The Topological Distribution of the Chromocenter in *Panstrongylus megistus* (Burmeister) Malpighian Tubule Cells Examined by Confocal Microscopy

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Summary *Panstrongylus megistus* (Burmeister) and *Triatoma infestans* (Klug), blood-sucking hemipterans in the Reduviidae family, are vectors of Chagas disease. Both species exhibit holocentric chromosomes and conspicuous heterochromatin bodies (chromocenters), but they differ in chromosome number and chromocenter structure and composition patterns. In the Malpighian tubule cells of *T. infestans*, the chromocenters are positioned close to the nuclear periphery throughout the life of the insect, with no apparent association with overall gene silencing. Because chromocenter topology may vary in different species of the same genus and the spatial distribution pattern of the chromocenter of *P. megistus* has not yet been described, we used confocal microscopy to evaluate the spatial organization of this heterochromatic body in the Malpighian tubule cells of this species during nymphal development and in the adult phase compared to data reported for *T. infestans*. Despite the differences in chromocenter composition, structural pattern, and size between *P. megistus* and *T. infestans*, the chromocenter of *P. megistus* nymphs and adults, similar to those of *T. infestans*, was found to occupy a nonincidental position close to the nuclear periphery. The topological pattern observed at the chromocenter of *P. megistus*, which is consistent with the previous report of highly concentrated dimethylated histone H3 lysine 9 residues at the nuclear periphery was not found to be directly related to gene silencing or modulation.

Keywords Heterochromatin, Spatial distribution, Hemipteran, Fluorescent Feulgen reaction.

Panstrongylus megistus (Burmeister, 1835) and *Triatoma infestans* (Klug, 1834) are blood-sucking insects in the Reduviidae hemipteran family that serve as important vectors of Chagas disease. *P. megistus*, which is native to Brazil, is particularly relevant for the spread of this disease because it has become associated with human habitations after the extensive destruction of its natural habitats (Silveira 2000).

Both *P. megistus* and *T. infestans* exhibit holokinetic (holocentric) chromosomes and constitutive heterochromatin bodies, known as chromocenters, containing AT-rich DNA (Ueshima 1966, Mello 1971, 1975, Mello *et al.* 1986, Alvarenga *et al.* 2011, 2012). In contrast, these species differ in the number of chromosomes and in chromocenter structure. While *P. megistus* contains nine autosomal pairs and multiple sex chromosomes (X_1X_2Y in males and $X_1X_1X_2X_2$ in females), *T. infestans* contains 10 autosomal pairs and XY/XX sex chromosomes (Ueshima 1966). While in *T. infestans*, the chromocenters are large, conspicuous structures (Mello 1971, 1979) encapsulating at least three pairs of autosomes and the sex chromosomes in *T. infestans* (Sch-

reiber *et al.* 1972, Solari 1979), in *P. megistus*, a single small chromocenter is detected only in male specimens and has been assumed to consist of the Y chromosome in somatic cells (Mello *et al.* 1986) and of the Y chromosome plus the associated X_1 and X_2 chromosomes in meiotic cells at the “diffuse stage” (Cossa *et al.* 2002). In contrast to *T. infestans*, no diversification of the chromocenter morphological pattern occurs in the Malpighian tubules of *P. megistus* (Mello 1975, Mello *et al.* 1986). Regarding the presence of histone epigenetic markers, while acetylated H3K9, H4K8, and H4K16 and mono-(H3K9me) and dimethylated H3K9 (H3K9me2) are absent in the Malpighian tubule chromocenters of both species, H3K9me2 markers are predominantly distributed at the nuclear periphery only in *P. megistus* (Alvarenga *et al.* 2016, 2018).

Chromatin organization and distribution in subdomains is relevant to nuclear physiology (Fritz *et al.* 2016, Jagannathan *et al.* 2019). The spatial distribution of heterochromatin in interphase, either as isolated loci or clustered together into conspicuous chromatin bodies known as chromocenters, has been associated with proximity to the nuclear periphery in several organisms. In certain cases, the nonincidental localization of chromocenters has been associated with a high content of

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gene-poor chromosome domains or gene silencing; in contrast, euchromatin is mostly positioned in the nuclear center and is rich in transcriptionally active genes (Croft *et al.* 1999, Bártova *et al.* 2008, Politz *et al.* 2013, Wijchers *et al.* 2015).

In the Malpighian tubules of *T. infestans*, the chromocenters are distributed relatively close to the nuclear edge but they are not endowed with overall gene silencing, because their boundaries contain 18S rDNA sites even in regions facing the nuclear periphery (Imperador *et al.* 2020). However, because the spatial distribution of chromocenters within the cell nucleus may vary between different species of the same genus (Ellison and Howard 1981, Hochstrasser *et al.* 1986), the data reported for *T. infestans* may not be extrapolated to other chromocenter-containing reduviid hemipterans, including *P. megistus*. Considering that the number of chromosomes and the size and chromosome content of the chromocenter of *P. megistus* differ from those of *T. infestans* and that there is an absence of data regarding the topological distribution of the chromocenter in the Malpighian tubule cell nuclei of *P. megistus*, the spatial distribution of the chromocenter in this species was investigated using confocal microscopy and was compared throughout insect development with data reported for *T. infestans* (Imperador *et al.* 2020).

Materials and methods

Insects

Male third- and fifth-instar nymphs and adults of a domestic population of *P. megistus* originating from natural populations of insects collected in the eastern region of the state of São Paulo were supplied by the insect facility of the Superintendence for Control of Endemic Diseases of the state of São Paulo (SUCEN) at Mogi-Guaçu (Brazil). In the SUCEN facility, the *P. megistus* specimens were reared at 28°C and 80% relative humidity and fed on hen's blood once per week following the animal care and ethical procedures registered at the National Council of Animal Experimentation Control (COBEA) from the Brazilian Ministry of Science, Technology and Innovation (accreditation protocol no. 01200.003280/2014-28). Three to five specimens of each developmental stage were used. The SUCEN Scientific and Ethics Committee approved this investigation (Protocol no. 64405/2015).

Fixation and cytochemistry

Whole-mounted Malpighian tubules were used because of their well-known cell biology, cytogenetics, fine structure, physiology, and chromatin epigenetics (Mello 1975, Dolder and Mello 1979, Wigglesworth 1984, Mello *et al.* 1986, Alvarenga *et al.* 2012, 2018). These organs were fixed in a mixture of absolute ethanol and glacial acetic acid (3:1, v/v) for 1 min, rinsed

in 70% ethanol for 5 min, and then either subjected to an ordinary Feulgen reaction, to obtain a global DNA cytochemical response or subjected to the fluorescent Feulgen reaction, which is required for DNA/chromatin localization using confocal microscopy (Mello and Vidal 2017). In both cases, DNA depurination by acid hydrolysis was performed in 4M HCl for 65 min at 25°C. For the fluorescent Feulgen staining, the Schiff reagent was diluted in sulfurous water (1:9, v/v) (Imperador *et al.* 2020). Following treatment with the Schiff reagent, the preparations were rinsed in three baths of sulfurous water (5 min each) and then in distilled water, air-dried, cleared in xylene for 10 min, and mounted in Canada balsam (ordinary Feulgen reaction) or VECTASHIELD (Vector Laboratories) (fluorescent Feulgen reaction).

Ordinary light microscopy

The preparations subjected to nonfluorescent Feulgen staining were examined using a Zeiss Axiophot 2 microscope (Carl Zeiss) equipped with a Neofluar 40×/0.75 objective, optovar factor 2, and a 0.90 condenser, and images of the nuclei were captured using an AxioCam HRc color video camera.

Confocal microscopy

The fluorescent images were examined using a Leica TS SP5 II broadband confocal microscope (Leica Microsystems) equipped with argon-ion and 543 nm and 633 nm helium-neon lasers, 63× and 100× objectives, and the Leica Application Suite AF software (Leica Microsystems) at the LaCTAD facilities (Unicamp, Campinas, Brazil). A spacing of 0.13 μm between optical sections was used for each nucleus. Because the nuclear size varies as a function of the insect developmental phase as polyploidization progresses (Mello 1975), the software provided further spacing compensation during 3D reconstruction. A total of 29 nuclei from third-instar nymphs, 26 nuclei from fifth-instar nymphs, and 15 nuclei from adults were analyzed.

Image analysis and statistics

The axial distances between the chromocenters and the nuclear periphery were measured in the X, Y, and Z axes using the Image-Pro Plus 7 software (Media Cybernetics, Inc., Rockville, MD, USA). The representation of the optical sections according to these axes followed the same schema previously reported for *T. infestans* (Imperador *et al.* 2020). For comparisons that required Kruskal–Wallis and Mann–Whitney tests, the Minitab18 software (State College, PA, USA) was used.

Results

The nuclei and chromocenter in the Feulgen-stained Malpighian tubule cells of *P. megistus* male nymphs and adults are ellipsoidal. The chromocenter stains deeply

Fig. 1. Feulgen-stained Malpighian tubule cell nuclei of a fifth-instar male nymph of *P. megistus* (a, b). The arrows indicate the chromocenter. Scale bars= $10\mu\text{m}$.

Fig. 2. Image gallery composed of a sequence of optical sections obtained using confocal microscopy of a fluorescent Feulgen-stained Malpighian tubule cell nucleus of a fifth-instar nymph of *P. megistus*. A spacing of $0.13\mu\text{m}$ between optical sections was used. Scale bar= $20\mu\text{m}$.

in the ordinary Feulgen reaction (Fig. 1) and exhibits a more intense response to the fluorescent Feulgen reaction than the rest of the chromatin (euchromatin) (Fig. 2). An example of a sequence of optically sectioned images captured with confocal microscopy from a fifth-instar Malpighian tubule cell nucleus stained with the fluorescent Feulgen reaction is presented in Fig. 2.

The measurement of the nuclear diameters and the distances between the chromocenter surface and the nuclear periphery in the opposite directions (distal long

and proximal short distances) revealed differences in all coordinates (Fig. 3, Tables 1 and 2). These differences indicate that the chromocenter is not centrally positioned in the cell nuclei of *P. megistus* and that there is relative proximity between the chromocenter and the nuclear periphery in all studied phases (Table 2). Despite the proximity of the chromocenter to the nuclear periphery, the proximal and distal distances between the chromocenters and the nuclear periphery in the X-, Y- and Z-axes demonstrated increased variability (Table 1). The

Fig. 3. Comparison of the distances in the X, Y, and Z axes between the chromocenter and the nuclear periphery in the fluorescent Feulgen-stained cell nuclei of Malpighian tubules of *P. megistus* (a–c). The nuclear diameters (axes) are also compared (d). The vertical lines on the bars indicate the mean standard deviations; the horizontal lines in the bars indicate the median values. *Differences significant at $P_{0.05}$ between the compared groups. $n=29$ (3rd-instar nymphs), 26 (5th-instar nymphs) and 15 (adults). The statistical comparisons are listed in Tables 2 and 3.

Table 1. Proximal and distal distances between the chromocenter and the nuclear periphery in Malpighian tubule cells of *P. megistus*.

Distances (μm)	Axes	Third instar nymphs ($n=29$)			Fifth instar nymphs ($n=26$)			Adults ($n=15$)		
		X	SD	CV (%)	X	SD	CV (%)	X	SD	CV (%)
Proximal	X	2.43	1.44	59.32	4.11	1.54	37.50	4.42	1.50	34.02
	Y	3.50	1.83	52.34	8.24	2.54	30.82	8.94	5.64	63.10
	Z	0.53	0.15	29.85	1.00	0.54	54.11	1.01	0.46	45.97
Distal	X	4.40	1.84	41.77	8.13	3.12	38.39	6.96	1.64	23.64
	Y	5.63	1.55	27.53	11.82	3.58	30.32	14.59	6.34	43.48
	Z	0.86	0.26	31.28	1.78	0.63	35.16	1.53	0.73	47.82
Nuclear diameter	X	8.68	3.12	35.95	14.41	3.87	26.80	14.21	2.90	20.16
	Y	11.18	3.04	27.24	23.14	5.68	24.54	25.90	12.23	47.23
	Z	2.60	0.59	22.80	4.85	1.33	27.59	4.52	2.05	45.23

CV, coefficient of variability; SD, standard deviation; X, arithmetic means

nuclear diameters and the distal and proximal distances between the chromocenter and the nuclear periphery in the X-, Y- and Z-coordinates were observed to increase throughout nymphal development, which is when polyploidization occurs in the Malpighian tubules, and were found to arrest in the adult phase, which is when polyploidization ceases (Mello 1975) (Table 3).

Discussion

The structural results obtained for *P. megistus* in the present study showed similarities with those previously reported for *T. infestans* (Imperador *et al.* 2020), despite their previously described differences in chromocenter composition, structural pattern, and size (Schreiber *et al.* 1972, Mello 1975, Solari 1979, Mello *et al.* 1986, Crossa *et al.* 2002). Because the Feulgen reaction is a reliable cytochemical test to reveal and quantify DNA (Mello

and Vidal 2017), the pattern of chromocenter distribution within the cell nuclei of *P. megistus* could be adequately assessed using confocal microscopy.

The function of the chromocenter and the reason for its close proximity to the nuclear periphery in the cells of *P. megistus* are questions that have also been raised regarding the constitutive heterochromatin of many other organisms and are considered not yet fully addressed (Allshire and Madhani 2018).

Heterochromatin self-association, such as that occurring in chromocenters, and satellite DNA repeats have been considered a driving force in the formation and maintenance of the 3D structure of the nucleus (Wijchers *et al.* 2015, Jagannathan *et al.* 2018, 2019, Ostromyshenskii *et al.* 2018). Chromocenters formed from bundling pericentromeric satellite DNA have been considered to play the role of packaging the genome within a single nucleus through the formation of a modu-

Table 2. Proximal and distal distances between the chromocenter (s) and the nuclear periphery in Feulgen-stained cell nuclei of Malpighian tubules of *P. megistus* compared in the X-, Y- and Z-optical axes.

Developmental stage	Parameters	Axis comparison	Test	P	Decision
Nymphal 3rd instar	Proximal distance	X vs. Y vs. Z	KW	0.0010	SS
		X vs. Y	MW	0.0349	S
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS
	Distal distance	X vs. Y vs. Z	KW	0.0000	SS
		X vs. Y	MW	0.0250	S
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS
	Nucleus axis	X vs. Y vs. Z	KW	0.0000	SS
		X vs. Y	MW	0.0166	S
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS
Nymphal 5th instar	Proximal distance	X vs. Y vs. Z	KW	0.0000	SS
		X vs. Y	MW	0.0000	SS
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS
	Distal distance	X vs. Y vs. Z	KW	0.0000	SS
		X vs. Y	MW	0.0005	SS
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS
	Nucleus axis	X vs. Y vs. Z	KW	0.0000	SS
		X vs. Y	MW	0.0000	SS
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS
Adult	Proximal distance	X vs. Y vs. Z	KW	0.0000	SS
		X vs. Y	MW	0.0000	SS
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS
	Distal distance	X vs. Y vs. Z	KW	0.0000	SS
		X vs. Y	MW	0.0000	SS
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS
	Nucleus axis	X vs. Y vs. Z	KW	0.0000	SS
		X vs. Y	MW	0.0000	SS
		X vs. Z	MW	0.0000	SS
		Y vs. Z	MW	0.0000	SS

KS, Kruskal–Wallis; MW, Mann–Whitney; NS, non-significant; S, significant; SS, highly significant; vs, versus.

lar network that results from associations between two sequence-specific satellite DNA-binding proteins bound to their cognate satellite DNAs (Jagannathan *et al.* 2018, 2019). Although a detailed examination of some of the published images of *T. infestans* somatic cell nuclei suggests the connection to their chromocenters of several chromosomes that do not belong to these heterochromatin bodies (Fig. 1a in Mello 1997; Figs. 2–4 in Mello 1979), no such evidence has been observed in *P. megistus* male somatic cells.

The nonincidental distribution of the chromocenter in the cell nuclei of *P. megistus* Malpighian tubules was consistent with observations in other animal species, including *T. infestans* (Imperador *et al.* 2020). In *Drosophila melanogaster*, the distribution of pericentromeric heterochromatin in specific nuclear spaces is assumed to be functionally related to the organization of the nucleolus (Wasserlauf *et al.* 2020). This event may also occur in *P. megistus* Malpighian tubules because a confocal-fluorescence *in situ* hybridization study has

revealed that both the peripheral and inner regions of the chromocenter contain 18S rDNA (Alvarenga *et al.* 2018), although 18S rDNA signals are also detected outside the chromocenter (Alvarenga *et al.* 2018), likely due to ribosomal genes present in one autosomal chromosome pair (Panzera *et al.* 2012).

Although there is no consensus on whether the topological organization of a chromocenter is associated with gene-poor chromosome domains and/or silenced chromatin, there are reports indicating that the nuclear periphery is not refractory to gene transcription and that it can contain concentrated histone epigenetic marks that participate in the modulation of certain genes and silencing factors (Ruault *et al.* 2008, Towbin *et al.* 2009, 2013, Meister and Taddei 2013). A deep concentration of H3K9me2 residues has been demonstrated at the nuclear periphery in the Malpighian tubule cells of *P. megistus* (Alvarenga *et al.* 2018). In other organisms, this epigenetic mark has been associated with the creation of a platform for the binding of the heterochromatin structural

Table 3. Statistical comparisons for X-, Y-, and Z-axes distance of chromocenters and nuclear axes of Feulgen-stained nuclei of Malpighian tubule cells of *P. megistus*.

Parameters	Stage comparison	Test	P	Decision
X-axis proximal distance	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0003	SS
	Third-instar nymphs vs. adults	MW	0.0004	SS
	Fifth-instar nymphs vs. adults	MW	0.8430	NS
X-axis distal distance	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0000	SS
	Third-instar nymphs vs. adults	MW	0.0004	SS
	Fifth-instar nymphs vs. adults	MW	0.2157	NS
Y-axis proximal distance	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0000	SS
	Third-instar nymphs vs. adults	MW	0.0002	SS
	Fifth-instar nymphs vs. adults	MW	0.7556	NS
Y-axis distal distance	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0000	SS
	Third-instar nymphs vs. adults	MW	0.0000	SS
	Fifth-instar nymphs vs. adults	MW	0.3790	NS
Z-axis proximal distance	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0005	SS
	Third-instar nymphs vs. adults	MW	0.0002	SS
	Fifth-instar nymphs vs. adults	MW	0.9892	NS
Z-axis distal distance	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0000	SS
	Third-instar nymphs vs. adults	MW	0.0012	SS
	Fifth-instar nymphs vs. adults	MW	0.1848	NS
Nucleus X-axis	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0000	SS
	Third-instar nymphs vs. adults	MW	0.0000	SS
	Fifth-instar nymphs vs. adults	MW	0.8817	NS
Nucleus Y-axis	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0000	SS
	Third-instar nymphs vs. adults	MW	0.0000	SS
	Fifth-instar nymphs vs. adults	MW	0.8603	NS
Nucleus Z-axis	Third- vs. fifth-instar nymphs vs. adults	KW	0.0000	SS
	Third- vs. fifth-instar nymphs	MW	0.0000	SS
	Third-instar nymphs vs. adults	MW	0.0032	SS
	Fifth-instar nymphs vs. adults	MW	0.5247	NS

KW, Kruskal–Wallis; MW, Mann–Whitney; NS, not significant; S, significant; SS, highly significant; vs, versus.

protein HP1 (Yokochi *et al.* 2009) and has been claimed to represent “a specific mark of nuclear peripheral heterochromatin that acts as a 3D architectural guidepost” (Poleshko *et al.* 2019). The maintenance of the spatial positioning of the chromocenter close to the nuclear periphery as polyploidization advances during the nymphal development of *P. megistus* may thus be related to the presence of H3K9me2-enriched genomic regions adjacent to this nuclear region (Alvarenga *et al.* 2018). The variability in the proximal and distal distances between the chromocenter and the nuclear periphery in *P. megistus* as well as in *T. infestans*, may suggest for both species that although the chromocenters are close to the nuclear periphery, they may exhibit a certain degree of mobility at this nuclear region (Imperador *et al.* 2020).

Although chromocenters are considered to comprise a repressive environment in the nucleus (Politz *et al.* 2013), the proximity of this heterochromatic body to the nuclear periphery in *P. megistus* may not be merely

associated with global gene silencing. The chromocenter containing several copies of the endoreplicated Y chromosome in the polyploid cells of the Malpighian tubules of this species may be not totally silent or empty of genes. Although not closely related to *P. megistus*, the reduviid *Rhodnius prolixus* contains nine genes in its Y chromosome, one with gut-restricted expression (Koricher *et al.* 2016).

In conclusion, the chromocenter of *P. megistus* shows a peripheral distribution pattern in the Malpighian tubule cell nuclei in which it may exhibit dynamic mobility, similar to the spatial organization of the single- and multi-chromocentered nuclei in the Malpighian tubules of *T. infestans* despite their differences in composition, structural organization, and size. The function of the *P. megistus* chromocenter in the context of the general nuclear organization in a somatic cell and the reason for its specific positioning close to the nuclear periphery, are questions that warrant further investigation. Molecular

biology studies, especially concerning genomic sequencing, types of repetitive DNA-cognate proteins, and immunocytochemical assays in conjunction with confocal microscopy and other advanced microscopies, may help to elucidate the nature of this chromocenter.

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