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Histone acetylation and methylation marks in chromatin of *Panstrongylus megistus* (Hemiptera, Reduviidae)

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ABSTRACT

Panstrongylus megistus, a potential vector of Chagas disease, currently occupies a wider geographic distribution in Brazil than *Triatoma infestans*, another member of the hemipteran Reduviidae family and a vector of the same disease. A small heterochromatic body (chromocenter) formed by the Y chromosome is evident in the somatic cells of *P. megistus*, differing in size and chromosome type contribution from the well-studied chromocenters present in *T. infestans*. While the overall distribution of histone epigenetic marks differ when comparing the heterochromatin and euchromatin territories in *T. infestans*, no similar data have been established for other hemipteran reduviids, including *P. megistus*. In the present work, histone acetylation and methylation marks were investigated in cells of Malpighian tubules of *P. megistus* 5th instar nymphs using immunocytochemical assays and compared to previously published data for *T. infestans*. Although similarities between these species were found regarding absence of acetylated H3K9, H4K8 and H4K16, and H3K9me and H3K9me$_2$ in the chromocenter, presence of these marks in euchromatin, and presence of H3K9me$_3$ in the chromocenter, no intimate association of acetylated H4K8 and 18S rDNA was revealed in the chromocenter of *P. megistus*. The elevated abundance of H3K9me$_2$ marks at the nuclear periphery in *P. megistus* cells, differing from data for *T. infestans*, is suggested to reflect differences in the interaction of lamina-associated chromatin domains with the nuclear lamina, methyl-transferase modulation and/or association with the last DNA endoreplication step in 5th instar nymphs, which is a matter for further investigation.

1. Introduction

Panstrongylus megistus (Burmeister) is a blood-sucking insect belonging to the hemipteran Reduviidae family. It has been in the focus of several investigations not only because of its role as potential vector of Chagas disease (Forattini, 1980; Perlowagora-Szumlewicz et al., 1988; Garcia et al., 2011) and wide geographic distribution in 22 out of 27 states in Brazil (Jurberg et al., 2015), but also because of its special biological characteristics. *P. megistus* developed ability to colonize artificial ecotopes, resistance to heat/cold shocks and long fasting periods, and tolerance to sequential insults by stressors (Forattini, 1980; Garcia et al., 2001a,b, 2002, 2003). Holokinetic (holocentric) chromosomes, high levels of somatic polyploidy, unusual meiosis process and nuclear and cell fusion under stress conditions are cell biology characteristics reported for this species, which contains 9 autosomal pairs and X_1X_2Y sex chromosomes in males and $X_1X_1X_2X_2$ sex chromosomes in females (Schreiber and Pellegrino, 1950; Barth, 1956; Mello, 1975; Mello and Raymundo, 1980; Mello et al., 1986; Tartarotti and Azeredo-Oliveira, 1999).

In *P. megistus*, a small chromocenter detected only in somatic cell nuclei of male specimens, has been considered a contribution by the Y chromosome (Mello et al., 1986). Chromocenter is a densely aggregated body of constitutive heterochromatin. Constitutive heterochromatin is classically defined not only in terms of chromatin condensation but as a condensed and heteropyknotic (=densely stained) chromatin throughout the cell cycle, containing highly repetitive DNA sequences and richness in non-coding DNA and transposons, and exhibiting a slowly replicating DNA in comparison with euchromatin. It is

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considered as genetically inactive. The finding that the Y chromosome is the only chromosome that shows heterochromatic regions in *P. megistus* (Crossa et al., 2002) supports the idea of its chromocenter being composed of the Y chromosome. The chromocenter identified in *P. megistus* differs in size and chromosome content from the chromocenters detected in *Triatoma infestans* Klug, another species of the Reduviidae family and a well-known vector of Chagas disease. In *T. infestans*, at least three pairs of large autosomes plus X and Y sex chromosomes contribute for its heterochromatin bodies (Schreiber et al., 1972). However, chromocenters in both species contain AT-rich, GC-poor DNA and apparently no significant contribution of DNA methylated cytosine, as detected immunocytochemically (Alvarenga et al., 2011, 2012).

Regarding the histone epigenetic marks that may differ, if comparing heterochromatin and euchromatin territories in animal cells (Cowell et al., 2002), there is no report for hemipteran reduviids, except for *T. infestans* (Alvarenga et al., 2016). According to Sharakhov and Sharakhova (2015), it is necessary to expand the current understanding of vector-pathogen-host interactions to the level that includes epigenetic modifications of the vector genome. The identification of epigenetic marks in cell nuclei of *P. megistus* would thus be important for establishing differences between heterochromatin and euchromatin territories and for comparison with *T. infestans* data. It would also provide a reference for further studies in which these marks could be investigated in association with the insect susceptibility to drugs or insecticides tested for insect control (Pita et al., 2017).

Histone modifications and heterochromatin organization in disease vectors have been proposed to play important roles in determining phenotypes with different vectorial capacity (Sharakhov and Sharakhova, 2015). *T. infestans* populations have been demonstrated as variably susceptible to deltamethrin, a potent and effective synthetic type II pyrethroid insecticide, with resistance being assumed to develop by contact with the insecticide (Depickère et al., 2012). In mice, perinatal deltamethrin exposure lead to decreased mRNA expression of specific genes (Armstrong et al., 2013). In humans, prenatal exposure to pesticides may lead to disease risk in later life with the involvement of epigenetic mechanisms (Declerck et al., 2017). Even in plants, the exposure of seedlings to deltamethrin also induce epigenetic changes (Taspinar et al., 2017). Therefore, the identification of epigenetic marks in disease insect vectors is a first step for further establishment of a potential correlation of these marks or their changes with different insect physiology and response to drugs or insecticides.

In this study, histone acetylation and methylation marks were investigated in Malpighian tubule cell nuclei of *P. megistus*, using immunocytochemical assays, and compared to previously reported data for *T. infestans*. A FISH assay was undertaken to establish the position of 18S rDNA with respect to the heterochromatic body, which could be suggestive of some relationship with one of the histone modifications under investigation.

2. Material and methods

2.1. Samples

Male 5th instar nymphs and adults of a domestic population of *Panstrongylus megistus* (Burmeister) (Hemiptera, Reduviidae) were provided by the insect facility of the Superintendence for Control of Endemic Diseases in the state of São Paulo (SUCEN) (Mogi-Guaçu, Brazil). The *P. megistus* specimens reared in SUCEN's facility originated from natural populations of insects collected in the east region of the state of São Paulo (approximately between latitudes 21°31′S and 21°58′S and close to longitude 46°40′W) since 1980. To avoid inbreeding, new specimens have periodically been collected and introduced to the previously established colonies. In SUCEN's facility, the insects are fed on hen's blood once a week and reared under a short light regime at 28 °C and 80% relative humidity following animal care and ethical procedures registered at the National Council of Animal Experimentation Control (COBEA) from the Brazilian Ministry of Science, Technology and Innovation under accreditation protocol no. 01200.003280/2014-28. The SUCEN Scientific and Ethics Committee (Protocol no. 64405/2015) approved the present study. Three to five specimens were used for each experiment.

2.2. Cell preparations and immunocytochemistry

Malpighian tubules were used for immunocytochemistry because of their well-known fine structure, cellular development and physiology, and nuclear phenotype pattern (Mello, 1975; Dolder and Mello, 1979; Wigglesworth, 1984; Mello et al., 1986). Immediately after removal from the insects, these organs were whole-mounted on slides and fixed in 4% paraformaldehyde (Lab Synth, Diadema, Brazil) diluted in 0.01 M sodium phosphate buffer (PBS) at pH 7.4 for 10 min at room temperature. Then, the preparations were rinsed in PBS, air-dried and subjected to immunocytochemical assays for histone H3 acetylated at lysine 9 (H3K9ac), histone H4 acetylated at lysine 8 and 16 (H4K8ac and H4K16ac, respectively), and histone H3 methylated, di-methylated and tri-methylated at lysine 9 (H3K9me, H3K9me$_2$, and H3K9me$_3$, respectively), following previously reported procedures (Alvarenga et al., 2016). After immunostaining, the preparations were rinsed in PBS, and mounted in Vectashield (Vector Lab, Burlingame, USA) containing 200 µg/mL DAPI (Sigma Chemical Co., St. Louis, USA). Examination was done using an Axiophot 2 microscope equipped for fluorescence, AxioCam HRc color video camera and Kontron KS-400-3 software (Carl Zeiss, Oberkochen/Munich, Germany). Image merging was processed using Image J software (NIH, Bethesda, USA).

2.3. Antibodies

The primary antibodies used were as follows: mouse monoclonal anti-H3K9ac (1:500) (Abcam, Cambridge, MA, USA – AH3-120), rabbit polyclonal anti-H4K8ac (1:50) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA – CI1033), mouse monoclonal anti-H4K16ac (1:200) (Abcam – ab109463 [EPR1004]), rabbit polyclonal anti-H3K9me (1:500) (Abcam – ab8896), mouse monoclonal anti-H3K9me$_2$ (1:500) (Millipore, Billerica, MA, USA – 05-1249 (clone CMA307)), and rabbit polyclonal H3K9me$_3$ (1:250) (Millipore – 07-442). For detection of the primary antibodies, goat anti-mouse IgG (H + L) conjugated to Texas Red (1:100) (Southern Biotech, Birmingham, AL, USA – 1031-07) and sheep anti-rabbit IgG conjugated to fluorescein (1:200) (Chemicon Int, Inc., Temecula, CA, USA – AP307F) were used as secondary antibodies.

2.4. Fluorescence in situ hybridization

DNA was isolated from leg muscles of three adult specimens of *P. megistus* using the Wizard Genome DNA Purification kit (Promega, Madison, USA). The protocol used for 18S rDNA FISH was previously described by Bardella et al. (2010). The primer sets for PCR were as follows: Sca18SF (5′ CCC CGT AAT CGG AAT GAG TA) and Sca18SR (5′ GAG GTT TCC CGT GTT GAG TC) (Cabral-de-Mello et al., 2010). Probe labeling was carried out *via* nick translation using biotin-14-dATP (Invitrogen, Carlsbad, USA).

Malpighian tubules of 5th instar nymphs of *P. megistus* were fixed in an absolute ethanol-acetic acid (3:1, v/v) solution, squashed in a drop of a 50% acetic acid aqueous solution, dehydrated through an EtOH gradient, and air-dried. Then, they were treated with 100 µg/mL RNase A (Sigma, St. Louis, MO, USA) for 1 h at 37 °C, rinsed in 2xSSC solution for 10 min, and post-fixed in 4% formaldehyde for 10 min at room temperature, followed by a new rinsing in 2xSSC solution for 10 min. Next, the preparations were treated with 30 µL of the hybridization mixture containing 100 ng of the labeled probe (4 µL of the probe per slide), 15 µL of 100% formamide (50%), 6 µL of 50% polyethylene glycol (10%), 3 µL of 20xSSC (2xSSC), 1 µL of calf thymus blockage

DNA (100 ng), and 1 µL 10% SDS (0.33%). The denaturation/renaturation cycles were performed at 90 °C for 10 min, 48 °C for 10 min, 38 °C for 5 min, and 37 °C in stand by using thermal cycler (MJ Research/Bio-rad Lab, Hercules, USA). The preparations were subsequently maintained in a moist chamber at 37 °C overnight for hybridization. Post-hybridization rinses were carried out using 2xSSC, 20% formamide in 0.1xSSC, 0.1xSSC, and 4xSSC/0.2% Tween 20 solutions, all of them at 42 °C. The probe was detected using a solution composed of 5 µg/mL avidin-FITC (fluorescein isothiocyanate) (Sigma) conjugate and 5% BSA/4xSSC/0.2% Tween 20 (1:100, v/v) for 1 h in a moist chamber. Next, the preparations were rinsed in 4xSSC/0.2% Tween 20 at room temperature, counterstained with DAPI and mounted in Vectashield (Vector, Burlingame, CA, USA).

Images were captured using an Olympus BX61 microscope equipped with appropriate filters and a DP70 digital camera. Image merging was performed using Adobe Photoshop CS5. At least 21 nuclei were examined.

3-D observations were also performed using a Leica TS SP5 II broadband confocal microscope (Wetzlar, Germany) equipped with argonium and helium-neonium lasers, 63x and 100x objectives, and the Leica Application Suite AF (Leica Microsystems) software.

3. Results and discussion

Fluorescent signals indicative of acetylation of lysine 9 in histone H3 and of lysine residues 8 and 16 in histone H4 appeared distributed throughout the euchromatin and not in the chromocenter of the Malpighian tubule cell nuclei of *P. megistus* (Fig. 1a–i). This characteristic resembles most patterns of distribution of such epigenetic marks in many other cell types, including *T. infestans* cells (Alvarenga et al., 2016). Regarding H3K9ac and H4K16ac marks, they are assumed as mostly associated with the chromatin accessibility and transcriptional activity expected for euchromatin (Kouzarides, 2007; Robinson et al., 2008; Zhang et al., 2017). Acetylation of K16 abolishes chromatin compaction dependent on N-terminal tail of histone H4 (Shogren-Knaak et al., 2006; Ngubo et al., 2011) and activates gene transcription by affecting chromatin structure and interplay with non-histone proteins (Zhang et al., 2017).

A few nuclei of *P. megistus* exhibited signals for H4K8ac concentrated at the nuclear periphery. Most cell nuclei showed signals that encircle the nuclear area devoid of DAPI staining (Fig. 1d–f), and that is filled by the nucleolus, if considering previously published electron microscopy images of the Malpighian tubule cells (Mello, 1987). In single-chromocentered nuclei of *T. infestans*, this epigenetic mark is concentrated around the large chromocenter, and is suggestive of association with the NOR region (Alvarenga et al., 2016; Imperador, 2018). However, such an association does not probably occur in *P. megistus* Malpighian tubule cells, because the FISH fluorescent signals that revealed 18S rDNA sites in the chromocenter were detected around and inside this structure (Fig. 2, Supplementary material – video), where no marks for H4K8ac were found (Fig. 1d–f). It is worth mentioning that the same rational cannot be applied with present methodology to the 18S rDNA signals that were identified in two to four small points in the euchromatin, probably inserted in copies of an autosome chromosome (Morielle-Souza and Azeredo-Oliveira, 2007; Panzera et al., 2012). The identification of 18S rDNA in the Y-chromosome-generated chromocenter in addition to copies of an autosome chromosome in the specimens of *P. megistus* studied here is in agreement with Morielle-Souza and Azeredo-Oliveira's data (2007). This observation, that is not in agreement with detection of 18S rDNA signal in only one autosome (Panzera et al., 2012), is possibly due to differences in geographical origins between the individuals analyzed in this study and those analyzed by Panzera et al. (2012).

The pattern of distribution of the fluorescent signals revealing H4K8ac (Fig. 1d–f) and H4K16ac (Fig. 1g–i) in euchromatin was not the same. While the signals for H4K8ac appeared as fine fluorescent granules (Fig. 1e and f), those for H4K16ac appeared as coarse granular elements that heterogeneously accumulated in the euchromatin (Fig. 1h–i). Whether a complex event occurs, such as the recognition by H4K8ac antibodies of poly-acetylated H4 substrates as proposed by Rothbart et al. (2012), more complex methodologies (ex.: mass spectrometry of isolated histones) would be required to demonstrate.

Broadly speaking, the results regarding H3K9 methylation are in agreement with reports for other cell types showing that mono-, di-, and tri-methylated H3K9 residues occupy different chromatin territories (Wu et al., 2005). Signals indicative of mono-methylated H3K9 residues were only evident in euchromatin (Fig. 1j–l). In some nuclei, where the nucleolar area is apparently reduced, the fluorescent signals for H3K9me are concentrated encircling the chromocenter area (Fig. 1j–l), similarly to the images displayed by *T. infestans* single-chromocentered cell nuclei, although less brightly and distributed over a narrower area (Alvarenga et al., 2016). H3K9me$_2$ residues, which were also revealed in euchromatin areas, appeared deeply concentrated at the nuclear periphery (Fig. 1m–o) and differed from the images previously reported for 5th instar nymphs of *T. infestans* (Alvarenga et al., 2016). The pattern of spatial distribution close to the nuclear periphery as exhibited by the H3K9me$_2$ marks in the cell nuclei of *P. megistus* may be related to the interaction of lamina-associated chromatin domains with the nuclear lamina (Yokochi et al., 2009; Bian et al., 2013; Kind et al., 2013). Di-methylation of lysine 9 of histone H3 at the nuclear periphery of mouse cells is usually undertaken by the G9a methyl-transferase within late-replicating and generally repressed chromatin (Yokochi et al., 2009). Additionally, H3K9me$_2$ participates in the creation of a platform for binding of the protein HP1 (Yokochi et al., 2009). Although HP1 has not yet been investigated in *P. megistus* cells, it is only detected encircling or as part of the chromocenters in 5th instar nymphs of *T. infestans*, where H3K9me$_2$ marks close to the nuclear periphery are not abundant (Alvarenga et al., 2016). On the other hand, predominance of H3K9me$_2$ signals close to the nuclear periphery may indicate sites of DNA synthesis primarily in mid-S phase (Wu et al., 2005). If that applies to the *P. megistus* Malpighian tubule cells, it would mean that the insect specimens examined here were still in the previously described process of the last DNA content doubling in this organ during the 5th nymphal instar (Mello, 1975), which is the stage we utilized for the immunofluorescence assay. In conclusion, comparison of H3K9me$_2$ and HP1α marks at the nuclear periphery, and detection of methyl-transferase (G9a?) levels along the various developmental stages where the growing process of polyploidization occurs in *T. infestans* and *P. megistus* would possibly bring some light to this question.

Regarding the fluorescent signals for H3K9me$_3$, most nuclei revealed a very intense fluorescence predominantly in the chromocenter body (Fig. 1p–r), whereas some nuclei displayed these marks in the chromocenter periphery and in some granular chromatin elements in euchromatin. No evidence of H3K9me$_3$ signals appeared concentrated at the nuclear periphery. H3K9me$_3$ is usually a marker linked to gene silencing (Lachner et al., 2001) and is associated with constitutive heterochromatin (Wu et al., 2005). However, it has also been reported for euchromatin in cells of some other species, including those of *T. infestans* (Wu et al., 2005; Alvarenga et al., 2016). In euchromatin, this occurrence may either be associated with repressed genes (when positioned in gene promoters) or with gene expression (when enriched within a gene body) (Vakoc et al., 2006; Black and Whetstine, 2011). For a better interpretation of the presence of H3K9me$_3$ marks in the euchromatin of *P. megistus*, 3-D confocal microscopy and chromatin immunoprecipitation assays would be probably more adequate.

The portion of the nuclear area devoid of DAPI staining and that is attributed to the nucleolar region varied in extension in the various cell nuclei that were examined in specimens of *P. megistus* at the same developmental stage and under the same nourishment conditions. In this species, the nucleolus appears in some cells as a narrow structure in intimate contact with the chromocenter, just like in *T. infestans* (Mello et al., 1990), whereas in other cells, supposed to be highly engaged in

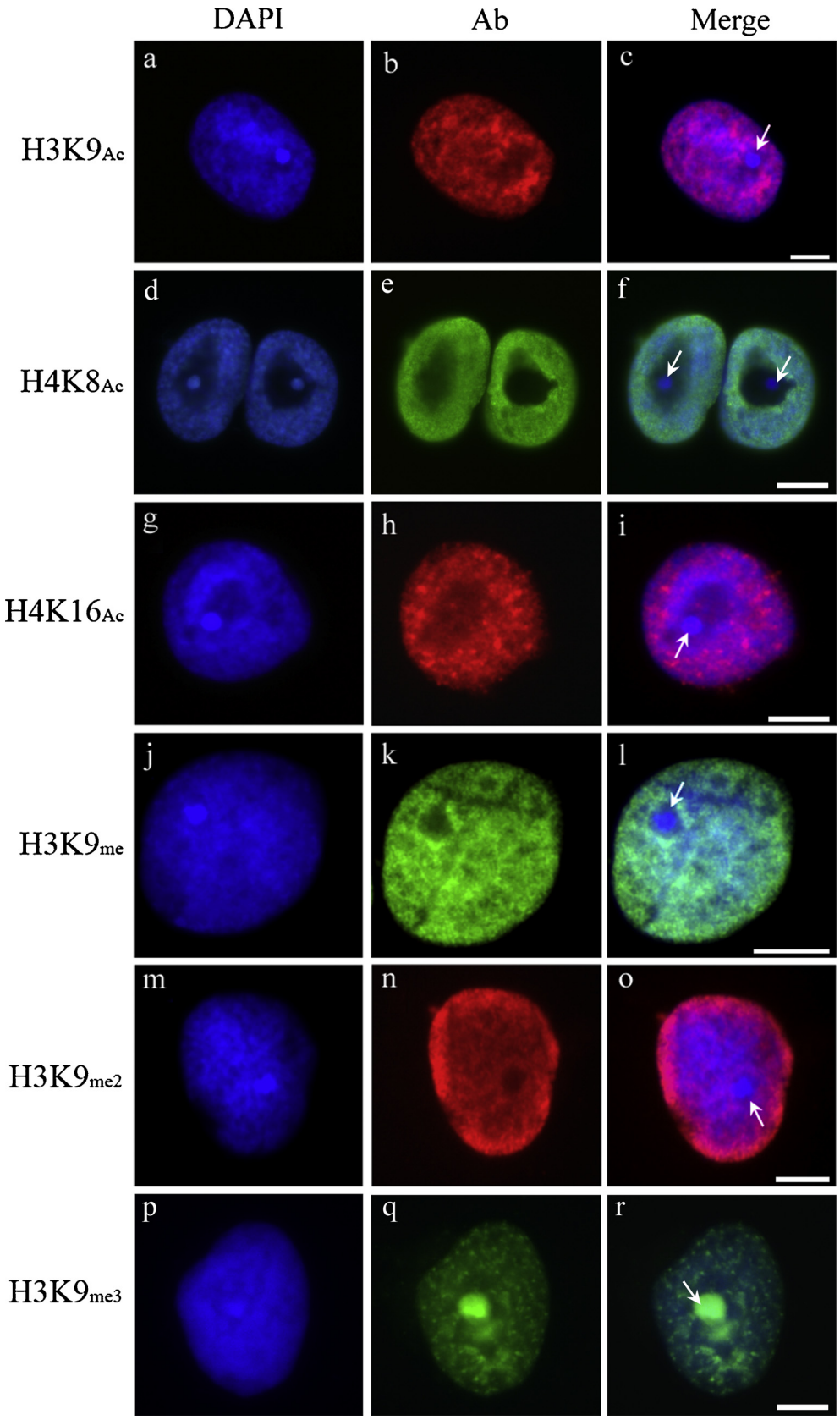

Fig. 1. Immunofluorescence signals for histone modifications in Malpighian tubule cell nuclei of *P. megistus* 5th instar male nymphs. Histone acetylation and methylation marks are identified in the panels (a–r). Counterstaining was performed with DAPI. The arrows indicate the chromocenter. Ab, antibody. The bars represent 10 μm.

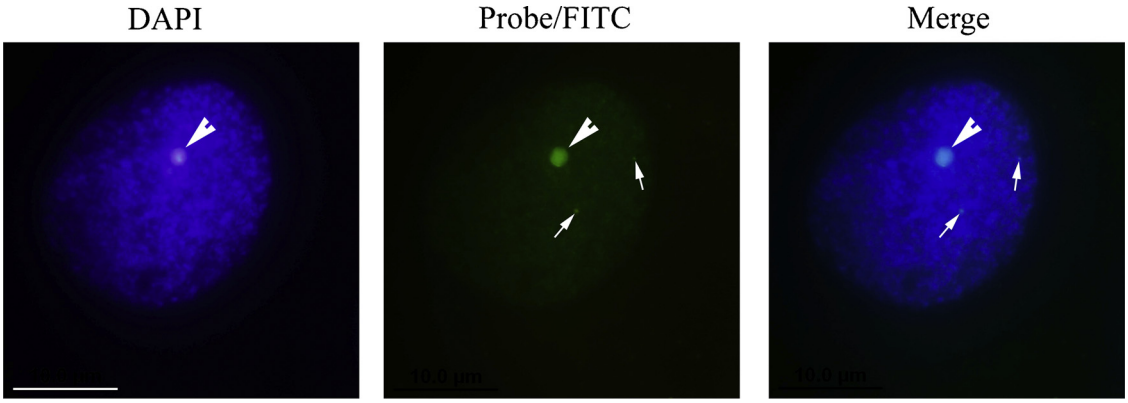

Fig. 2. FISH of 18S rDNA reveals signals (green) in the chromocenter (arrowheads) and in two points of euchromatin (arrows) in a Malpighian tubule cell nucleus of *P. megistus*. The bar represents 10 µm.

ribosome biogenesis, it occupies a much larger volume (Fig. 1d–f). This finding indicates variability in nucleolar activity when considering different cells of the Malpighian tubules of *P. megistus,* although no functional cellular diversity involved has so far been described for this excretory organ.

4. Conclusion

Although the formation of chromocenters in *P. megistus* and *T. infestans* involves contribution of different chromosome types and the chromocenter size greatly differs in these species, fluorescent signals that reveal acetylated H3K9, H4K8 and H4K16, and mono- and dimethylated H3K9 marks are absent in the heterochromatic bodies but present in the euchromatin of the Malpighian tubule cells of both species. Regarding the spatial distribution of H4K8ac marks in the cell nuclei, no intimate association with the NOR region that is predominantly found in the periphery of the chromocenter, was detected in *P. megistus*, thus differing from reports for single-chromocentered cell nuclei of *T. infestans*. H3K9me$_3$, a mark usually present in constitutive heterochromatin, appeared concentrated in the chromocenters in *P. megistus* or encircling them, similarly as in *T. infestans* cells. However, there is a clear evidence of differences between the spatial distributions of the H3K9me$_2$ marks when comparing 5th instar nymphs of these species. A comparative investigation between *P. megistus* and *T. infestans* is still required on H3K9me$_2$ and HP1α marks at the nuclear periphery and on methyl-transferase levels along the growing process of polyploidization during these insects' nymphal development.

Considering how important *P. megistus* is as a vector of Chagas disease, the overall distribution of the epigenetic marks revealed here may also inspire further studies as a search for possible differences at these marks in response to drugs and/or insecticides.

Author statement

Conceptualization: MLSM
Data curation: –
Formal analysis: ASM, MLSM
Funding acquisition: MLSM
Investigation: EMA, CHLI, VBB, ASM
Methodology: MM, VBB, DCCM
Project administration: MLSM
Resources: VLCC, VBB, DCCM, MM, MLSM
Software: –
Supervision: MLSM
Validation: –
Visualization: EMA, CHLI, VBB, ASM
Roles/Writing – original draft: EMA, CHLI, VBB, VLCC, MM, DCCM, ASM, MLSM
Writing – review and editing: ASM, MLSM

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.acthis.2018.07.002.

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