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Tissue analysis of Brazilian gracile opossum digestory tube (*Gracilinanus microtarsus* Wagner, 1842) (Marsupialia: Didelphidae)

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Abstract. The alimentary habit reflects in the morphology of digestive organs. In this sense, microscopic studies are useful to analyze the tissue composition of each organ and allow greater comparisons with other marsupials described in the literature. For this reason, the aim of this study was realize a microscopic description of the digestive tube in *Gracilinanus microtarsus* Wagner, 1842 (Marsupialia: Didelphidae). Six adults specimens were used (3 males and 3 females). Samples from organs of digestive tube were fixed in 10% folmaldehyde solution. The samples were dehydrated in increasing concentrations of ethanol (70%-100%), diaphanized in xylol, paraffin-embedded, cut into a microtome and stained by H. E., Picrossirius, Schiff periodic acid, Toluidine Blue and Masson's Trichrome. The material was analyzed and photographed through a Nikon Eclipse E-400 Photomicroscope. This study demonstrated, for the first time, the histological constitution of the digestive tube of *G. microtarsus*. It has been demonstrated that the digestive tube is formed by the esophagus, stomach, small intestine (duodenum, jejunum and ileum) and large intestine (cecum, colon and rectum). These organs were constituted by five distinct layers, a serosa coating externally the organ, a muscular, a submucosa, a muscular of the mucosa, and a mucosa with epithelium that varied between squamous keratinized in the esophagus to prismatic-type in the other organs. This study demonstrated that the digestive tube of animals studied is similar to that described in other marsupials Didelphidae described in the literature with the same food habit. Future immunohistochemical studies may demonstrate the enzymatic complex involved in digestion and the cellular and molecular

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mechanisms involved in the uptake of nutrients by the digestive tube in this species.

Keywords: Didelphidae; Digestive tract; Digestory apparatus; Marsupialia; Omnivorous.

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Introduction

It is known that animals need food energy to grow, maintain and reproduce. According to Hildebrand (1995) the functions of the digestive apparatus are to capture and to seize the food; reduce mechanically this food; receive and to store temporarily the food ingested; reduce it physically and chemically; absorb the products resulting from digestion, retain unabsorbed remnants for a period of time; and, finally, eliminate them. To perform these functions, several organs are necessary, which constitute the digestive apparatus (Lobo et al., 2014). To perform this process, the digestive organs are adapted and specialized according to the food habit of the species. These adaptations were demonstrated in comparative studies among several species of marsupials in the New World (Santori et al., 2004; Cáceres, 2005).

The Brazilian gracile opossum *Gracilinanus microtarsus* Wagner, 1842 (Marsupialia: Didelphidae) is a small marsupial of nocturnal and arboreal habit (Martins and Araújo, 2008). It belongs to the Family Didelphidae (Orr, 1986; Eisenberg and Redford, 1999) and is popularly known in Brazil as guaiquica or guaiquiquinha (Lima et al., 2013). It has an omnivorous habit. It feeds on insects, small vertebrates and fruits (Lobo et al., 2014, 2015). It is able to travel long distances inside the forests in search of food (Fontes et al., 2007) and for this reason it is considered important seed disperser (Pires et al., 2010; Lima et al., 2013). Some studies have shown that the seeds that pass through the digestive tract undergo dormancy break, increasing the germination potential. For this reason, *G. microtarsus* can be used in recovery programs of degraded area with a focus on seed dispersal of pioneer plants (Cáceres and Monteiro-Filho, 2007; Campos et al., 2012; Lobo et al., 2014).

Previous studies conducted by our research group have demonstrated the anatomical constitution of the digestive tract in *G. microtarsus* (Lobo et al., 2014). This study revealed that the digestive tube in this species consists of a tubular esophagus formed by the cervical, thoracic and abdominal portions; by a simple single-chamber, composed stomach, with the presence of gastric folds. The small intestine of this species has three parts: duodenum, jejunum and ileum. And, the large intestine is composed of the cecum, colon and rectum. The rectum opens in the cloaca (Lobo et al., 2014). In another study we demonstrated the morphology of the tongue in the species (Lobo et al., 2015), revealing a similar pattern in the distribution of the lingual papillae among others Didelphidae. We also described the dental formula and morphology (Lobo et al., 2016), which also presents similarities with other species of Didelphidae.

In order to improve the knowledge about the alimentary habit, reflected in the digestive organs, we believe that microscopic studies could be useful to analyze the tissue composition of each organ and would allow greater comparisons with other marsupials described in the literature. For this reason, the aim of this study was realize a microscopic description of the digestive tube in *G. microtarsus*.

Material and methods

Six animals (3 males and 3 females) were used. All animals were donated by the Laboratory of Morphological Sciences of the University Center of the Octávio Bastos Teaching Foundation, authorized by IBAMA with Process 02001.007176/03-69. The study was also authorized by the Institution's Bioethics Commission.

These animals were already fixed in 10% solution of formaldehyde and stored in 70% ethanol. For microscopic analysis the organs of the digestive tube were fragmented and dehydrated in a series of ethanols in increasing concentrations (70%-100%) and diaphanized in xylol, followed by inclusion in paraffin. The blocks were cut into a microtome, with a thickness of 5μm. The sections were stained following the techniques of H. E. Toluidine Blue, Masson's Trichrome, Picrossirius and histochemical reaction by Schiff periodic acid with hematoxylin background. After the stains, the slides were mounted with coverslips and glue.

The material was analyzed, mapped and photographed in Nikon Eclipse E-400 Photomicroscope.

Results

Esophagus

The esophagus in its different portions (cervical, thoracic and abdominal) presented a serosa as more external layer. This layer was constituted by squamous epithelium composed of mesothelial cells supported on a thin layer of loose connective tissue. Internally the serous layer was the muscular layer, divided into external longitudinal and internal circular portions. More internally it was the submucosal layer, consisting of loose connective tissue. The muscular layer of the mucosa was formed by smooth musculature arranged longitudinally. Related to the lumen of the organ was the mucosal layer. The mucosa consisted of keratinized stratified squamous epithelium (Figure 1).

Stomach

The stomach presented the layers common to all constituent portions of the digestive tube (serosa, muscular, submucosa, muscular of mucosa and mucosa). The presence of several gastric folds and gastric pits were observed. The muscular layers were composed of smooth muscular fibers. The muscular layer showed to be thicker in the pyloric region in relation to the same layer of the fundic region. The muscular layer of the stomach was also thicker than the same layer of the other constituent portions of the digestive tract. The mucosal epithelium was of the simple prismatic type where cells secreting mucus and gastric or pyloric glands were located at the base of the epithelium (Figure 2).

Small intestine: duodenum jejunum and ileum

The duodenum presented (from the outer layer to the lumen of the organ) a serosa layer; a muscular layer, divided into external longitudinal and the internal circular portions; a submucosa with muscular lamina; a muscular of the mucosa; and the mucosa. The simple prismatic epithelium with goblet cells was present in the mucosa. The villi were elongated and narrow (digitiform). Absorptive cells, Paneth cells, and myenteric ganglia were observed (Figure 3).

The jejunum and the ileum presented the same layers found in the duodenum. The ileum had a lower number of villi. The external longitudinal and internal circular muscular layers of the ileum were thicker. The myenteric ganglia were also observed in the muscular layer, between the external longitudinal muscular and the internal circular muscular layer (Figure 4).

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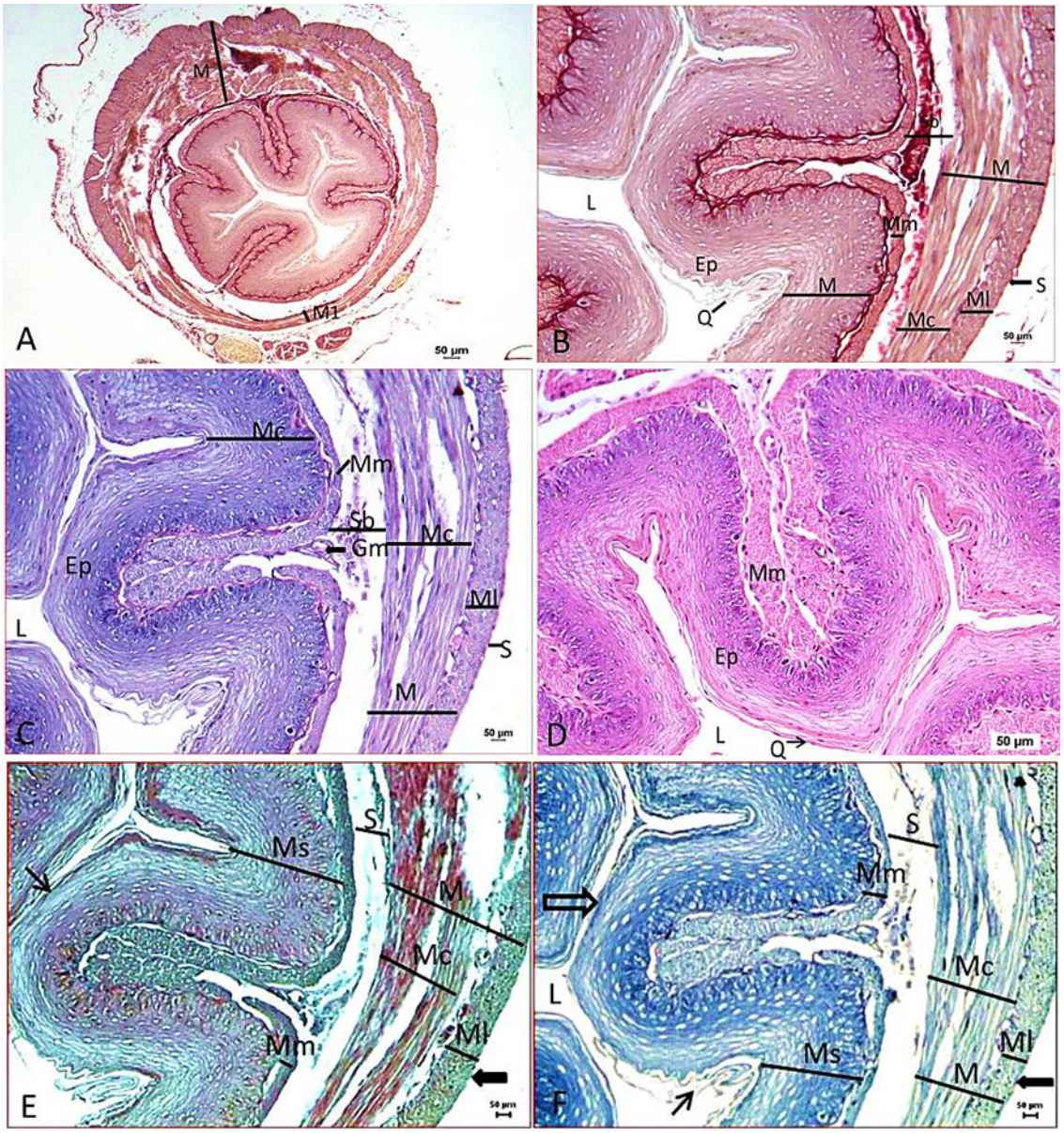

Figure 1. Photomicrography of the esophagus. A: thickness differences of the muscular layer (M and M1), on the opposing walls of the organ. Picrossirius. B: serous (S); muscular (M), divided into: external longitudinal (Ml) and internal circular (Mm); submucosa (Sb); muscular of the mucosa (Mm); mucosa (M); keratinized (Q) squamous stratified epithelium (Ep) lumen (L). Picrossirius. C: serous (S); muscular (M), divided into: external longitudinal (Ml) and internal circular (Mm); submucosa (Sb); muscular of the mucosa; mucosa (Mc); squamous stratified epithelium (Ep); mucous gland in the submucosa (Gm); lumen (L). Schiff periodic acid. D: keratinized squamous stratified epithelium (Ep); muscular of the mucosa (Mm); keratin (Q); lumen of the organ (L). H.E. E: serous layer (Full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mc); submucosa (S); muscular of the mucosa (Mm); mucosa (Ms); keratinized squamous stratified epithelium (thin arrow). Masson's trichrome. F: serous layer (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mc); submucosa (S); muscular of the mucosa (Mm); mucosa (Ms); keratinized squamous stratified epithelium (thin arrow); lumen (L). Toluidine Blue. Bars: 50 µm.

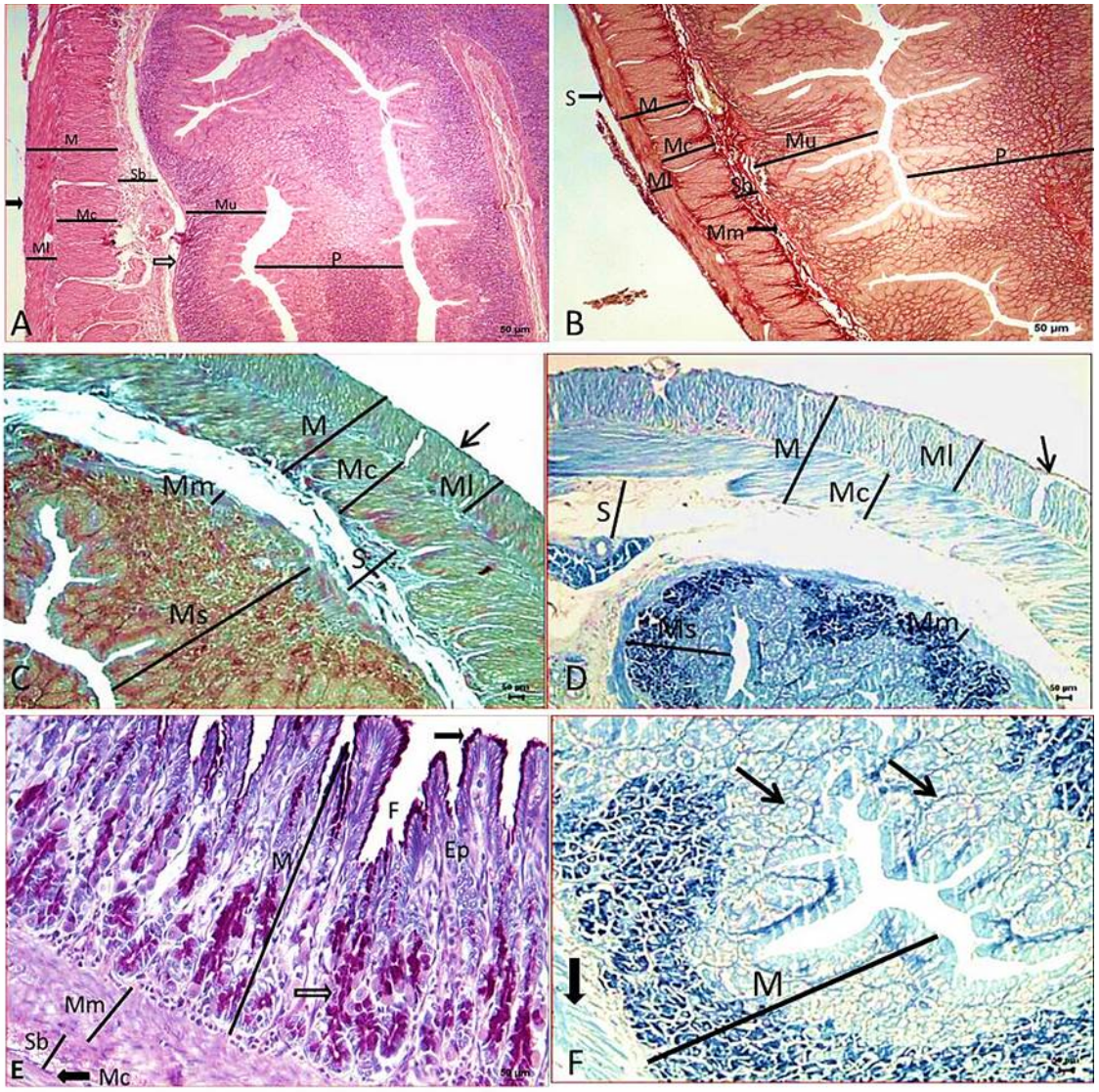

Figure 2. Photomicrograph of the stomach. A: Serous layer (full arrow); muscular (M), composed of the external longitudinal (Ml) and internal circular (Mc); submucosa (Sb); mucosa (empty arrow); mucosa (Mu); gastric fold (P). H. E. B: serous layer (S); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (Sb); muscular of the mucosa (Mm); mucosa (Mu); gastric fold (P). Picrossirius. C: serous layer (thin arrow); muscular layer (M), divided into external longitudinal (Ml) and internal circular (Mc); submucosa (S); muscular of the mucosa (Mm); mucosa (Ms). Masson's trichrome. D: serous layer (fine arrow); muscular (M), divided into internal longitudinal and internal circular; submucosa (S); muscular of the mucosa (Mm); mucosa (Ms). Toluidine Blue. E: mucus secreting cells (full arrow) on the surface of the epithelium (Ep); gastric or pyloric glands (empty arrow) at the bottom of the gastric pits (F); mucosa (M); muscular of the mucosa (Mm); submucosa (Sb); internal circular muscular. Schiff periodic acid. F: gastric mucosa (M) of the muscular mucosa (seta) and mucus secreting cells (thin arrows). Toluidine Blue. Bars: 50 µm.

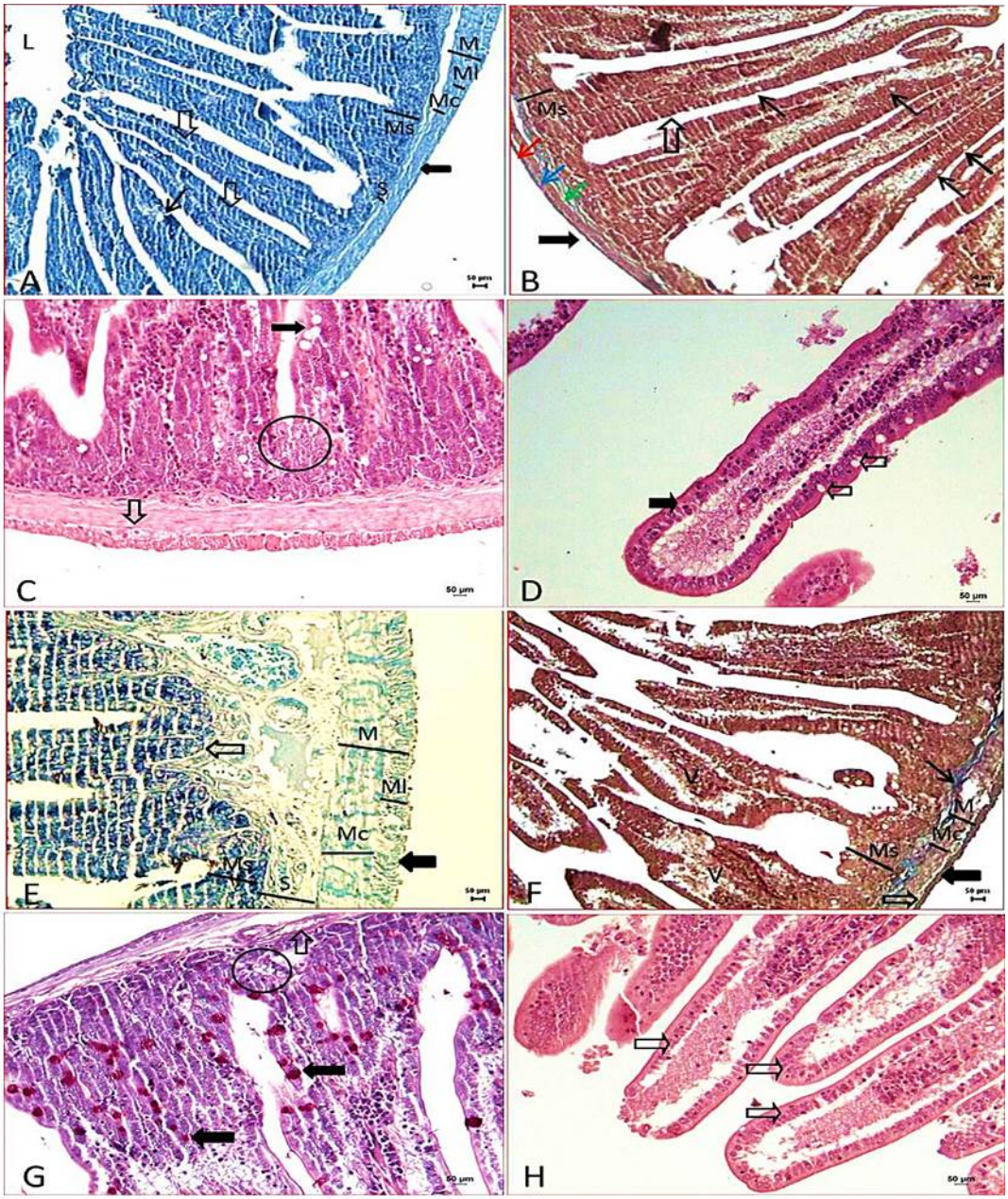

Figure 3. Photomicrograph of the duodenum (A, B, C, D) and jejunum (E, F, G, H). A: serosa (full arrow); muscular, divided into external longitudinal (Ml) and internal circular (Mc); submucosa (S); mucosa (Ms); villus digitiform (empty arrows); goblet cell (thin arrow). Toluidine Blue. B: serosa (full arrow); external longitudinal muscular (red arrow); internal circular muscular (blue arrow); submucosa (green arrow); mucosa (Ms); vilo (Empty arrow); goblet cells (thin arrows). Masson's trichrome. C: goblet cells (full arrow); Paneth cells (circle); myenteric ganglion (empty arrow). H. E. D: digitiform villi with the absorptive epithelium (full arrow) and goblet cells (empty arrow). E: serous (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); muscular of the mucosa (empty arrow); mucosa (Ms). Toluidine Blue. F: serous (Full arrow); muscular (M), divided into external longitudinal (empty arrow) and internal circular (Mc); submucosa (thin arrow); mucosa (Ms). Masson's trichrome. G: Peyer's plaque (empty arrow); Paneth cells (circle); goblet cells (Full arrow). Schiff periodic acid. H: villi with absorptive epithelium, composed of columnar cells (empty arrows) and goblet cells (full arrows). H. E. Bars: 50 µm.

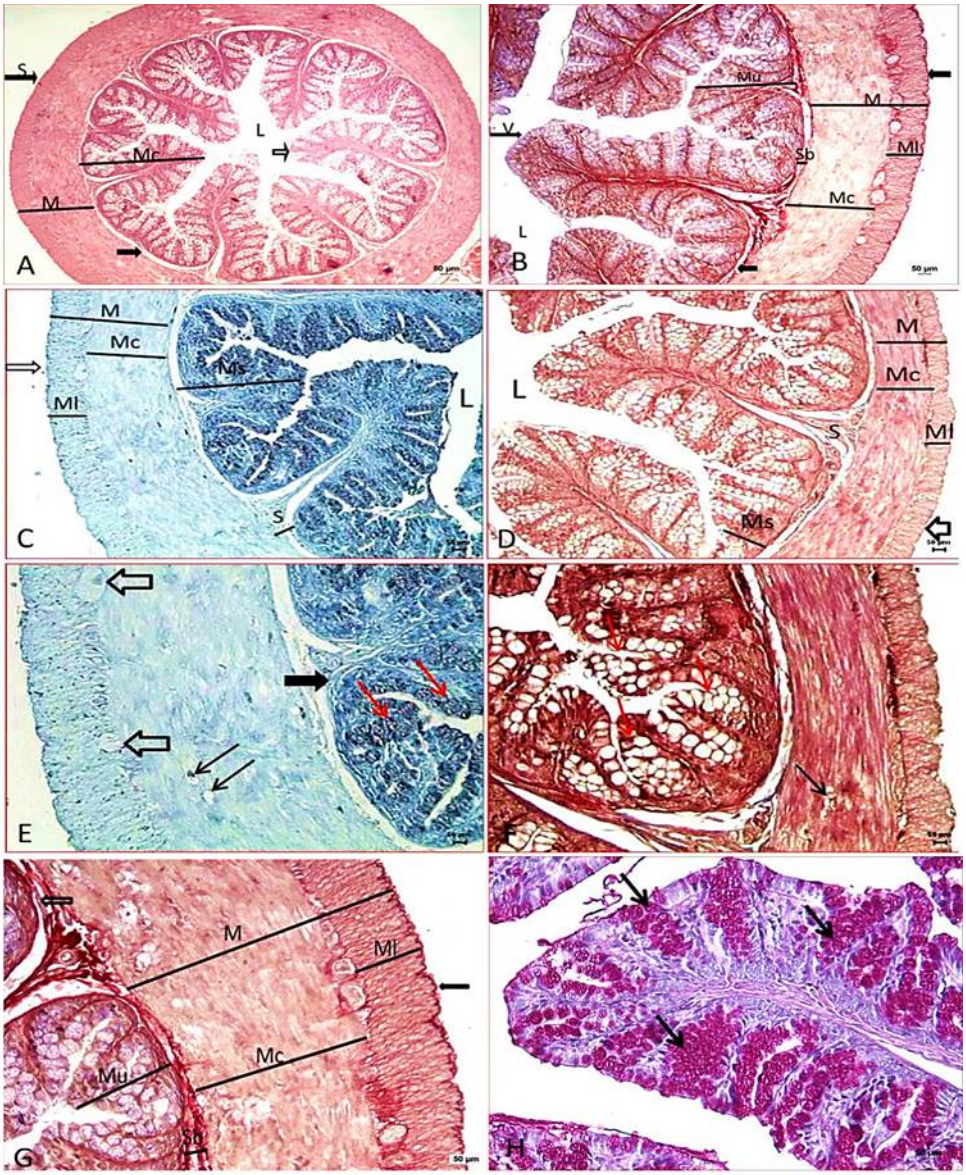

Figure 4. Photomicrograph of the ileum. A: serosa (S); muscular (M); submucosa (full arrow); mucosa (Mc); villi (empty arrow); lumen (L). H.E. B: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (Sb); muscular of the mucosa (Mm); mucosa; myenteric nerve ganglion (empty arrow); (V); lumen (L). Picrossirius. C: serosa (empty arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); mucosa (Ms); lumen (L). Toluidine Blue. D: serosa (empty arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); mucosa (Ms); lumen (L). Masson's trichrome. E: myenteric ganglia (empty arrows); bodies of neurons (black arrows); muscular of the mucosa (full arrow); goblet cells (red arrows). Toluidine Blue. F: neuron bodies (thin arrow); goblet cells (red arrows). Masson's trichrome. G: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (Sb); mucosa (empty arrow); mucosa (Mu). Picrossirius. H: mucous cells in the villi (arrows). Schiff periodic acid. Bars: 50 μm.

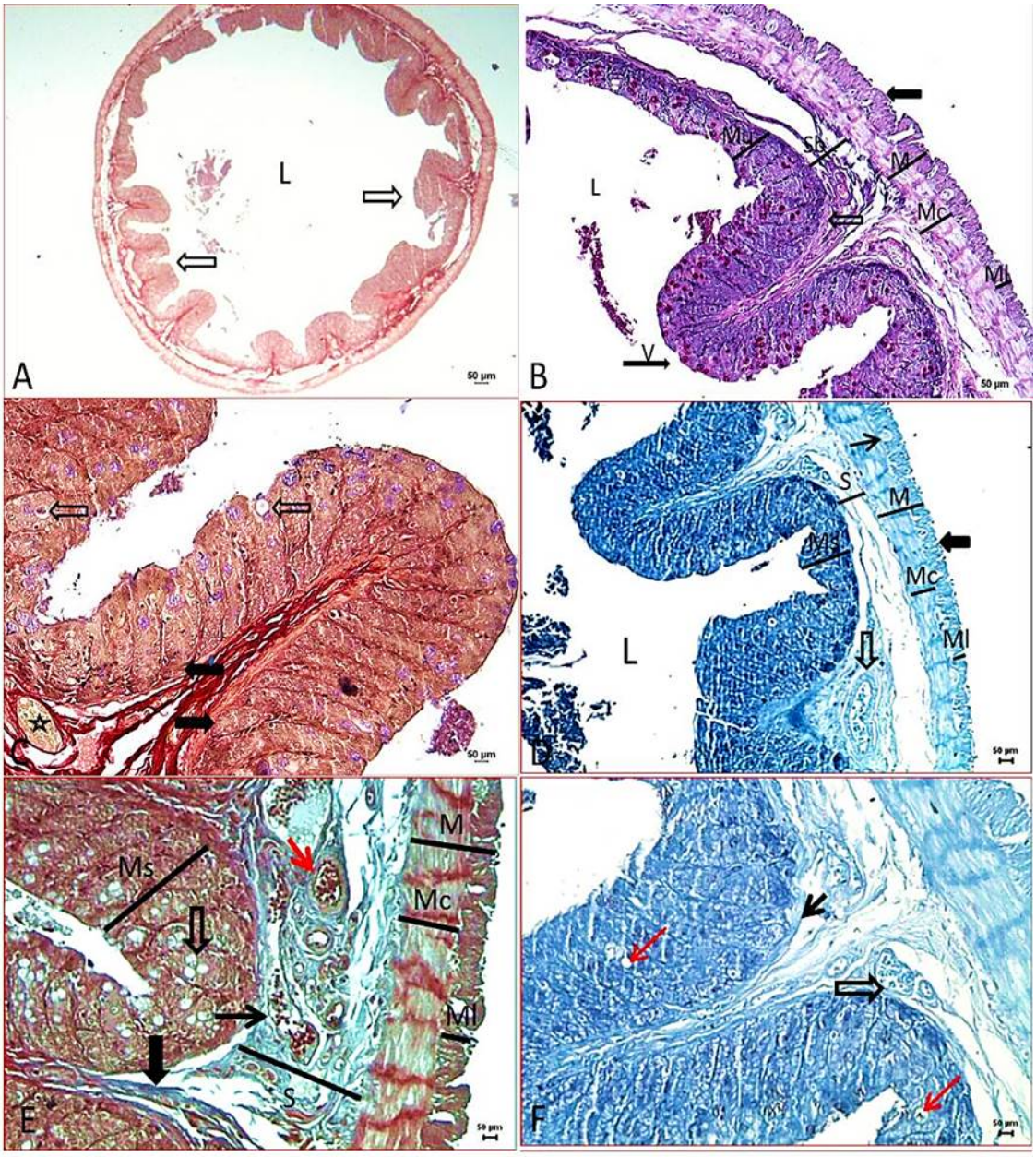

Figure 5. Photomicrography of the cecum. A: villi and folds (arrows); lumen (L). Picrossirius. B: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (Sb); muscular of the mucosa (empty arrow); mucosa (Um); (V); lumen (L). Schiff periodic acid. C: simple prismatic epithelium; goblet cells (empty arrow); intestinal glands (full arrow); Peyer's plaque (star). Picrossirius. D: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); mucosa (Ms); myenteric ganglion (thin arrow); Peyer's plaque (empty arrow). Toluidine Blue. E: muscular (M), divided into external longitudinal (Ml) and internal circular (Mc); submucosa (S); muscular of the mucosa (full arrow); mucosa (Ms); goblet cells (empty arrow); artery (red arrow) and vein (black arrow). Masson's trichrome. F: goblet cells (red arrows); Peyer's plaque (empty arrow); muscular of the mucosa (black arrow). Toluidine Blue. Bars: 50µm.

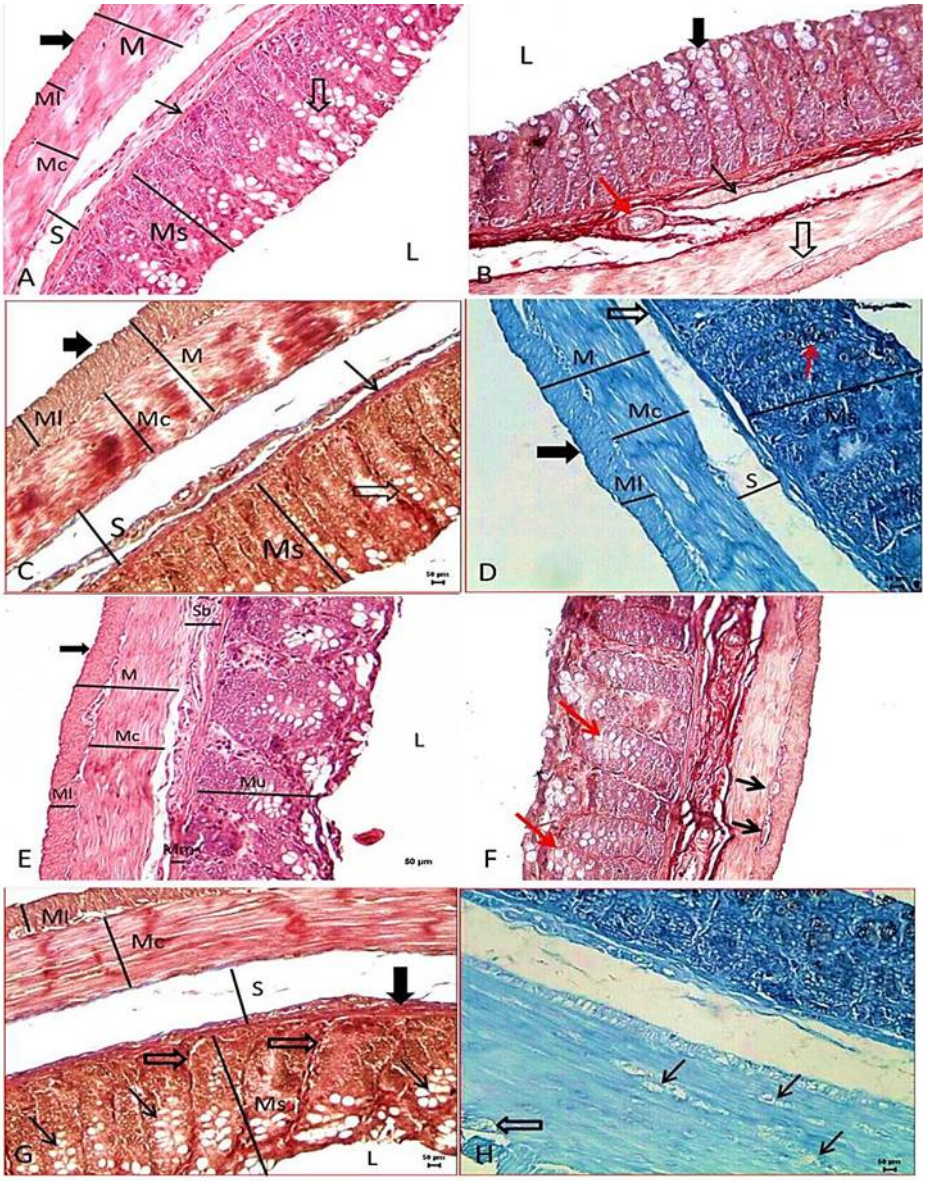

Figure 6. Photomicrograph of the ascending (A, B, C and D) and descending (E, F, G and H) colon. A: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); muscular of the mucosa (thin arrow); mucosa (Ms); lumen (L). H. E. B: myenteric ganglion (empty arrow); Peyer's plaque (black arrow); artery (red arrow); goblet cells (full arrow); lumen (L). Picrossirius. C: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); muscular of the mucosa (thin arrow); mucosa (Ms); goblet cells (empty arrow). Masson's trichrome. D: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); muscular of the mucosa (empty arrow); mucosa (Ms); goblet cells (red arrow). Toluidine Blue. E: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); muscular of the mucosa (Mm); mucosa (Mu); lumen (L). H.E. F: myenteric ganglia (black arrow); goblet cells (red arrow). Picrossirius. G: external longitudinal muscular (M1); internal circular muscular (Mc); submucosa (S); muscular of the mucosa (full arrow); mucosa (Ms); goblet cells (fine arrows) with columnar absorptive epithelium (empty arrows). Masson's trichrome. H: myenteric ganglion (empty arrow); bodies of neurons (thin arrows). Toluidine Blue. Bars: 50 μm.

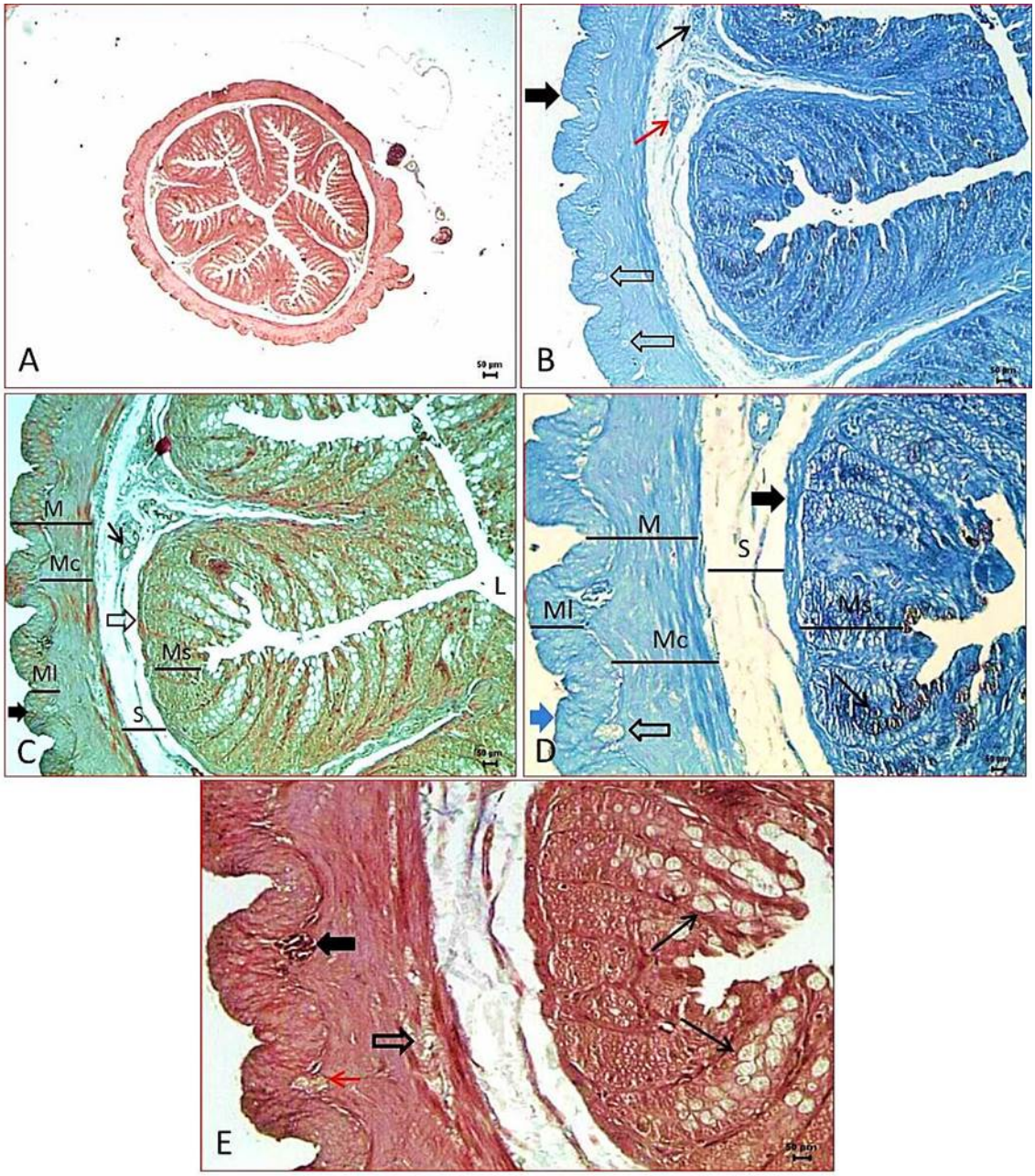

Figure 7. Photomicrography of the rectum. A: general aspect of the rectum in cross section. Masson's trichrome. B: Peyer's plaque (black arrow); blood vessels (red arrow); myenteric ganglia (empty arrows). Toluidine Blue. C: serosa (full arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); muscular of the mucosa (empty arrow); mucosa (Ms); blood vessels (thin arrow) in the submucosa. Masson's trichrome. D: serosa (blue arrow); muscular (M), divided into external longitudinal (Ml) and internal circular (Mm); submucosa (S); muscular of the mucosa (full arrow); mucosa (Ms); myenteric ganglia (empty arrows); goblet cells (thin arrow). Toluidine Blue. E: goblet cells (thin arrows); bodies of neurons (empty arrow); myenteric ganglion (red arrow and full arrow). Masson's trichrome. Bars: 50 µm.

The ascending and descending colon had the same layers found in the cecum. The mucosa was smooth, without folds or villi. Goblet cells, Peyer's plaques, and myenteric ganglia were present. The external longitudinal muscular and internal circular muscular was thinner, compared to the same layer present in the cecum (Figure 6).

Large intestine: cecum, colon and rectum

Cecum was composed of the layers: serosa, muscular, submucosa, muscular of the mucosa and mucosa. It presented folds and villi. The mucosal epithelium was simple prismatic-type with goblet cells and intestinal glands. Peyer's plaques were located in the submucosa (Figure 5).

The rectum also presented the layers common to the other organs of the digestive tube, but the external longitudinal muscular layer was rough. It presented myenteric ganglia, Peyer's plates and folds or villi, where goblet cells were present. It was shown to be smaller in diameter, in relation to the cecum and the colon, however, the muscular layers were thicker (Figure 7).

Discussion

In the present study, it was demonstrated that the digestive tube consists of the esophagus (cervical, thoracic and abdominal portions), a single stomach, the small intestine divided into duodenum, jejunum and ileum, and the large intestine divided into a large cecum, the colon and rectum. Santori et al. (2004) studied 10 different marsupial species of the Didelphidae Family and found that all animals in this group have a gastrointestinal tract similar to that described in *G. microtarsus* in the present study. However, the authors look for variations in the anatomical conformation regarding the different proportions between the small intestine and the large intestine, especially the variations in the proportions of organs of the large intestine.

In another study (Cáceres, 2005), the comparison between seven different species of marsupials was carried out. In this study a wide variation of anatomical conformations in the organs of the digestive tube was described, being that the greater variations were found in the large intestine, mainly in the cecum. Cáceres (2005) points out that cecum has different functions in each species and that it is often not directly related to its food habit. One of the reasons would be the different adaptation strategies between species of marsupials. Martins et al. (2007), describes that even within the species *G. microtarsus*, seasonal variations were found according to the food supply throughout the year.

In our previous studies on the macroscopic anatomy of the digestive tract, we reported that the dimensions of the organs of the digestive tract, especially the cecum in *G. microtarsus* were associated with their preferentially insectivorous habit, although the species could also feed on fruits and small vertebrates (Lobo et al., 2014; 2015), as also suggested previously by Pires et al. (2010). In relation to the tissue conformation, we found that the constituent layers of the *G. microtarsus* digestive tube were in agreement with what was described in *D. aurita* (Santos et al., 2013). The same histological layers may be found in humans (Junqueira and Carneiro, 2008) and in domestic animals (Samuelson, 2007). The characteristic that resembles these different groups would be the presence of distinct layers that compound the organs of the digestive tube (serosa, muscular, submucosa, muscular of the mucosa and mucosa).

The descriptions for the esophagus of the animals studied seem to agree with that described for ruminants (Banks, 1992). The keratinization of the esophageal mucosa in the animals of the present study may be related to the omnivorous eating habits and a less selective feeding (Fontes et al., 2007), such as that found in ruminants. George et al. (1998) report that the esophagus has a mucosa consisting of non-keratinized squamous stratified epithelium, but this characteristic was not observed in the animals of the present study. Illanes et al. (2006)

observed presence of non-keratinized squamous stratified epithelium in the esophagus of ostrich (*Struthio camelus domesticus*). Junqueira and Carneiro (2008) noted the presence of esophageal glands in the submucosal layer of the esophagus of humans. This structure was not observed in the animals of present study. The submucosa tunica is typical and exhibits many branched tubule-alveolar glands along the esophagus in dogs. In cats, they are present only in the cervical region. In dogs the muscular layer is formed entirely by striated musculature. In cats it consists of striated muscle up to the middle part and from there it is formed by smooth musculature (Banks, 1992).

The characteristics found in the stomach of *G. microtarsus* resemble those described in dogs (Banks, 1992). The muscular layer showed to be thicker in the pyloric region in relation to the same layer of the fundic region, as described by Jin (1988), in *Caiman crocodillus yacare*. Cáceres (2005) in his comparative study in marsupials Didelphidae describes that the stomach undergoes variations according to the food habit of the species. The small stomach found in the *G. microtarsus*, reveals that this species has an omnivorous, but preferably insectivorous habit (Lobo et al., 2014). In species of carnivorous habit the stomach is large and elastic to temporarily store large amounts of meat (Cáceres, 2005). Fontes et al. (2007) reports that *G. microtarsus* is a species of considerable importance in the dispersal of seeds of some plants. Thus, it is possible that the gastric juice, produced by the mucus-secreting cells of the gastric glands of the animals analyzed in this study may help in the dormancy break of the fruits ingested.

The histological constitution of the duodenum, jejunum and ileum of *G. microtarsus* is in accordance with that observed by Ribeiro et al. (2011) on *D. aurita* and by Banks (1992) in carnivorous domestic animals, such as dogs and cats. The digitiform villi have also been described by Banks (1992) in the small intestine of horses and by Ribeiro et al. (2011) in the small intestine of *D. aurita*. Aleixo et al. (2011) observed bifurcate and irregular villi in the Pantanal's alligator. The myenteric ganglia were also observed in the muscular layer, between the external longitudinal and internal circular portions in the present study.

These data are in agreement with that observed by Ribeiro et al. (2011) in *D. aurita* and by Illanes et al. (2006) in ostriches. In our previous study (Lobo et al., 2014), we demonstrated that the small intestine is much larger than the large intestine, as also found in omnivorous marsupials *Didelphis albiventris*, *D. aurita*, *Metachirus nudicaudatus*, *Philander frenatus*, *Lutreolina crassicaudata*, *Monodelphis sorex* and *Caluromys lanatus*. Species with a frugivorous habit such as *Caluromys lanatus* present a large intestine more developed than species with carnivorous habits such as *D. albiventris* and *D. aurita* (Cáceres, 2005)

In the large intestine of *D. aurita* (Santos et al., 2013) the villi found in *G. microtarsus* were not found. Nevertheless the other characteristics are similar between the two species of marsupials. The constitution of villous in the cecum in *G. microtarsus* is in accordance with what has been described in *D. aurita* (Santos et al., 2013). The presence of the cecum in most of the marsupials of the Didelphidae family is attributed to the omnivorous habit of the group. A large and dilated cecum is used to store and to ferment food residues, especially those from vegetables (Santos et al., 2013). The well-developed cecum in this species may also be related to seed stock and fermentation and to the absorption of water, vitamins, electrolytes and carbohydrates, as described in other species of insectivorous habit (Cáceres, 2005). Illanes et al. (2006) in his study on the ostrich digestive system did not notice the presence of villi in the cecum

of this animal. This difference may be related to the different eating habits of both.

The absence of villi in the colon of *G. microtarus* agrees with that described in *D. aurita* (Santos et al., 2013). Illanes et al. (2006) in ostrich and Aleixo et al. (2011) in the Pantanal's alligator observed the presence of folds or villi in the large intestine. This characteristic was not observed in the present study. Peyer's plaques, goblet cells and myenteric ganglia were present in agreement with that observed in *D. aurita* (Santos et al., 2013) and in dogs and cats (Banks, 1992). The muscular layers: internal circular and external longitudinal were thinner when compared to the same layer present in the cecum. The well-developed colon can be related to water and electrolyte absorption, storage of faecal cake and fermentation of organic matter that escapes the digestion and absorption in the small intestine (Cunningham, 2004).

The rectum presented the five layers common to another organs of the digestive tube and similarities to that found by Illanes et al. (2006) in ostrich and by Banks (1992) in domestic carnivorous as dogs and cats. It had a smaller diameter in relation to the cecum and the colon, but the muscular layers were thicker, as observed in *D. aurita* (Santos et al., 2013) and ostrich (Illanes et al., 2006). According to Cubas et al. (2006), the digestive and urogenital system of marsupials ends in a common cavity, called the cloaca, with the anal opening lying dorsally to the genital opening as found in present study.

Conclusion

This study demonstrated, for the first time, the histological constitution of the digestive tube of *G. microtarsus*. It has been demonstrated that the digestive tube is formed by the esophagus, stomach, small intestine (duodenum, jejunum and ileum) and large intestine (cecum, colon and rectum). These organs were constituted by five distinct layers: a serosa coating externally the organ; a muscular; a submucosa; a muscular of mucosa; and a mucosa with epithelium that varied between stratified squamous keratinized in the esophagus to prismatic-type in the other organs. This study demonstrated that the digestive tube of animals studied is similar to that described in other marsupials Didelphidae described in the literature with the same food habit. Future immunohistochemical studies may demonstrate the enzymatic complex involved in digestion and the cellular and molecular mechanisms involved in the uptake of nutrients by the digestive tract of the species along the year.

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Conflict of interest statement

Authors declare that they have no conflict of interests.

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