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STRUCTURAL CELLS IDENTIFICATION IN ARMADILLO STOMACH: AN OPPORTUNITY TO KNOW ABOUT ITS DIGESTION

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ABSTRACT

This study presents the morphological characteristics of the stomach in *E. sexcinctus* focusing on the composition and distribution of gastric cells, relating the function of each region of the armadillo's stomach. Three stomachs of adults were used in this study. Each stomach was examined by macroscopy. Samples from various parts of the stomach were taken for routine histological examination. The stomach of E. sexcinctus is similar to the stomach of a carnivore, but it has macroscopic and microscopic characteristics that differentiate it from these. The stomach interposes between the esophagus and the small intestine occupying four abdominal regions, predominantly in the umbilical and left lateral regions. It is intensely pleated and composed of a glandular surface. The cells present in the gastric mucosa can be found in the three anatomical regions of the stomach: cardia, body and pylorus. However, they are statistically different within the regions of the stomach ($p > 0.05$). These data corroborate the concept that cells are distributed differently depending on the region of the stomach. Thus, studies on the physiology, eating habits and behavior of this animal in the ecosystem are still needed to provide knowledge that best elucidates gastric function and the relationship with its morphology.

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INTRODUCTION

The Euphractus sexcinctus, Linnaeus 1758 also known as a six-band armadillo or yellow armadillo (Abba and Superina, 2010; Anacleto *et al.*, 2006; Balamayooran *et al.*, 2015), is a small mammal of the order Xenarthra, Dasypodidae native to South America, (Redford and Wetzel, 1985) and inhabiting a wide variety of biomes, including the Amazon, Caatinga, Cerrado, Pantanal, Chaco and the Atlantic Forest (Fonseca *et al.*, 1996; Paglia *et al.*, 2012; Silva-Junior and Nunes, 2001). The six-band armadillo feeding is opportunist with great diversity of items, classifying them as carnivore / omnivore (Redford, 1985). They consume many types of animal prey, invertebrates, anurans, carrion and plant matter such as fruits and tubers (Bezerra et al., 2001; Dalponte and Tavares-Filho, 2004; McDonough and Loughry, 2001; Vaz I 2012). Euphractus diets vary according to the geographical and seasonal distribution of the food, (Dalponte and Tavares-Filho, 2004), vegetables and invertebrates are the most representative items in the diet (Redford, 1985; Wetzel, 1982).

The species has a solitary habit, but several individuals can gather around some dead animal to feed on meat and larvae (Nowak and Walker, 1999). Omnivores have a digestive system very similar to carnivores, in which the stomach is an organ of the digestive tract where important steps of the digestion process occur (Dyce et al., 2010). The organ, located on the left side of the abdominal cavity, below the diaphragm, represents the most dilated portion of the gastrointestinal tract and communicates proximally via the cardiac orifice with the esophagus, and distally with the duodenum via the pylorus. Three other gastric regions are described: the proximal distensible fundus, the distal funnel-like antrum, and the corpus of the stomach between the fundus and antrum (Landa et al., 2019; Soybel, 2005). Histologically, the stomach is composed by four layers. From the inside out they are mucosa, submucosa followed by muscular externa and serous layer, each respectively contributing in digestive functions. These layers vary little in their development, not only between species but also within different areas of the stomach (Banks, 1991), specifically in carnivores the stomach is made up of a glandular layer (Zahariev et al., 2010). In a cross section of the mucosa it is possible to observe the main cellular components of the epithelium: oxyntic (parietal) cells, zymogenic (chief) cells, surface and neck mucous foveolar (pit) cells, and hormone-secreting enteroendocrine. Each has its prevalent location and specific function. Foveolar cells protect and produce mucus on the mucosal surface, parietal or oxyntic cells secrete gastric acid, neck mucous cells secrete viscous mucus, and zymogens are capable of producing and secreting digestive enzymes (Fothergill et al., 2019; Mills and Shivdasani, 2011). The aim of this study was to describe the anatomy, histology and histochemistry of E. sexcinctus stomachs, as well as the composition and distribution of gastric cells in the organ lumen, relating the function of each stomach region, in order to produce information that enables comparative analysis with other omnivores. Therefore, the morphological knowledge of armadillos is necessary in order to allow the proper management of these animals in the environment and to substantiate clinical procedures that may result in any assistance in this species.

MATERIALS AND METHODS

Ethical and legal aspects: Three female adult armadillos were selected, which came to death victims of predatory hunting in the Serra da Capivara region, northeast state of Piauí (between latitudes 8° 26' 50" and 8° 54' 23" south and longitudes 42° 19' 47" and 42° 45' 51" west) seized by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio-PI). The animals were donated and sent frozen to the Morphology Laboratory of the Morphology Departament of the Federal University of Piaui (UFPI), where they were corretcidentificed necropsied. This research was authorized by SISBIO (No. 53303) and approved by the Committee on Ethics in Animal Experimentation with protocol nº 136/16.

Animals: After defrosting, the specimens were fixed with subcutaneous, intracavitary 10% buffered formaldehyde and immersed in a reservoir with the same solution for 24 hours. Subsequently, the animal was positioned in dorsal decubitus position to be dissected with the aid of a blade scapel handle no. 4 or 5. To access the organs of interest at digestory system, an incision was made in the ventral midline for opening the abdominal cavity and pelvis; followed by partial removal of the sternum and some ribs of the left antimer. The muscles and structures attached to stomach were individualized and removed, allowing the removal of stomach. The stomachs were dissected in order to evidence the anatomical constituents of this structure. Subsequently, an incision was made in the great curvature to obtain fragments for other techniques.

Light microscopy

For analysis by light microscopy, after obtaining the macroscopic results of each organ, fragments with about 0.5 cm of each, from of the stomach regions (cardia, body and pylorus) one was obtained which were kept in 10% formaldehyde buffered for 48 hours. After this minimum period, the semithin sections (0.5 μm) of the stomachs were placed on glass slides and stained with submitted to the histological routine for staining by Hematoxylin-Eosin, Toluidine Blue and Masson's Trichrome, which are routinely used in our laboratory for overview staining because of its simple implementation and easy to interpret results. The sections were examined and photodocumented was done using the Leica DM 2000 microscope (Leica Microsystems®, Germany). The analyzed histological slides were submitted to differential counting of mucosal cells (foveolar, parietal, cervical and zymogenic cells) in four fields visualized in histological sections of each region of the stomach (cardia, body and pylorus) in each animal performed with the aid of the software Microscope Imaging Leica X- LAS X (Leica Microsystems®, Germany).

Preparation of overview drawings

An overview diagram was created summarizing the structural information of a representative panorama that consists of overlapping images. Relevant structures, such as cell organelles, were designed in an overview scheme, digitized and modified in Adobe Illustrator (Adobe Creative Suite CS4).

Statistical Analysis

Descriptive statistics and inferential tests were performed. The Friedman non-parametric test was used to compare the different cells distributed in the cardia, body and pylorus regions of the stomach with the Simes-Hochberg multiple comparison tests. The level of significance was 5%.

RESULTS

The *E. sexcinctus* stomach is located in four abdominal regions: xiphoid, left hypochondriac, left umbilical and lateral, with predominance in umbilical and left lateral regions (Fig 1B). The organ is limited on its parietal surface by the liver and esophagus, on its visceral surface by the small and large intestines, and on the left end of the major curvature by the spleen (Fig 1A). It was observed that the armadillo's stomach is fixed to the liver through the minor omentum and hepatogastric ligament, with the spleen by the gastroesplenic ligament, and with the diaphragm by the gastrophrenic ligament. The major omentum appears affixed to the greater curvature of the stomach (Fig 1A). The armadillo's stomach is a saccular organ with its whitish, smooth and shiny outer surface coloring. It has a variable diameter, being the smallest in the transition regions with the esophagus (cardial part) and the small intestine (pyloric part), regardless of the degree of gastric repletion. The largest diameter is limited to the region of the gastric body, comprised between the cardial and pyloric parts, the latter being marked by an annular structure of firm consistency, the pyloric sphincter. The organ has two faces, one parietal and the other visceral, respectively, positioned dorsally and ventrally. In the anatomical contour, two curvatures were observed, one smaller concave and one larger convex extending from the cardia to the pylorus (Fig 1C, 1D, 1E). The cardic and pyloric notches have shallow angulation, making the fundus region smaller, and the body region of the stomach larger.

The internal view of the stomach shows that the armadillo *E. sexcinctus* gastric mucosa is intensely creased, and that these gastric folds are clearly tortuous with longitudinal orientation, parallel to the largest axis of the organ (Fig 1D). The stomach of *E. sexcinctus* is all glandular. Histologically presented the following layers: mucosa, submucosa, muscularis externaand serosa. In the mucous layer were present a columnar simple epithelium (epithelial mucous surface cells) that lined the organ's lumen, and this epithelium was interrupted by the gastric pits (or gastric foveolas), consisting of the faveolar cells in a single layer, mucous surface cells. Cells covering the stomach exhibited abasal spherical nucleus of loose chromatin, with an evident nucleolus. The pits continue the gastric glands. In the isthmus of the glands, parietal (oxyntic) and mucous cells were presented. In the neck of the gland, which has a constitution similar to that of the isthmus, we observed mucous neck cells, progenitor cells and, finally, at the base of the gastric gland, which houses, besides mucous cells, the zymogenic cells (or Chief cells). Most of the lamina propria of the mucous layer is occupied by mucous glands. The lamina propria was made up of loose connective tissue all regions. The muscle layer lies deep to the lamina propria and is made up of smooth muscle tissue with circular and longitudinal fibers. These three layers together constitute the

Table 1-Compare the different cells distributed in the cardia, body and pylorus regions of the stomach. Friedman rankings of the different cells, after multiple comparison test - FWER (Simes- Hochberg). Significance level 5%.

Group Leaderboards		
Factors	***Amount (Rank)***	***Groups***
Parietal	12	A
Pit	9	B
Mucous	5	C
Zymogenic	4	C

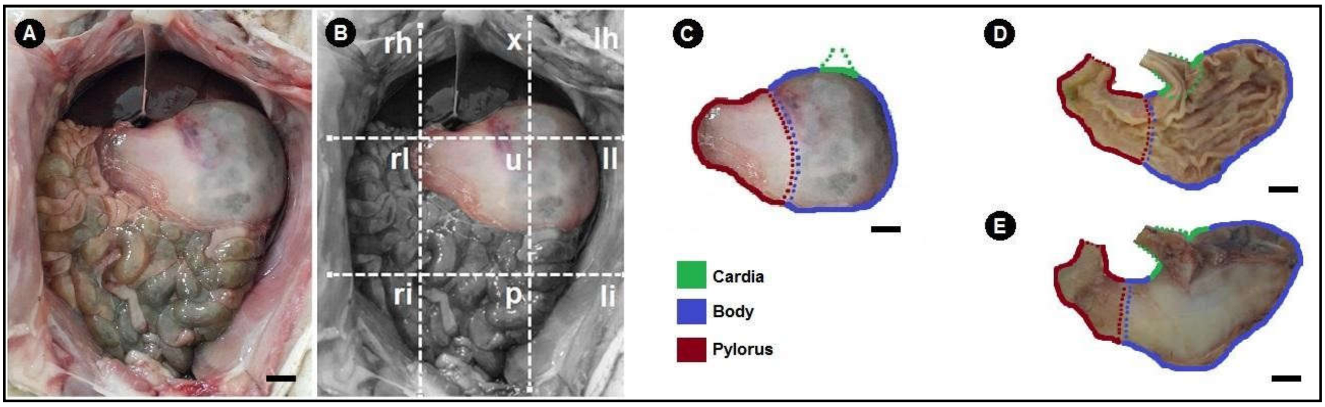

Fig. 1- Macroscopic board of the topography of the organs of the abdominal cavity of the armadillo E. sexcinctus in ventral view (A) and diagram (B) of the regions that compose the cavity: rh (right hypochondriac), x (xiphoid), lh (left hypochondriac), rl (right lateral), u (umbilical), ll (left lateral), ri (right inguinal), p (pubic) and li (left inguinal). The ex situ stomach (C), its mucosa organized in gastric folds (D) and external wall (E), in the ventral position, are also observed, with emphasis on the three anatomical regions of the organ: cardia (green), body (blue) and pylorus (red). Bar: 1cm

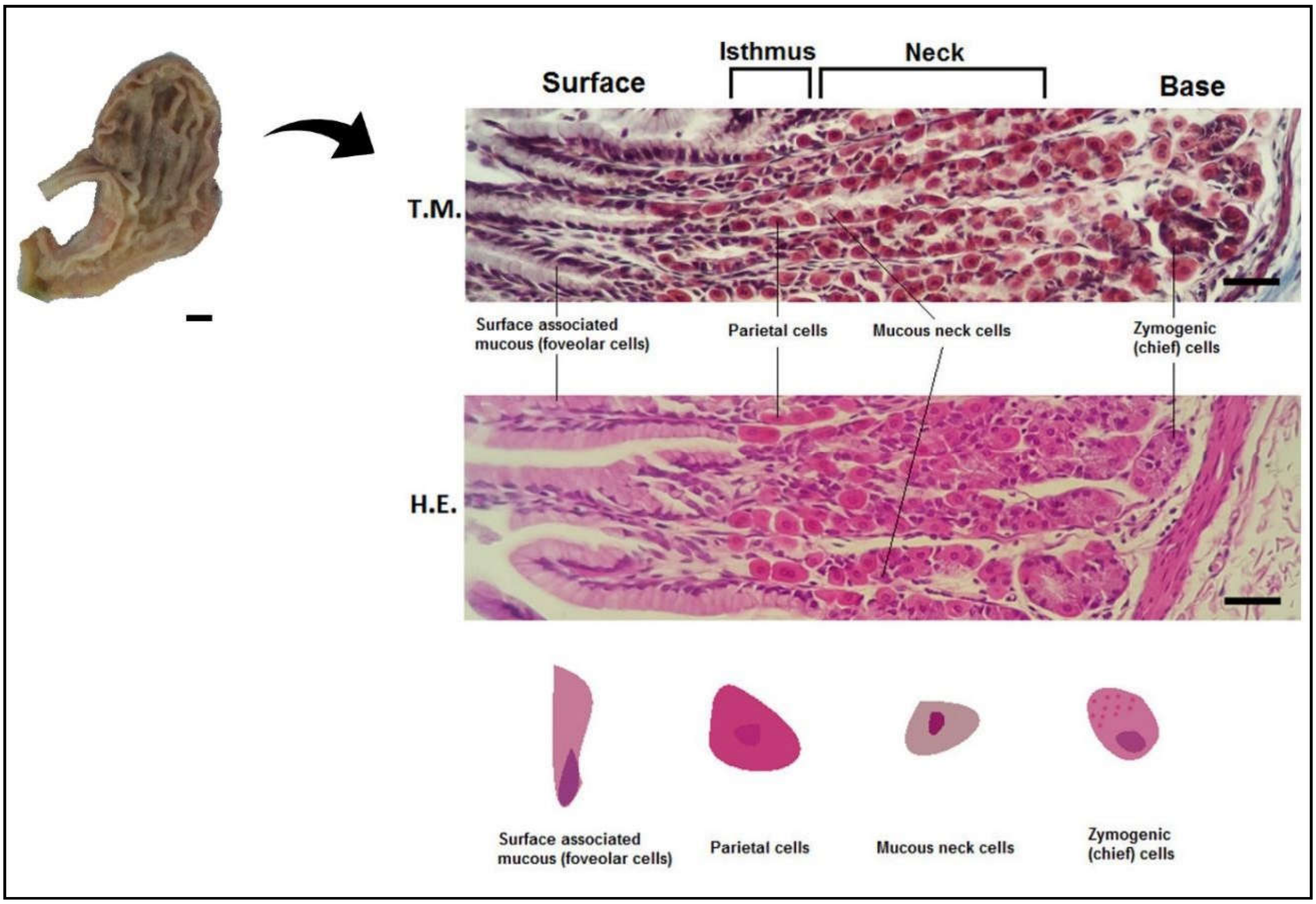

Fig. 2- Photomicrograph of the gastric mucosa of the E. sexcinctus armadillo in a longitudinal section of the stomach stained in Hematoxylin-Eosin (HE) and Masson's Trichrome (TM) highlighting the surface regions of the mucosa, isthmus, neck and base, with schemas of the cell types that make up these regions: foveolar, parietal, mucous neck, and zymogenic cells. Bar: 50µm.

mucous layer of the stomach. The submucosa, made up of connective tissue, is much thicker than the lamina propria and has many vessels and nerves. The muscle layers are typical, forming one more inner, inner circular muscle, and one more outer, outer longitudinal muscle, both of smooth muscle tissue. Externally lining the stomach, the serosa, consisting of simple paved epithelium and connective tissue, was observed. Regarding the serous tunic, it was verified by microscopy that it is constituted by loose connective tissue, blood vessels and mesothelium. There are four distinct regions in the stomach pits of the armadillo: mucosal surface, isthmus, neck and base. The mucosal surface of the stomach of the *E. sexcinctus* was composed of simple columnar epithelial cells, producing a viscous mucus; the isthmus, the thinnest region of the crypts is composed of parietal, and mucosal cells; the base presenting a constitution identical to the isthmus still presents progenitor cells and finally the base that houses besides mucosal cells, the zymogenic cells (Fig. 2). The cells present in the gastric mucosa can be found in the three anatomic regions of the stomach (cardia, body and pylorus). In the *E. sexcinctus* there is the predominance of parietal cells in the pylorus, to the detriment of the other regions (Fig 3). The mucous neck cells are more prevalent in the cardia region, while zymogenic cells are found in greater numbers in the body region (Fig 3). Friedman's test of the cell count was significant, with p-value equal to 0.042, which ratifies the existence of at least two groups of statistically different cells within the stomach regions at the 5% level of significance. After analysis of multiple Simes-Hochberg comparisons one can assemble

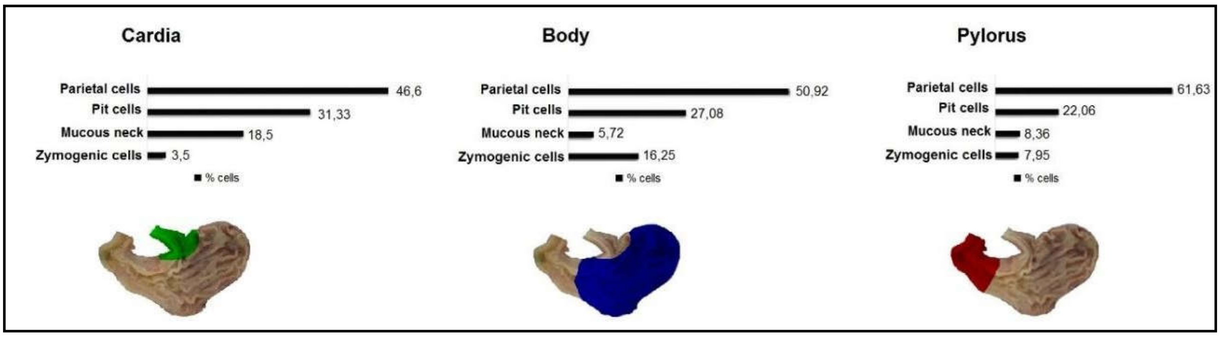

Fig. 3. Percentage of parietal, mucous, cervical and zymogenic cells of the armadillo in the anatomical regions of the stomach: cardia, body and pylorus, and the schematic representation of the referred region in the stomach.

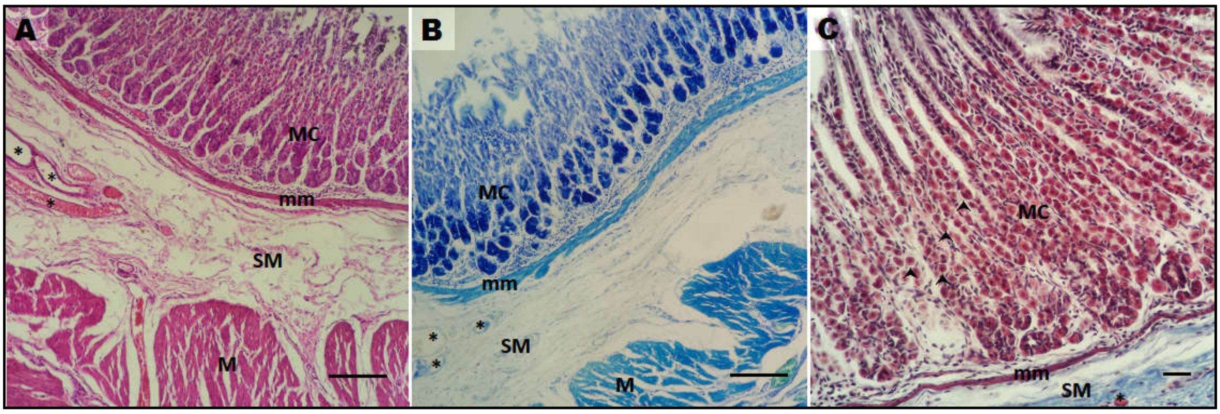

Fig. 4. Photomicrography of the cardia region of the stomach of the armadillo, in different histological stains, exposing the several layers: Mucosa (MC), muscular of mucosa (mm), Submucosa (SM), Muscularis (M) and blood vessels. A- Different layers of the stomach, in the coloration H.E, 50x, with the bar equivalent to 100µm. B- Different layers of the stomach, in the coloration of Toluidine Blue, 50x, with bar equivalent to 100µm. C- Mucosa and part of the submucosa, evidencing the largest amount of parietal cells (arrowhead) in its composition, in the color of Masson's Trichrome, 200x, with the bar equivalent to 50µm.

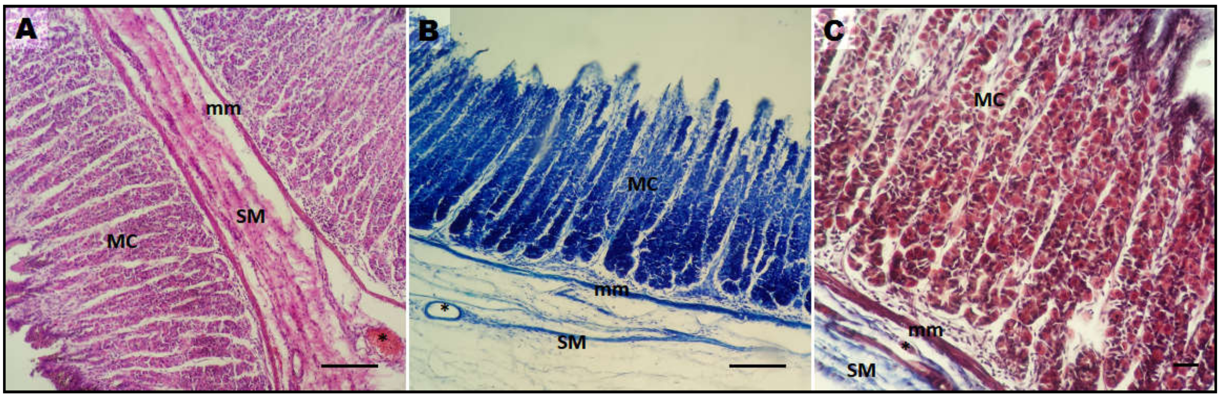

Fig. 5. Photomicrographic board of the fundus region of the stomach of the armadillo, in different histological stains, determining the Mucosa (MC), muscular of mucosa (mm), Submucosa (SM), Muscularis (M) and blood vessels (*). A- Different layers of the stomach, in the coloration H.E, 100x, with the bar equivalent to 100µm. B- Different layers of the stomach, in the coloration of Toluidine Blue, 100x, with bar equivalent to 100µm. C- Mucosa and part of the submucosa, showing the equivalence of parietal and chief cells, in the coloration of Masson's Trichrome, 200x, with the bar equivalent to 50µm

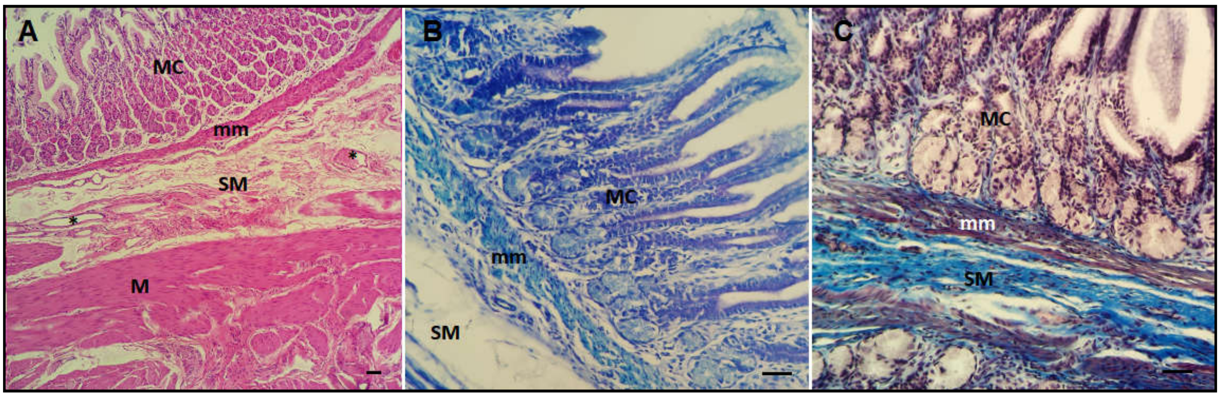

Fig 6-Photomicrography of the pyloric region of the stomach of the armadillo, in different histological stains, constituted of Mucosa (MC), muscular of mucosa (mm), Submucosa (SM), Muscularis (M) and blood vessels (*). A- Mucosa and the predominance of musculature in the submucosa, in the coloration H.E, 100x, with the bar equivalent to 100µm. B- Different layers of the stomach, in the coloration of Toluidine Blue, 100x, with bar equivalent to 100µm. C- Mucosa and pyloric glands, in the coloration of Masson's trichrome, 200x, with bar equivalent to 50µm.

the rankings table (Table 1), verifying that only the zygomatic and mucosal cells are statistically equal within the counts in the different areas of the stomach. These data corroborate with the concept that the cells distribute differently depending on the stomach region. In the cardia region of the armadillo, the gastric foveoli are not deep and the glands have a broad lumen. Notably, the predominance of parietal cells in the general constitution of their mucosa can be seen (Fig 4). In the funicular region (Fig 5), there is a proportionality between the number of parietal and mucous cells. Gastric fovéoles are seen more deeply than those of the cardia, with branched tubular glands at the bottom of each foveal. The pyloric region (Fig 6) has a greater number of isolated glands in relation to the cardia and fundus regions. The pylorus foveoli are longer and the glands shorter. This region is constituted of chief cells that secrete mucus, interspersed by parietal cells. There is also a thick layer of musculature, which is present between all the layers of the stomach.

DISCUSSION

Morphological research on the digestive system of species of the Xenarthra order, such as *Tamandua tetradactyla* and *Bradypus torquatus*, brought important clarifications about their architecture and cellular arrangement(Carvalho *et al.,* 2014; Mesquita *et al.,* 2015; Pinheiro *et al.,* 2014; Rezende *et al.,* 2011). However, there are still few studies produced on the subject in the family Dasypodidea. The realization of morphological and morphometric research in wild animals makes it possible to obtain more accurate results in order to subsidize studies on the knowledge of digestive processes and the adequate food for each species. The stomach is a large dilation in the alimentary canal (Dyce *et al.,* 2010). The topographic location of this organ between the esophagus and the small intestine is related to the performance of its functionality, temporary storage and chemical digestion of food for later absorption of nutrients (Dyce *et al.*, 2010). In *E. sexcinctus*, the synthetic characteristics agree with the majority of those reported in domestic and wild monogastric mammals(Barone, 2001; Barros Moraes *et al.,* 2002; Borsari *et al.,* 2010; Dyce *et al.,* 2010; Machado *et al.,* 2015; Mesquita *et al.,* 2015; Pinheiro *et al.,* 2014). However, there are gastric anatomical particularities described in other mammals(Mesquita *et al.,* 2015; Pinheiro *et al.,* 2014; Rezende *et al.,* 2011). Mammals have a considerable diversity in shape and structure of the digestive system (Pinheiro *et al.,* 2014; Rezende *et al.,* 2011). It is known that the various formats of gastric compartments are specialized to meet the specific needs of animal digestion, depending on the form of feeding (herbivore, carnivore or omnivore) (Kim and Shivdasani, 2016; Rezende *et al.,* 2011). Therefore, *E. sexcinctus*, being an omnivorous animal, has a small, saccular and unicavitary stomach, similar to that of carnivores and Tamandua tetradactyla, to digest food more easily (Dyce *et al.,* 2010; Pinheiro *et al.,* 2014; Vaz *et al.*, 2012).

Physiology and morphology go together when the goal is to know a certain organ. In the armadillo and anteater, the internal surface of the stomach is rough to increase the mucosal surface area, allowing gastric expansion with food intake (Hsu *et al.,* 2020). The stomach wall of animals and humans is formed by four layers of different tissues: mucous, submucosa, muscular and serous (Banks, 1991; Borsari *et al*., 2010; Pinheiro *et al*., 2014). The structural pattern of tubular organs contributes to the knowledge of the specific characteristics of each organ related to food, helping to differentiate each species (Ofusori *et al.,* 2008). The histological pattern of the mucous membrane is similar to that found in the anteater *(Pinheiro et al.,* 2014), in the mocó(Borsari *et al.,* 2010), in the paca(Machado *et al*., 2015) and domestic animals (Zahariev *et al.,* 2010). The armadillo has a totally glandular mucosa like that of the capybara (Barros Moraes *et al.,* 2002) and *Lagostomus maximus* (Tano de la Hoz *et al.,* 2020). The anteater (Pinheiro *et al.,* 2014), although it is phylogenetically related to the armadillo, has an aglandular region in its stomach mucosa. These discrepancies may be associated with the fact that several species are forced to adapt to consume alternative diets in environments where the preferred food is not available (Kotzé *et al.,* 2006). Therefore, it can be inferred that the stomach mucosa is adapted to supplies available in nature without this implying any damage to food and to the good nutrition of the species. Thus, it is suggested that animals with different eating habits may have similarities in the constitution of the stomach mucosa, such as *L. maximus* and *E. sexcinstus* (Pereira *et al.,* 2016; Tano de la Hoz *et al.,* 2020; Walters *et al.,* 2014). The structure of the gastric mucosa is composed of invaginations that have continuity with the gastric glands that receive their secretions (Eurell and Frappier, 2012). The depth of glands in mammals varies according to the region in which it is located (Eurell and Frappier, 2012). In the armadillo, the pyloric glands are branched and with a narrow lumen when compared to the cardic glands, simple and with ample light, differing from the pyloric glands of the monkey's stomach (Fayed *et al.,* 2010). In the armadillo, the pyloric glands are branched and with a narrow lumen when compared to the cardiac glands, simple and with ample light, differing from the pyloric glands of the monkey's stomach (Fayed *et al.,* 2010). Such glands are divided into four regions (surface, isthmus, neck and base) which are lined by four stomach cell types: parietal, pit, cervical mucosa and zymogenic cells that are distributed throughout the entire organ with independent functions(Barros Moraes *et al.,* 2002; Choi *et al.*, 2014; Garcia *et al.,* 2000; Junqueira and Carneiro, 2013; Karam and Leblond, 1993, 1992; Machado *et al.,* 2015; Mills and Shivdasani, 2011; Pinheiro *et al.,* 2014). This arrangement can also be found in domestic carnivores (Bacha and Bacha, 2003), agouti (Garcia, 1989; Garcia *et al.,* 2000), capybara (Barros Moraes *et al.,* 2002) and paca (Machado *et al.,* 2015). However, domestic ruminants do not have the same cellular distribution, since the presence of multiple chambers gives the mucosa an increase in extracts: horny, granular and spiny; based on their functional needs (Eurell and Frappier, 2012; Langer, 1988). The cells present in the gastric mucosa can be found in the three anatomical regions of the stomach (cardia, body and pylorus). However, the distribution varies according to digestive needs (Ofusori *et al.,* 2008). Therefore, each of the stomach segments has histological differences that are involved in unique roles in the digestion process (Landa *et al.,* 2019).

The count of stomach cell types is variable according to the species analyzed, with the highest concentration of parietal cells in the cardia, body and pylorus in humans and domestic carnivores (Choi *et al.,* 2014; Helander, 1981; Ito, 1981) while in mice, there are a greater number of zymogenic cells in the body region of the stomach(Karam and Leblond, 1992). Mucous cells are more prevalent in the armadillo's cardia region. The large amount of these cells in the cardia and pylorus of mammals is correlated with the secretion of high levels of mucins to neutralize the corrosive and harmful effect of HCl and pepsin from gastric juice, thus helping to maintain normal pH levels in this organ (Fayed *et al.,* 2010; Igbokwe and Obinna, 2016). There is a predominance of parietal cells in the constitution of the mucosa, which differentiates it from other animals in which the occasional cells constitute the main type in the glandular region of the cardia (Bacha and Bacha, 2003). This difference may be probably due to the phylogenetic distance between the mammals evaluated. Possibly, the hegemony of parietal cells in the cardiac region is related to the type of food that the armadillo has, largely rustic (Vaz *et al.,* 2012). Due to the greater release of hydrochloric acid in the passage of food through the cardia, favoring a faster and more efficient digestion(Choi *et al.,* 2014). The absence of parietal cells in the cardiac and pyloric glands in E. sexcinctus would indicate that the production of HCl would not be necessary to favor digestion in both regions of the stomach. Comparing the muscular layer of the mucosa, it is observed that, like the paca, this layer is thin in relation to that of mice, hamsters and gerbil (Ghoshal and Bal, 1989; Machado *et al.,* 2015). The muscular tunic is typical, formed by layers of internal circular muscle and external longitudinal muscle, both of smooth muscle tissue (Pinheiro *et al.,* 2014). This musculature contracts continuously generating waves of contraction that help in gastric motility and in the formation of the chyme (Banks, 1991).

Conclusion

In summary, the stomach of the six-banded armadillo (*E. sexcinctus*) is divided into three regions, the cardia, the fundus and the pylorus, which is lined internally by a simple columnar epithelium. This epithelium presents different cell types epiteilais, parietal, pit, mucous and zimogenic that can be found in the three stomach regions in variable amounts. Among cell groups, there are at least two that differ statistically in the regions of the stomach. Parietal cells occupy a large portion of the mucous layer of the gastric compartment, being more prevalent in the pyloric region than in the other regions. The topographic location, as well as the anatomical architecture and cellular arrangement, contribute to the knowledge of E. sexcinctus digestive processes and eating habits. The armadillo's stomach is peculiar in relation to syntopy, macro and microscopic morphology and how much functionality, when compared to other species. Studies on the physiology, eating habits and behavior of this animal in the ecosystem are still needed to provide knowledge that best elucidates gastric function and the relationship with its morphology.

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