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Endocrine and Regenerative Cells in the Midgut of Chagas' Disease Vector *Triatoma vitticeps* During Different Starvation Periods*

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The midgut of an insect has three main cell types, viz., digestive, endocrine and regenerative cells, and a relationship between cell morphology and blood meal has been suggested. This study evaluated the occurrence of the regenerative and endocrine cells in different regions of the midgut of female and male *Triatoma vitticeps* during different periods after a blood meal. Adults of both sexes were dissected at 4, 72, 120, 168, 288, 360 and 600 hours after feeding and the midgut was pulled apart. The midgut was divided into the anterior, middle and posterior regions and the fragments were analyzed under a light microscope. The results showed the presence of endocrine cells of the open type and positive for FMRFamide as well as regenerative cell nests in the midgut of *T. vitticeps*. An association was observed between the starvation period and frequency of FMRFamide positive and regenerative cells in the midgut of *T. vitticeps*. Differences in the pattern of distribution of these cells between the males and females as well as in the different regions of the midgut were also discussed.

Key words: FMRFamide, digestion, Triatominae, Chagas' disease.

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The midgut of insects is lined by simple columnar epithelium, where three cell types are distinguished: digestive, endocrine and regenerative cells (CRUZ-LANDIM 1985; SERRÃO & CRUZ-LANDIM 2000; NEVES *et al*. 2002; ROCHA *et al*. 2010).

Endocrine cells are mainly characterized by the presence of granules in the infra-nuclear region. They can be found either as cells of the closed type, which do not reach the midgut lumen or as open-type cells, whose apices reach the midgut lumen (ENDO & NISHIITSUTSUJI-UWO 1981; NEVES *et al*. 2003; TAKASHIMA *et al*. 2011).

The presence of endocrine cells has been reported in the midgut of Orthoptera (LANGE & ORCHARD 1998; ZUDAIRE *et al*. 1998; LANGE 2001), Phasmatodea (ANDRIÉS & BEAUVILLAIN 1988; ZITNAN *et al*. 1993), Blattaria (ENDO & NISHIITSUTSUJI-UWO 1981; ANDRIÉS & TRAMU 1985), Lepidoptera (ZITNAN *et al*. 1995; AN *et al*. 1998; HUANG *et al*. 1998), Diptera (VEENSTRA 1999; MOFFETT & MOFFETT 2005) and Hymenoptera (RAES & VERBEKE 1994; SERRÃO & CRUZ-LANDIM 1996; NEVES *et al*. 2003). In Hemiptera, endocrine cells were described in the midgut of the predator *Brontocoris tabidus* (FIALHO *et al*. 2009) and in the hematophagous *Rhodnius prolixus* (BILLINGSLEY & DOWNE 1986; ZITNAN *et al*. 1993), *Cimex hemipterus* (AZEVEDO *et al*. 2009) and *Acyrthosiphon pisum* (DOWN *et al*. 2011).

The endocrine cells in the midgut of these insects are responsible for the production of neuropeptides such as FMRFamide, cholecystokinin, tachykinins, allatostatin, glucagon-like peptide, gastrin, P substance and urotensin I (ANDRIÉS & TRAMU 1985; ANDRIÉS & BEAUVILLAIN 1988; ZITNAN *et al*. 1993; VEENSTRA & LAMBROU 1995;

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HUANG *et al*. 1998; ZUDAIRE *et al*. 1998; LANGE 2001; HARSHINI *et al*. 2002; NEVES *et al*. 2003; MOFFETT & MOFFETT 2005; TAKASHIMA *et al*. 2011; LEE *et al*. 2012). To date, the biological role of these peptides is not clear, although it is suggested that the modulation of their release can be related to physiological changes caused by feeding, starvation and parasitism (ZITNAN *et al*. 1995; ZUDAIRE *et al*. 1998; LANGE 2001, SIMPSON & RAUBENHEIMER 2011). SEHNAL & ZITNAN (1996) stated that midgut endocrine cells of insects could probably have an important role in peristalsis, digestion, growth and reproduction control.

Isolation and identification of FMRFamide as a cardio-stimulator peptide was first performed in a coelenterate (PRICE & GREENBERG 1977) and later identified in other invertebrates, including insects (ZITNAN *et al*. 1995; LANGE 2001; HARSHINI *et al*. 2002; NEVES *et al*. 2002; HILL & ORCHARD 2004). The feeding cycle and feeding habits affect the occurrence of FMRFamide in the gut of *Locusta migratoria* (Orthoptera), suggesting a link between this peptide and the maintenance of nutritional content balance (LANGE 2001; HILL & ORCHARD 2004). NEVES *et al*. (2002) have studied the presence of different regulatory peptides in the endocrine cells of the midgut during metamorphosis in the bee, *Melipona quadrifasciata anthidioides*, among them FMRFamide, suggesting that this peptide can play a role in controlling digestion processes. In *Opisina arenosella* larvae, a peptide related to FxRFamide stimulates the release of amylase and protease in the midgut (HARSHINI *et al*. 2002) and in *Manduca sexta* larvae parasitized by *Cotesia congregata*, FMRFamide was found in a higher quantity than in larvae not parasitized, due to the suppression of the peptide release rate or its synthesis due to the presence of the parasitoid (ZITNAN *et al*. 1995).

In insects, as well as in all metazoans, homeostasis is essential for the morphogenesis and maintenance of tissue architecture and physiology. In different animal groups, the gastrointestinal tract is characterized by high recycling of cells that compose its epithelium; thus, homeostasis is assured by the control of complex activities such as programmed cell death, cell proliferation and differentiation (POTTEN *et al*. 1997; BRITTAN & WRIGHT 2002; TEIXEIRA *et al*. 2013).

Studies related to gut epithelium maintenance from the gastrointestinal stem cells use different animal groups as the experimental model, among which are mammals (MARTIN *et al*. 1998; SLORACH *et al*. 1999; BJERKNES & CHENG 2001; LEEDHAM *et al*. 2005), amphibians (ISHIZUYA-OKA 2007) and insects (OHLSTEIN & SPRADLING 2007; MARTINS *et al*. 2006; ILLA-BOCHACA & MONTUEGA 2006; TEIXEIRA *et al*. 2013).

The midgut epithelium of the fly *Drosophila melanogaster* has multipotent gut stem cells which can differentiate into both digestive and endocrine cells (OHLSTEIN & SPRADLING 2007). In the midgut of insects, stem cells are called regenerative cells, and are either located in clusters (nests) or isolated in the epithelium.

Endocrine cells and the identification of the regulator peptides produced by them, as well as the aspects of the midgut epithelial regeneration in Triatominae, are restricted to those described for *R. prolixus* (BILLINGSLEY & DOWNE 1986; ZITNAN *et al*. 1993).

This work has assessed the distribution of the regenerative and endocrine cells in the different midgut regions of the hematophagous hemipteran, *Triatoma vitticeps,* and tested the hypothesis of changes in their distribution according to the time following a blood meal.

Material and Methods

Animals

Thirty adults of both sexes of *T. vitticeps* (Stal, 1859) (Hemiptera: Reduviidae) were obtained from the Instituto René Rachou – FIOCRUZ, Belo Horizonte, state of Minas Gerais, Brazil. The animals were kept in colonies in the Entomology Laboratory of the Centro Universitário de Caratinga, Caratinga, state of Minas Gerais, Brazil, at 28°C and 60% relative humidity. After 15 days of starvation, the insects were permitted to feed on previously anesthetized rats (*Rattus norvegicus*). After the blood meal, the insects were dissected in 125 mM NaCl solution, at 4, 72, 120, 168, 288, 360 and 600 hours after feeding, the midgut was pulled apart and divided into anterior, middle and posterior regions. These times were chosen from a pilot study which demonstrated that 20 days after a blood meal, the midgut was empty.

Immunohistochemistry

For the identification of midgut endocrine cells, we performed immunohistochemistry analyses for the identification of cells positive to the presence FMRFamide, a current marker for insect endocrine cells (ZUDAIRE *et al.* 1998). The fragments of the different regions of the midgut were fixed in Zamboni solution (STEFANINI *et al*. 1967) for 30 min. After washing in 0.1M sodium phosphate buffer solution with 1% Tween-20 (PBST), the samples were immersed in 1% phenyl hydrazine solution for 40 min to block endogenous peroxidase. Fragments were then washed in PBST, transferred to 1.5% bovine serum albumin in PBST for

10 min and incubated in 2% goat serum in PBST for 20 min. Next, the samples were washed in PBST and incubated in the anti-FMRFamide primary antibody 1:100 in PBST (Peninsula Laboratories, San Carlos, CA) for 24 h at 4°C. Following this, the samples were incubated in anti-IgG conjugated secondary antibody with peroxidase (1:20) in PBST (Sigma-Aldrich, Saint Louis, MO) for 3 h at room temperature and the peroxidase was revealed with diaminobenzidine. Dehydration in a graded ethanol series and inclusion in glycol methacrylate Historesin (Leica, Heidelberg, Germany) were carried out subsequently. Sections 2 m in thickness were counter-stained with fast red for 10 min and analyzed under a light microscope. Negative control was performed by the omission of the anti-FMRFamide antibody.

Number of endocrine and regenerative cells

For cell count, 10 sections were analyzed per insect, per midgut region and for each time after a blood meal (n = 4), considering 10 fields per section, totaling 1000 fields per region of each insect and in each starvation period. The sections analyzed were at a minimum distance of 60 m from each other, to avoid counting the same cells twice. In counting the positive cells, nuclei for anti-FMRFamide, nuclei of the digestive cells, regenerative cells and others (muscle layer, trachea and Malpighian tubules) were considered. The sections were analyzed under a light microscope, with 40x objective and a reticulated eyepiece with 100 points.

Statistics

For statistical analysis, the starvation periods were distributed into classes. The association periods:sex, sex:midgut region, sex:cell types were subjected to chi-square testing. The ratio of the variable responses (nuclei of the digestive cells, regenerative cells, endocrine cells and other parameters) obtained in the midgut regions were tested for equality of ratio by the Chi-square test.

Results

The presence of the endocrine open-type cells was determined in the anterior, middle and posterior regions of the midgut of *T. vitticeps* (Table 1, Figs 1-4). The specificity of the reaction for FMRFamide was obtained by a comparison with the negative control.

Digestive cells were the most frequent, followed by regenerative and endocrine cells. Cell types and tissue structures in the midgut portions were distributed with a different frequencies in each region. In addition, the middle portion of midgut had a higher frequency of digestive cells *versus* the other regions, whereas the highest frequency of the regenerative cells was found in the posterior region. The frequencies of the endocrine cells were similar in all three midgut regions (Table 1).

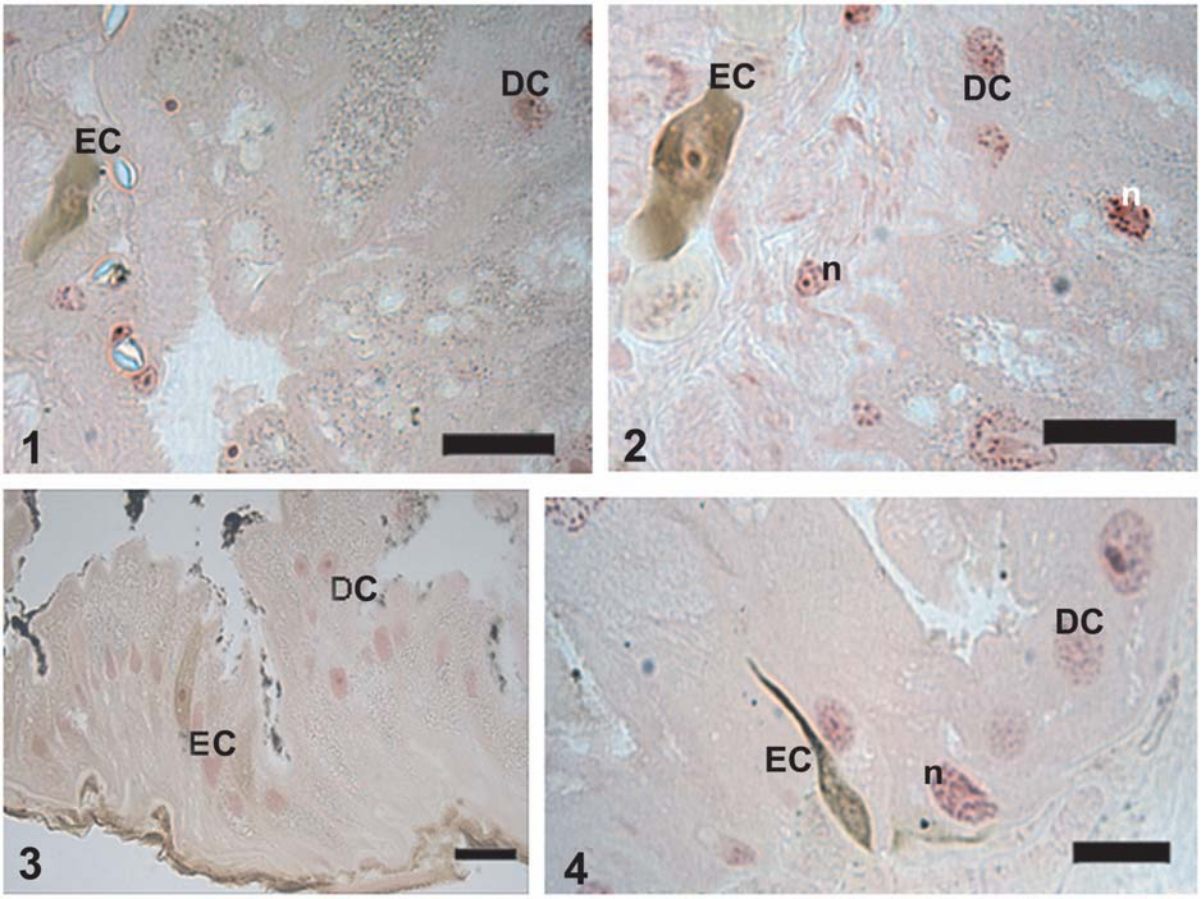
In the midguts of the females and males, different types of epithelial cells and other structures that integrate this organ occurred with different frequencies. The highest values were found for the parameter identified as “others” as well as for the digestive cells. A comparison between the sexes showed different frequencies of the digestive and regenerative cells, with males presenting a higher frequency of digestive cells than females and females having a higher frequency of regenerative cells than males (Table 2). Differences in the frequencies of the endocrine cells associated with sex were found when the different periods after a blood meal were analyzed, with the females presenting greater frequency of endocrine cells than the males in the intervals of 4 to 143 and 312 to 600 hours after a blood meal (Table 3).

Table 1

Number (n) and frequency (%) of different cell types and tissues (other) in the three midgut regions of *Triatoma vitticeps* from a total area of 12 x 10^4 mm^2 per midgut region

Cell type	Midgut region (N = 4)			Chi square
	Anterior	Median	Posterior	
	n (%)	n (%)	n (%)	
Endocrine	32 (0.03)^{Da}	42 (0.04)^{Da}	21 (0.02)^{Da}	9.5
Digestive	9599 (8.0)^{Bb}	10787 (8.99)^{Ba}	9563 (7.97)^{Bc}	97.2
Regenerative	293 (0.24)^{Cc}	363 (0.30)^{Cb}	411 (0.34)^{Ca}	19.8
Other	110076 (91.73)^{Aa}	108812 (90.67)^{Aa}	110002 (91.67)^{Aa}	9.1

Values followed by different small letters in the lines and capital letters in the columns are significantly different (Chi-square test). N – number of insects studied. Subscriptions under Tables 2-6 are identical to subscription under Table 1.



Figs 1-4. Immunohistochemistry of *Triatoma vitticeps* midgut showing FMRFamide positive endocrine cells in different starvation periods. 1 – Anterior midgut 72 hours after meal. 2 – Anterior midgut 72 hours after meal. 3 – Middle midgut region 168 hours starved. 4 – posterior midgut region 168 hours starved. EC – endocrine cells, DC – digestive cells, n – nucleus. Bars – 10 μm.

Table 2

Number (n) and frequency (%) of different cell types and tissues (other) in males and females of *Triatoma vitticeps* from a total area of 6 x 10^4 mm^2 per sex

Cell type	Male (N = 4)	Female (N = 4)	Chi square
	n (%)	n (%)	
Endocrine	47 (0.03)^{Da}	48 (0.03)^{Da}	0.011
Digestive	15474 (8.59)^{Ba}	14475 (8.03)^{Bb}	33.32
Regenerative	460 (0.26)^{Cb}	607 (0.34)^{Ca}	20.25
Other	164075 (91.12)^{Aa}	164865 (91,60)^{Aa}	1.92

In *T. vitticeps* males and females, different midgut regions showed different frequencies of endocrine cells. Males had a higher number of endocrine cells in the middle midgut region, whereas in females a higher number occurred in the anterior region. The posterior midgut had a lower number of endocrine cells in the entire midgut, with similar frequencies between the males and females (Table 4).

The regenerative cells were found at a higher frequency from 312 to 600 hours after a meal. From 4 to 143 hours after the blood meal, the females and males did not show any differences in the frequencies of the regenerative cells. From 144 hours after the blood meal, an increase in the number of this cell type in both sexes occurred, with the females presenting a higher frequency than the males (Table 5).

Table 3

Number (n) and frequency (%) of endocrine cells in males and females of *Triatoma vitticeps* after different blood meal periods, from a total area of 6 x 10^4 mm^2 per sex and interval

Hours after blood meal	Male (N= 4)	Female (N=4)	Chi square
	n (%)	n (%)	
4-143	7 (14.9)^{Bb}	19 (39.6)^{Ba}	6
144-311	40 (85.1)^{Aa}	23 (47.9)^{Ab}	5
312-600	0 (0)^{Cb}	6 (12.5)^{Ca}	6

Table 4

Number (n) and frequency (%) of endocrine cells in the three midgut regions of males and females of *Triatoma vitticeps* from a total area of 6 x 10^4 mm^2 per sex and midgut region

Midgut region	Male (N = 4)	Female (N = 4)	Chi square
	n (%)	n (%)	
anterior	8 (17.0)^{Bb}	24 (50.0)^{Aa}	8
median	31 (66.0)^{Aa}	11 (22.9)^{Bb}	9.5
posterior	8 (17.0)^{Bb}	13 (27.1)^{Ba}	1.2

Table 5

Number (n) and frequency (%) of regenerative cells in males and females of *Triatoma vitticeps* after different blood meal periods, from a total area of 6 x 10^4 mm^2 per sex and interval

Hours after blood meal	Male (N = 4)	Female (N = 4)	Chi square
	n (%)	n (%)	
4-143	174 (37.83)^{Aa}	149 (24.54)^{Ca}	1.9
144-311	130 (28.26)^{Cb}	208 (34.27)^{Ba}	18
312-600	156 (33.91)^{Bb}	250 (41.19)^{Aa}	21.8

Table 6

Number (n) and frequency (%) of digestive cells in males and females of *Triatoma vitticeps* after different blood meal periods, from a total area of 6 x 10^4 mm^2 per sex and interval

Hours after blood meal	Male (N = 4)	Female (N = 4)	Chi square
	n (%)	n (%)	
4-143	6199 (40.06)^{Aa}	5558 (38.4)^{Ab}	34.9
144-311	4206 (27.18)^{Ca}	3895 (26.91)^{Cb}	11.9
312-600	5069 (32.76)^{Ba}	5022 (34.69)^{Ba}	0.2

The frequency of the digestive cells varied in the males and females according to the different periods after feeding. In both sexes, these cells had lower frequencies in the time class 144-311 hours of starvation, but showed an increase after 312 hours after the meal. In addition, the males had higher frequencies of digestive cells during the starvation periods of 4-143 and 144-311 hours. After 312 hours, this difference between sexes was no longer observed (Table 6).

Discussion

Our findings showed the occurrence of endocrine cells of the open type which were positive for FMRFamide. The presence of FMRFamide in the endocrine cells of the midgut was also found in other insects (ZITNAN *et al*. 1995; NEVES *et al*. 2002; HARSHINI *et al*. 2002). In the hemipteran *R. prolixus,* the presence of FMRFamide endocrine cells of the open type was found by ZITNAN *et al*. (1993), but BILLINGSLEY & DOWNE (1996), stated that there are closed-type FMRFamide endocrine cells in the posterior midgut region of this insect. The endocrine cells in the midgut of *T. vitticeps* are of the open type with a higher frequency in the middle midgut region. Variation between our results and those obtained by BILLINGSLEY and DOWNE (1996) may be due to the difference in the divisions of the midgut regions. In light of this, we divided the midgut into three regions, which differ structurally and physiologically, whereas BILLINGSLEY & DOWNE (1996) divided the midgut into only two regions, the anterior and posterior, thus not considering the different morphophysiology of the middle and posterior regions.

In *T. vitticeps,* the frequencies of the endocrine cells varied according to the feeding cycle, midgut region and sex, suggesting functional diversity of these cell types during digestion. The midgut endocrine cells in *Aedes aegypti* have various shapes, forming a heterogeneous population, which could act in the paracrine pathway, regulating the digestive functions of the adjacent epithelial cells and muscle layers, as well as in the endocrine pathway, releasing its secretion into the hemolymph (MOFFETT & MOFFETT 2005). The open type cells, through their projections, may release substances into the midgut lumen, acting as modulators of the digestive processes; else, as sensory cells when regulating the release of peptides from other cell surfaces. Considering the different endocrine cell types and their role in digestion, FUJITA and KOBAYASHI (1977) stated that endocrine open type cells act as primary sensors which registered the presence of food and nutrients in the midgut lumen, whereas the closed-type would respond to variation in the gut volume.

The frequency of the different cell types in the midgut epithelium of *T. vitticeps* was similar to those described by BILLINGSLEY & DOWNE (1986), with the digestive cells being the most numerous, whereas lower frequency was detected for the endocrine cells in the midgut of *R. prolixus*. In light of this, the endocrine cells should be present in all insects, so that reports on the absence of this cell type in the midgut of insects should be interpreted with caution, due to their low frequency.

In *A. aegypti*, the posterior midgut region (posterior stomach) of the females is two-fold bigger than in males and has a higher endocrine cell frequency, density and size when compared with males (MOFFETT & MOFFETT 2005). LANGE and ORCHARD (1998) found higher positivity for FMRFamide in the midgut of the *L. migratoria* female when compared with the male; however, they did not discuss these results. Unlike these insects, *T. vitticeps* females and males had a similar frequency of endocrine cells along the midgut, in spite of the different frequencies found when the sex of *T. vitticeps* was associated with the starvation periods. The lower frequency of the positive FMRFamide endocrine cells in the middle region of the midgut of *T. vitticeps* females may be associated with differences in the reproductive metabolism and in the role of feeding in this process in both sexes, because the nutritional status of Triatominae has little effect on male reproductive activity (KHALIFA 1950; RÉGIS *et al*. 1985), but the lack of important blood nutritional factors affects egg production (WIGGLESWORTH 1936).

The middle midgut region is the main site of midgut enzyme synthesis in Hemiptera, followed by the posterior region, that plays a role in the final digestion and also secretes some enzymes (WIGGLESWORTH 1975; HOUSEMAN & DOWNE 1983; BILLINGSLEY & DOWNE 1983; BILLINGSLEY & DOWNE 1986; BILLINGSLEY 1990; BORGES *et al*. 2006; GUEDES *et al*. 2007; FIALHO *et al*. 2009, 2012). The similar number of endocrine cells in all midgut regions of *T. vitticeps* suggests the participation of these cells in enzyme synthesis and/or release in the midgut as suggested for other insects (BROADWAY & DUFFEY 1986; LEHANE *et al*. 1996; NACHMAN *et al*. 1997; NEVES *et al*. 2003). FMRFamide regulates *in vitro* secretion of trypsin and amylase in midgut cultured cells of *O. arenosella* (Lepidoptera) (HARSHINI *et al*. 2002).

In spite of the predominance of immune reactive cells for peptides similar to those of the family FMRFamide in the midgut of most insects studied, its function is not yet known (MERTE & NICHOLS 2002). The influence of these peptides in the digestive processes can be related to myotropic action on the visceral muscles (SCHOOFS *et al*. 1993; GEARY *et al*. 1999; LAJEUNESSE *et al*. 2010; MARCINIAK *et al*. 2011), in addition to their role in enzyme secretion, water and electrolyte control and nutritional content balance (VEENSTRA *et al*. 1995; KINGAN *et al*. 1997; ZUDAIRE *et al*. 1998; MERTE & NICHOLS 2002; HILL & ORCHARD 2004; BECHTOLD & LUCKMAN 2007; NÄSSEL & WINTHER 2010). Thus, different frequencies of FMRFamide endocrine cells of *T. vitticeps* with different starvation periods in the regions of the midgut, suggest the occurrence of various types of regulatory peptides

of the family FMRFamide, which have different functions in the midgut regions of this hemipteran.

The decrease in the number of digestive cells in *T. vitticeps* in the interval from 144 to 311 hours after a blood meal may be due to the loss of the midgut epithelial cells resulting from wear suffered by the digestive and absorptive processes. Enzyme secretion by the digestive cells in the midgut of Hemiptera may be similar to that of apocrine cells (BILLINGSLEY & DOWNE 1986) or by the release of small secretory vesicles (AZEVEDO *et al*. 2009; FIALHO *et al*. 2013), characterized by constant cell death and the necessity of replacing them by the regenerative cells (CRUZ-LANDIM *et al*. 1996). In addition, blood digestion generates derivatives such as free radicals and the heme group, which are toxic for cells, promoting their death (OKUDA *et al*. 2005; JARIAL 2005; CRUZ-LANDIM *et al*. 1996; BILLINGSLEY & LEHANE 1996; NEVES *et al*., 2003; TETAMANTTI *et al*. 2007; ROST-ROSZKOWSKA 2008; FIALHO *et al*. 2009; AZEVEDO *et al*. 2009). The highest frequency of the regenerative cells in the midgut of *T. vitticeps* from 312 h after a blood meal is probably determined by the high necessity of cell replacement due to increasing cell death along the feeding cycle due to enzyme secretion and/or exposure to oxygen-free radicals.

Whether the middle and posterior regions of the midgut are the sites of synthesis of the higher digestive enzymes, in addition to exposure to toxic substances, necessitates a stronger presence of the regenerative cells for the maintenance of tissue homeostasis, as shown herein. In light of this, in the regenerative process of the midgut epithelium in *T. vitticeps,* the regenerative cells generally assure the production of digestive cells from cellular differentiation, which is supported by the increase in frequency of digestive cells in the feeding cycle end, i.e. in the period required for digestion in the next blood meal.

Only the endocrine cells of the three midgut regions, in both sexes, have lower frequencies in long-starved *T. vitticeps*. These findings support the data obtained in *L. migratoria* with the regenerative cells giving rise to all cell types of the midgut epithelium, although the origin of the endocrine cells from the regenerative ones is yet to be resolved due to the longer time spent in the differentiation cycle for this cell type when compared to the time spent for the digestive cells (ILLA-BOCHACA & MONTUENGA 2006).

Overall, the present study showed a link between digestive processes and the frequency of the FMRFamide-positive endocrine, digestive and regenerative cells in the midgut of *T. vitticeps*. The findings also showed differences occurring in the distribution pattern of midgut cell types between the males and females and in different regions of the *T. vitticeps* midgut. More generally, this study presents insight in the complexity of the digestive process of the Chagas' disease vectors.

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