixus* (Stål, 1859), stained by the following cytochemical methods: 13, 19, lacto-acetic orcein; 14, 20, silver ion impregnation; 15, 21, Feulgen reaction; 16, 22, Toluidine Blue; 17, 23, Variant method of critical electrolyte concentration; 18, 24, C-banding. Arrows indicate heterochromatic corpuscles and arrowhead the nucleolar ones. Notice the presence of heterochromatic corpuscle on males (13, 15, 16, 18) and some on females (1). Magnifications: 1344x.

R. neglectus and *R. prolixus*. The most intense activity was found in chromatin and in nucleolus (ANHÊ *et al.*, 2007).

For both nucleolar and chromatin staining, the technique of Variant of CEC was used (MELLO *et al.*, 1993). In this technique, slides were first treated with a Toluidine Blue solution, which stains DNA and RNA in blue (control technique). After, the material was submitted to a magnesium chloride 0.05M solution until the metachromasy of DNA be abolished and it stained in green, while RNA

continues bluish. So, the results showed small nucleolar corpuscles (bluish) surrounded by halos of heterochromatin (greenish), which confirmed association between DNA and RNA, proposed by other techniques utilized. This very close association, classically observed in other tissues and organisms, evidences the functional relationship between these structures, very important to the maintenance of nucleolar architecture (CARMO-FONSECA *et al.*, 2000). Staining with just Toluidine Blue was employed as control

for the technique, when DNA and RNA showed the same metachromatic blue coloration.

Besides, different forms and sizes of nuclei were observed, which could be related to the saliva secretion. According to BARTH (1954), there are four to six phases, including transport of substances, secretion of the saliva and cell regeneration. BARTH (1954) and LACOMBE (1999) also observed that the insect nutrition changes the cell morphology. To avoid this variable, in this study the insects were dissected seven days after feeding.

Cytoplasmic staining evidenced by the techniques of silver ion impregnation, Toluidine blue and variant of CEC indicated the presence of cytoplasmic RNA (mRNA, tRNA and rRNA). This suggested the high protein synthesis in the gland, necessary to produce saliva.

Thus, these results clearly suggest that polyploid binucleated cells with bulky nuclei, large nucleoli and the high metachromasy in cytoplasm were related to the intense activity of salivary gland cells. These characteristics reflect the high protein synthesis, necessary to the composition of saliva and contribute to a successful blood feeding. In addition, chromatin staining techniques enable to differentiate the sexes and the species.

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