

HISTOPATHOLOGY OF ESOPHAGUS AND STOMACH OF A NEW BRAZILIAN SILURIFORMES HYBRID TRICROSS SURUBIM

Lucimar Rodrigues Vieira Curvo^{1,2*}, Milena Wolff Ferreira¹, Joseane Moreira Mangelot¹, Ulisses Simon da Silveira³ and Gisele Brazilliano de Andrade¹

¹Department of Post-Graduate Program in Environmental Sciences and Agricultural Sustainability, Dom Bosco Catholic University, Campo Grande – MS, Brazil

²Federal Institute of Education, Science and Technology of Mato Grosso, Campus Cuiabá, Mato Grosso 78.005-200, Brazil

³Department of Zootecnia, State University of Mato Grosso do Sul, Campo Grande – MS, Brazil

E-mail: lucimar.curvo@cba.ifmt.edu.br

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ABSTRACT

Hybrid tricross surubim are products of triple crosses among native surubim (Pimelodidae: *Pseudoplatystoma corruscam*), cachara (*Pseudoplatystoma reticulatum*), and the Amazonian jundiá (Pimelodidae: *Leiarius marmoratus*). To the best of our knowledge, the histology of the novel hybrid has not been reported. In the present study, we describe the histological and histochemical aspects of the esophagus and stomach of hybrid tricross surubim. In this research One hundred and sixty juvenile specimens were euthanized, fixed, and subjected to automated histological and histochemical procedures, and then microscopically examined. Three cell types were observed in the esophageal stratified epithelial mucosa: squamous to cuboidal superficial cells, oval claviforms and elongated goblets. The stratified epithelial mucosa was fused to the submucosa and lacked a mucous muscular layer. The mucosa tunic of the stomach presented a simple prismatic epithelium with acidic, neutral, and mixed mucus substances within goblet cells of mucosae of the esophagus and stomach. Organizational variations associated with the structures of the digestive tract were observed in the histological constituents, suggesting that hybrid tricross surubim are carnivores. However, hybridization has led to greater food diversity and flexibility for vegetable diets, which could facilitate improved nutritional management of the hybrids in captivity, as well as for other aquaculture fish.

KEYWORDS: fish gut, gastrointestinal tract, fish histology, Pimelodidae

HISTOPATOLOGIA DO ESÔFAGO E ESTÔMAGO DO NOVO HÍBRIDO BRASILEIRO TRICROSS SURUBIM

RESUMO

O tricross surubim híbrido é produto de cruzamentos triplos entre surubim nativo (Pimelodidae: *Pseudoplatystoma corruscam*), cachara (Pimelodidae: *Pseudoplatystoma reticulatum*) e o jundiá amazônico (Pimelodidae: *Leiarius*

marmoratus). Até onde sabemos a histologia do novo híbrido não foi relatada. No presente estudo, descrevemos os aspectos histológicos e histoquímicos do esôfago e estômago do híbrido tricross surubim. Três tipos de células foram observados na mucosa epitelial estratificada do esôfago: células superficiais escamosas a cuboidais, claviformes ovais e cálices alongados. A mucosa epitelial estratificada estava fundida à submucosa e carecia de uma camada muscular mucosa. A túnica mucosa do estômago apresentou um epitélio prismático simples com substâncias mucosas ácidas, neutras e mistas dentro das células caliciformes das membranas do esôfago e estômago. Variações organizacionais associadas às estruturas do trato digestivo foram observadas nos constituintes histológicos, sugerindo que os híbridos tricross surubim são carnívoros. No entanto, a hibridização de espécies tem proporcionado maior diversidade e flexibilidade alimentar para dietas vegetais, o que pode facilitar o melhor manejo nutricional dos híbridos em cativeiro, bem como de outros peixes da aquicultura.

PALAVRAS-CHAVE: intestino de peixe, trato gastrointestinal, histologia de peixe, Pimelodidae

INTRODUCTION

The hybrid tricross surubim group is one of the most important fish groups in Brazil (MAYUMEOSHIRO *et al.*, 2012). It is comprised of fish of different genera of the order Siluriformes and family Pimelodidae. In fish farming, the major representatives of the surubim are in the genus *Pseudoplatystoma* (SILVA *et al.*, 2015), which are crossed with cacharas (*Pseudoplatystoma reticulatum*) and Amazonian jundiá (*Leiarius marmoratus*) (ALVES *et al.*, 2014; SILVA *et al.*, 2015). The production of hybrid surubim in Brazil in fish farming is expanding (PEIXE BR, 2021), and the hybridization of such native species has enabled the breeding of catfish in captivity (JERÔNIMO *et al.*, 2016). The surubim pintado (“surubim”) tricross is a novel hybrid, a product of the crossing of pintado with cachara. The cross results in a hybrid called pintachara (or “ponto e vírgula” due to its markings), which, when crossed with the Amazonian jundiá, yields the surubim tricross hybrid (ALVES *et al.*, 2014; BAGGIO *et al.*, 2016).

Hybrid surubim have been bred in Brazil and provide great potential for fish farming owing to their zootechnical, organoleptic, and carcass yield characteristics (VENTURA *et al.* 2013; SILVA *et al.*, 2015). In addition, Silva *et al.* (2015) reported other potential applications of interspecific hybrid and non-hybrid surubim, which have high quality meat, high commercialization value, and are popular in commercial fishing. These authors highlighted that these fish are considered table fish and are used in sport fishing or even exported as ornamental fish.

Although there is great economic interest in the creation of hybrid tricross surubim and non-hybrids in captivity, our understanding of the basic biology and morphophysiology of the gastrointestinal tract of hybrids remains poor. In this respect, Santos *et al.* (2015) reported that anatomical, histological, and histochemical studies on the structure of the digestive tract enhance our understanding of their biology and facilitate the appropriate management of the species. Thus, the lack of information on such aspects of fishes, whether hybrid or non-hybrid, makes advanced fish farming impossible, especially with regard to the preparation of feed and efficient management (KUBITZA 1999; 2000; 2004).

Further histochemical and histomorphological studies of the esophagus and stomach of hybrid fish such as surubim are required to fill the many knowledge gaps.

To ensure adequate food for hybrids originating from native species (*i.e.*, for studies of the nutrition of any organism), it is critical to understand the anatomy and histology of the digestive system. Understanding the morphophysiology and dietary aspects increases our ability to meet the nutritional needs of each fish and to improve the quality of production in aquaculture (GONÇALVES *et al.*, 2012).

Another points to be observed are the ecosystem relationships (biotic and abiotic factors) that involve breeding in captivity, as they can cause morphophysiological changes and cause abrupt death (ANDRADE-PORTO *et al.*, 2018). In many cases, high concentrations of Toxic Ammonia and alkaline pH increase mortality and consequently compromise production performance and damage production. Since fish are bred in confinement, they allow the increase of undesirable substances and organisms, which can compromise the homeostasis of fish, causing them diseases, which compromise their health and well-being (MARTOS-SITCHA *et al.*, 2020). It is believed that it is necessary to study the relationships of the physical-chemical parameters of the water as a function of possible morphological changes in fish organs (MOTA *et al.*, 2019).

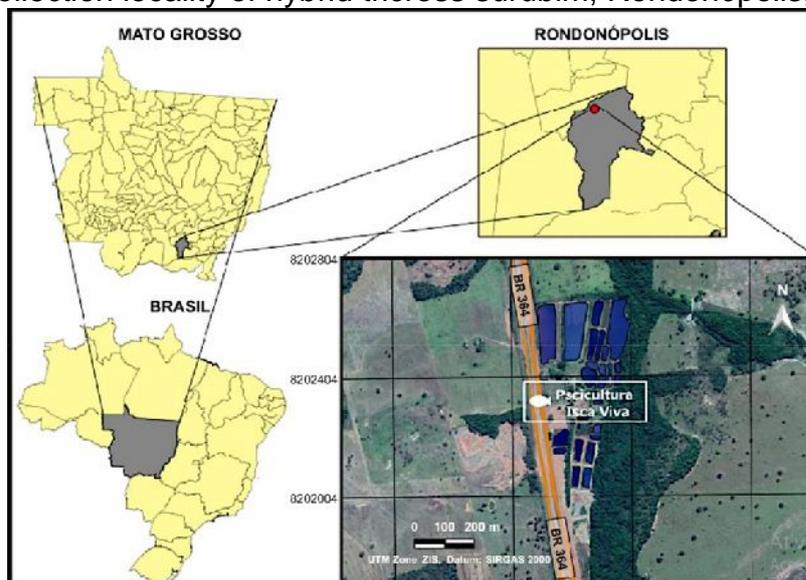
Previous studies have reported that cachara (RODRIGUES *et al.*, 2009) and pintado (Seixas Filho *et al.* 2001) have digestive systems typical of carnivorous species, but the organs have some particularities that indicate a certain degree of flexibility in the diet; these are common only for omnivorous Siluriformes. Pintachara hybrids, the result of crossing these two species with Amazonian jundiá, may present greater flexibility in eating habits since these are omnivorous fish (SOUZA *et al.*, 2005). Due to the scarcity of morphological studies of the gastrointestinal tract of interspecific hybrid fish, the morphological characterization of the esophagus and stomach of hybrid tricross surubim is necessary in order to compare with other population groups, especially Siluriformes. Thus, the aim of the present study was to characterize the histology of the esophagus and stomach in order to describe the histomorphology of the digestive organs of hybrid tricross surubim.

MATERIALS AND METHODS

This research was registered and approved by the Ethics Committee on the Use of Animals of Universidade Católica Dom Bosco (UCDB) under protocol no. 0021/2019. The precepts of Law No. 11,794, of October 2008, and of Decree No. 6.899, of July 15, 2009, were strictly adhered, as were the regulations issued by the National Council for the Control of Animal Experimentation (CONCEA) (BRASIL, 2013). The experiments were carried out at the Fish Production and Health Laboratory and the Pathological Anatomy Laboratory, UCDB, in the city of Campo Grande, MS, Brazil.

In the present study, 160 specimens of hybrid tricross surubim acquired from the Isca Viva Fish Farming Station, in Rondonópolis, MT, Brazil were used (see Figure 1).

FIGURE 1. Collection locality of hybrid tricross surubim, Rondonópolis, MT, Brazil.



Source: Research Data

The water quality maintained desirable standards for the experimental cultivation of fish based on the presupposed conditions of Wedemeyer (1997); (EMBRAPA, 2013). The fish were bred in a Water Recycling System (RWS), packed in ten polyethinene boxes with a capacity of 80 L and a 1000 L renewal box, with a ½ CV propeller pump, with a 6000 L / h renewal rate renewal of 4 to 5x / hour,. Two filters were used, biological and sand, with aeration with replacement of 1% of water per day or when necessary day. Dissolved Oxygen (DO mg / L), total and non-ionized ammonia (toxic), and electrical conductivity with the aid of a HANNA HI 9146 multifunctional device, pH (portable digital pH meter model WTW pH 330i) and temperature (bulb thermometer) were measured once a day.

The water quality parameters were measured every day. The average temperature was $25.47 \pm 1.40^{\circ}\text{C}$, pH was 8.66 ± 0.53 , dissolved oxygen was 5.14 ± 1.40 mg/L, total ammonia was 3.11 ± 0.6 mg/L and ionized (toxic) ammonia was 1.28 ± 0.25 mg/L. The fish were fed four times a day with commercial extruded 1 mm Guabi® ration with 42% protein content and 4.200 Kcal of gross energy until satiety (*ad libitum*) for 30 days. Food was restricted for 24 hours to completely eliminate residues from the digestive tract, before the necropsy.

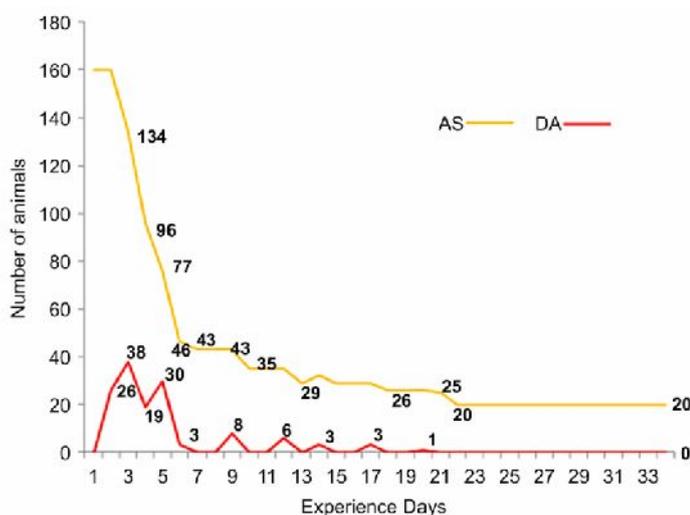
The fish were anesthetized in benzocaine solution (50 parts per million). Afterward, they were euthanized by rapid cooling in benzocaine solution at 4°C, according to Fujimoto *et al.* (2015) and the Euthanasia Practice Guidelines of the Ministry of Science, Technology and Innovation (BRASIL, 2019). Necropsy was performed after complete cessation of opercular movement. A superficial ventral incision was made in the abdominal cavity to remove the esophagus and stomach, which were collected and immediately fixed in a neutral 10% buffered formalin solution. The procedures for histological slides followed the protocols described by Caputo *et al.* (2010). The material was submitted to increasing concentrations of alcohol, clarified by xylol, and included in liquid paraffin. Then, microtome cuts were made at 4-µm thickness, and the slides were stained with hematoxylin and eosin with

Alcian blue (HE-AB), Mallory's trichrome, and periodic acid-Schiff (PAS pH 2.5) for morphological examination and histochemical testing for mucosubstances in the goblet cells of mucosa. The slides were observed using an Axio Scope A1 light composite optical microscope (MOC) (Carl Zeiss GmbH, Jena, Germany) and Zen software. The tissue images were photographed with an Axiocam 503 color camera (Carl Zeiss GmbH) coupled to the MOC.

RESULTS AND DISCUSSION

Out of a total of one hundred hybrid tricross surubim died during the initial phases of the experiment, because of exposure to high concentrations of toxic ammonia and alkaline pH (see Figure 2, below).

FIGURE 2. Number of dead animals and survivors during thirty-four days of experiment.



Source: Research Data. Surviving Animals (SA); Dead Animals (DA)

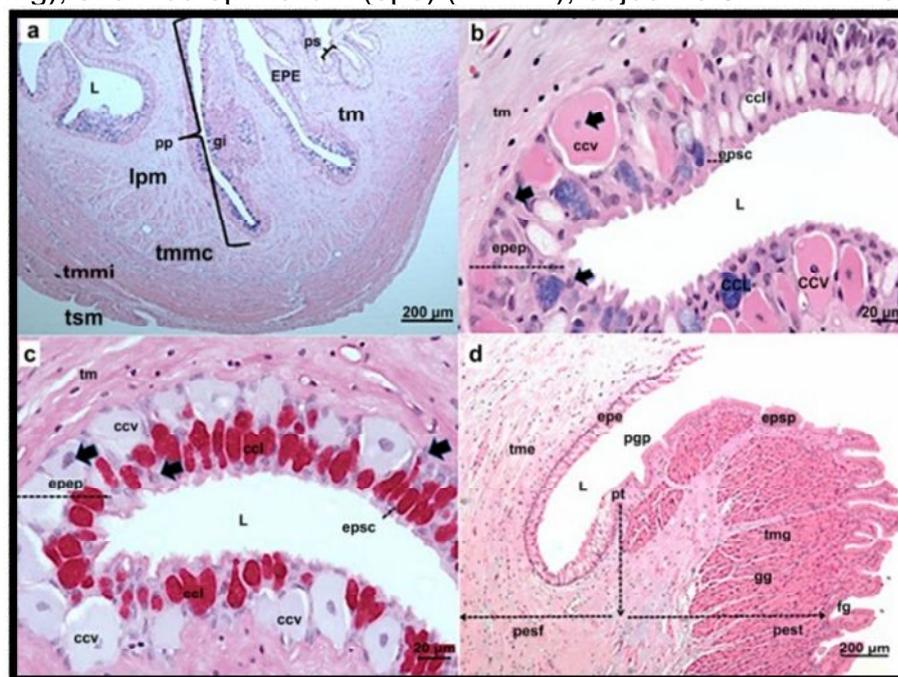
These results were different to those reported by Mardones *et al.* (2019) for *Eleginops maclovinus*, which did not die following exposure to varying concentrations of toxic ammonia (24.09 ± 2.1 g) and alkaline water ($\text{pH } 7.89 \pm 0.2$), showing behavioral variations (lethargy) and mortality after 72 hours.

Aquatic organisms are often physiologically adapted to the aquatic environments they inhabit; therefore, shifts in water quality parameters can adversely influence their development and survival (MELLO *et al.*, 2012). Aquatic ecosystems are complex environments and water quality characteristics vary daily and seasonally, influencing biota and other environmental factors (WINTON *et al.*, 2019). According to Kousar *et al.* (2019), in environments where fish are raised, water quality is influenced by ecosystem interactions in the aquaculture sites, which can cause environmental deterioration, mainly due to the accumulation of metabolic residue, decomposition, deposition of unconsumed food in benthic environments, in addition residual nitrogen compounds.

Similar histopathological death, under different concentrations and pH (above 1 ppm and pH 7.5), was reported by Ribeiro *et al.* (2017) in lambaris (*Bryconops caudomaculatus* - Caricidae), Cantanhêde *et al.* (2014) in *Centropomus undecimalis* (Centropomidae), and Velcheva *et al.* (2010) in three fish species, *Alburnus alburnus*, *Scardinius erythrophthalmus*, and *Perca fluviatilis*.

In the histological study we observed that the structural morphology of the esophagus and stomach of hybrid tricross surubim was similar to those of other teleosts and vertebrate fishes. Each segment of the digestive tract exhibited specialization of the mucosa and other tunicae for optimal efficiency of secretion, absorption, and digestive functions (NASRUDDIN *et al.*, 2014). It was also possible to visualize in the esophagus of hybrid tricross surubim, three independent or mixed tunicae with different thicknesses and constituents. The esophageal tissues were organized in layers or overlapping tunicae as follows: i) mucosa, ii) muscularis, and iii) serosa, forming primary and secondary digitiform folds, which were high and thin at the apex and wider at the base (as can be seen in Figure 3).

FIGURE 3. Photomicrograph of the esophagus of the hybrid tricross surubim. (a) primary fold (pp), secondary fold (ps), lumen (L); tunica mucosa (tm) with stratified epithelium (epe), lamina propria (lpm), intra-epithelial gland (gi), longitudinal and circular tunica muscularis (tmml and tmmc); tunica serosa (tse), (HE-AB) objective 5x; (b) and (c) simple cuboidal epithelium (epsc); prismatic stratified epithelium (epep), goblet cells (CCI - PAS+), claviform cells (ccv) (B - HE-AB staining); cell nucleus (arrow) (C - PAS stain), objective 40x. (d) Esophagus–stomach transition region. Transition point (pt), esophageal portion (pesf), and stomach portion (pest) - primary gastric folds (pgp), lumen (L); esophageal mucosa and gastric tunica mucosa (tme and tmg); stratified epithelium (epe) (HE-AB), objective 5x. HE-AB and PAS.



Source: Research Data.

Köprücü and Yaman (2016) observed in the jundiá (Pimelodidae: *Rhamdia quelen*) and the European catfish (Siluridae: *Silurus glanis*), respectively, that the esophagus had four distinct tunicae. Nasruddin *et al.* (2014), in their study of a subspecies of wild eels (Anguilliformes: Anguillidae: *Anguilla bicolor bicolor*), mentioned that there may be differences between carnivorous and herbivorous fish.

Three tunicae were observed in the esophagus of hybrid tricross surubim: mucosa, muscularis, and serosa. Just below the epithelium layer, the lamina propria of the tunica mucosa was fused with the submucosa and a relatively thick but

variable striated skeletal muscle layer dispersed in loose connective tissue with little vascularity (see Figure 2a–d). Several fish histology reports have described four tunicae for the entire gastrointestinal tract, often differing in their constituents, quantity, thickness and height, which may be related to characteristic of the species or even to their eating habits, physiological nutritional needs, gender, and size (SANTOS *et al.*, 2007; FACCIOLI *et al.*, 2014; SANTOS *et al.*, 2015; GHOSH; CHAKRABARTI, 2015; MOAWAD *et al.*, 2017; KALHORO *et al.*, 2018).

Pazzim and dos Santos (2011) clarified that the absence of a distinct muscular layer in the cachara (Pimelodidae: *Pseudoplatystoma fasciatum*) esophageal tunica mucosa indicates that the submucosa is also absent; this is because the muscular layer of the mucosa marks the boundary and allows the two layers to be distinguished.

The muscular layers in the hybrid tricross surubim is formed by an internal circular layer and an external longitudinal layer. Canan *et al.* (2012), Hopperdietzel *et al.* (2014), and Purushothaman *et al.* (2016) examined the histology of the digestive tract of peixe-donzela or dusky damselfish (Pomacentridae: *Stegastes fuscus*), zebrafish (Cichlidae: *Amatitlania nigrofasciata*), and Asian seabass (Latidae: *Lates calcarifer*), respectively, and found that the two muscular layers were organized into an external circular layer and an internal longitudinal layer, different from hybrid tricross surubim. Hopperdietzel *et al.* (2014) found that the internal muscular layer was dispersed in the lamina propria of the mucosa (lamina propria-submucosa), and the external longitudinal muscle was organized independently and in contact with the tunica serosa.

The esophageal serosa was positioned more externally with a thin aspect and contained blood vessels (Figure 2a–c above), as reported by Köprücü and Yaman (2016). These authors identified a simple pavement epithelium in the tunica serosa, which could not be visualized in the present study. The esophageal mucosa was variable on the surface from simple to pavement cuboidal epithelium and was non-reactive to HE-AB and PAS in the most superficial layers facing the lumen (the Figure 2b previous). In the deeper layers, the continuity of the prismatic stratified epithelium was visualized with varied cells reactive or not to PAS and HE-AB in different shapes, stages of development, and sizes (Figure 2a, b, c above). Two types of goblet cells containing acidic and neutral mucus secretions were visualized in histochemical tests using AB/PAS pH 2.5, as reported by Kumari *et al.* (2009), in a histochemical analysis of glycoproteins in the secretory cells in the gill epithelia of a catfish, *Rita rita* (Siluriformes: Bagridae). Northcott and Beveridge (1988) and Arellano *et al.* (2004) reported findings similar to those of the present study. They described two types of goblet cells in the gills with different mucus secreting cells in *Oreochromis niloticus* and *Solea senegalensis*.

Notably, the proliferation of goblet cells in the esophagus and stomach regions of hybrid tricross surubim is related to a potential response to environmental stressors. As mentioned by Mello *et al.* (2012), mucus secretion by goblet cells in the gills acts as the first barrier against foreign agents in aquatic environments.

In the present study it was possible to identify three cell types in the epithelium of the esophageal mucosa: i) cuboidal or pavement superficial cells, which were not reactive to PAS and HE-AB; ii) mucus-secreting cells or goblet cells, which were superimposed in different amounts, positions, developmental stages, and shades. These cells were HE-AB positive [ranging from white or transparent (neutral mucopolysaccharides) to different shades of blue (acidic mucopolysaccharides) and

PAS positive, and presented secretion granules in the median–apical portion and spherical or oval nuclei in the lower portion of the cells; and iii) large claviform cells distributed throughout the entire tunica mucosa, which were positioned below the superficial layer, with oval to elongated shapes, and with cytoplasm stained by eosin and central nuclei by hematoxylin (Figure 2b, c above).

In the histological images of the esophagus analyzed here, the lamina propria was formed by loose vascularized connective tissue, which was fused with the submucosa layer (the Figure 2a, b previous), as reported by Rotta (2003), Gonçalves *et al.* (2012), Faccioli *et al.* (2014), Mello *et al.* (2019), and Cardoso *et al.* (2015) in studies on lambari yellow tail, or two-spot tetra, (Characidae: *Astyanax bimaculatus*). The muscle layer of the esophageal mucosa was absent from the hybrid tricross surubim in the current study; this is similar to observations for African catfish (Clariidae: *Clarias gariepinus*) by Moawad *et al.* (2017). Kalhorro *et al.* (2018) reported similar results for the esophagus mucous membrane of the yellow corvina (Sciaenidae: *Larimichthys crocea*), referring to the mucous membrane cells positive for PAS and HE-AB staining. In a morphological study of the gastrointestinal tract of wild eels (*Anguilla bicolor bicolor*), Nasruddin *et al.* (2014) reported results similar to those of our research, mentioning the presence of goblet cells on the surface of the esophageal mucosa in the stratified epithelium. These same authors described the surface of the esophageal mucosa as being tall and digitiform.

The three epithelial cell types (predominantly acidophilic and neutral mucous or goblet cells and small squamous surface cells turned for the lumen), described in the hybrid tricross surubim of the present study, were also reported in previous morphological studies of the digestive system of puffer fish (Tetraodontidae: *Sphoeroides testudineus*) (FAGUNDES *et al.*, 2016). Although these authors had the same results in histochemical analyses, and positive or negative reactions to PAS and HE-AB for acidic and neutral mucins, neither of these studies mentioned the presence of claviform cells, as observed here.

It is important to point out that claviform cells in the mucous layer were initially described as large, single-celled pheromone glands involved in the release of secretions (alarm substances, or “Schreckstoff”) (CHIVERS *et al.*, 2007). These cells were also related to inducing escape (Ralphs and Benjamin 1992) and act in defense and protection against mechanical injuries in the esophagus caused by swallowing (Santos *et al.* 2007). The defense mechanisms, aimed at claviform cells, have been described as protein substances that are released into the water and perceived by the sensory organs of possible aggressors or predators (HAFEZ *et al.*, 2013). This characteristic, described here in the hybrid tricross surubim, was investigated by Hafez *et al.* (2013) who reported the presence of claviform cells and mucous cells in the esophagus in a comparative study of the morphology of the gastrointestinal tract of the African catfish (Clariidae: *C. gariepinus*) and grass carp (Cyprinidae: *Ctenopharyngodon idella*). According to these authors, these claviform cells are known by several names, including “giant cells,” “alarm substance cells,” or “club cells.”

The epithelium of tricross surubim classified here as simple squamous or cuboid was different from the type identified by Oliveira *et al.* (2019), in the esophageal mucosa of the pirapitinga (Characidae: *Piaractus brachypomus*), which was classified as pseudo-stratified epithelial mucosa. The large amount of mucus-producing cells (acidic and neutral goblet cells) evidenced here have also been described by Yang *et al.* (2019) as secretory columnar epithelium, in the esophageal

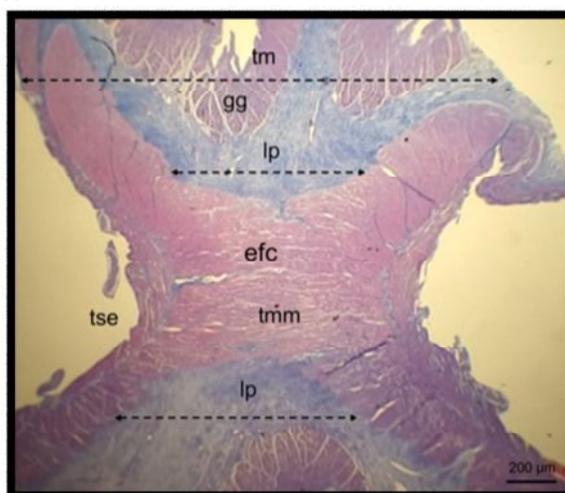
mucosa of Dabry's sturgeons (*Acipenseridae: Acipenser dabryanus*), as well in hybrid jundiarias (*Pseudoplatystoma reticulatum/Leiarius marmoratus*) by Pereira *et al.* 2019 which demonstrated the presence of a large amount of goblet cells decreasing in the caudal direction. Likewise, as highlighted by Oliveira *et al.* (2019), the esophageal mucosa of the pirapitinga, was reported to be morphologically similar to that of our study as well the intense synthesis of neutral and acidic mucous substances in the esophagus.

The posterior portion of the hybrid tricross surubim esophagus is located in the J-shaped stomach. The transition region between the esophagus and stomach was histologically observed with an unexpected variation in the structural characteristics and cell types (Figure 2d above). The type of mucosal epithelium in the transition region from the esophagus to the stomach changed from stratified to simple prismatic epithelial cells, which were reactive to PAS and HE-AB, respectively. The tunica submucosa was not observed in the esophageal portion, and the serosa was very thin; this was in contrast to the stomach portion, in which gastric glands occurred in large amounts along the entire length of the lamina propria (see Figure 2d). These findings were described for other native and non-native teleost fish (hybrids), as mentioned by Pereira *et al.* (2019) for tambaqui (*C. macropomum*) and jundiarias (*P. reticulatum x Leiarius marmoratus*).

The mucous membrane of the esophagus in hybrid tricross surubim was continuous with no apparent pits, diverging from the first portion of the stomach where the folds and shallow pits, short along the mucous membrane, were observed to increase gradually in the cranio-caudal direction, as reported by Yang *et al.* (2019). The muscular layer was formed by striated skeletal fibers, gradually reducing the thickness of the esophageal portion to the stomach in one or two layers of different thicknesses in transverse and longitudinal directions.

The transition region from the esophagus to the stomach is anatomically and histologically separated by a muscular sphincter (see Figure 4, below), with smooth muscle fibers arranged in transverse and longitudinal laminae.

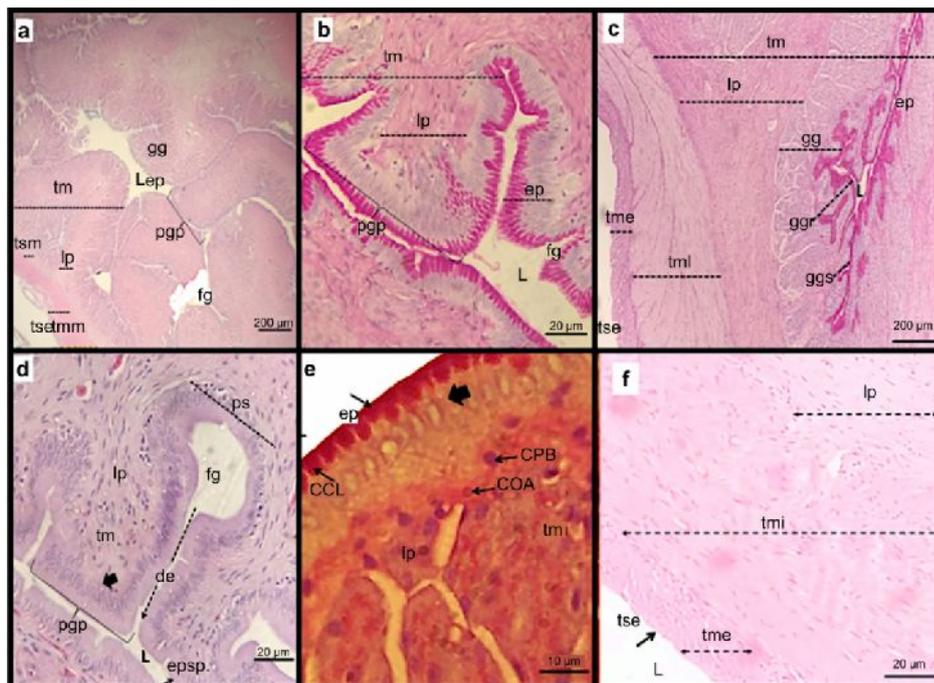
FIGURE 4. Longitudinal cross-sectional photomicrograph showing the cardiac sphincter (efc) of the hybrid tricross surubim: Lumen (l); tunica mucosa (tm); lamina propria (lp), gastric glands (gg); tunica muscularis (tmm) and tunica serosa (tse), objective 10x, TM stain



Source: Research Data.

The layers were thicker in the median and transverse regions and separated from the mucous membrane with thick lamina propria consisting of loose connective tissue rich in collagen fibers (Figure 5) as reported by Fagundes *et al.* (2016) in the pufferfish.

FIGURE 5. Photomicrographs of the hybrid tricross surubim stomach. (a) lumen (l); primary gastric fold (pgp), tunica mucosa (tm) with epithelium (ep), lamina propria (lp), gastric glands (gg); gastric pit (fg), tunica submucosa (tsm), tunica muscularis (tmm); tunica serosa (tse), (HE-AB), objective 5x. (b) Details of the gastric mucosa (mm) objective 40x (PAS⁺); (c) simple and branched tubular gastric glands (ggs and ggr) objective 5x (PAS⁺) (d) Details of the simple gastric gland: simple prismatic epithelium (epsp), excretory duct (de), secretory portion of the gastric gland (ps) and primary gastric fold (pgp) (AB), objective 40x. (e) Details of the cardiac mucosa: epithelium (ep), cell nucleus (wide arrow), acidophilic parietal or oxyntopeptic cell (COA), main or basophil cell (CPB) objective 100x (PAS⁺). (f) Details of the internal tunica muscularis (tmi) and external tunica muscularis (tme) and objective tunica serosa (tse) 40x (HE). HE/AB = hematoxylin and eosin with Alcian blue; PAS = periodic-acid-Schiff.



Source: Research Data.

The histomorphology of the stomach tissues of the hybrid tricross surubim was similar to that observed in the esophageal region (Figure 5), as verified by Atenciogarcía *et al.* (2007) for rubio (Characidae: *Salminus affinis*), by Köprücü and Yaman (2016) for European catfish (Siluridae: *Silurus glanis*), by Kalhor *et al.* (2018) for yellow corvina (Sciaenidae: *L. crocea*), and by Yang *et al.* (2019) for Dabry's sturgeon (Acipenseridae: *A. dabryanus*). As revealed in the present study, the stomach was subdivided into cardiac, fundic, and pyloric regions, differing from each other in the organization of the mucous membrane and the small number of gastric glands in the pyloric region. The same division of regions was observed by Mello *et al.* (2019) in pacamã (Pseudopimelodidae: *Lophiosilurus alexandri*)

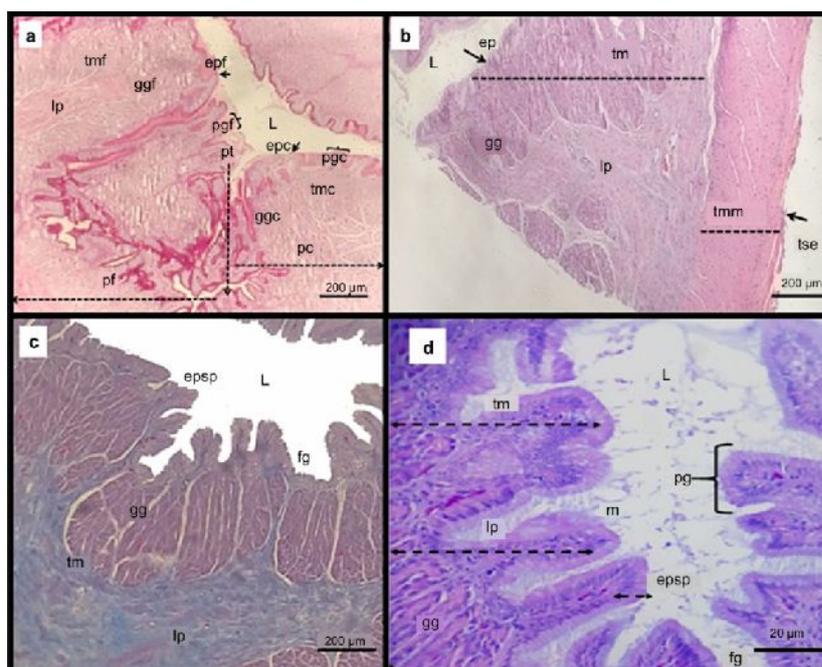
The cardiac region of the hybrid tricross surubim stomach showed wide, shallow, and sometimes rectilinear folds, occupying almost all the stomach lumen and shallow gastric pits where the ducts of a large quantity of simple or branched tubular gastric glands discharged (Figure 5b, c).

These findings were also observed by Köprücü and Yaman (2016), Kalhoro *et al.* (2018), and Riddle *et al.* (2018) in other species of teleosts. It was possible to determine that the epithelial cells have spherical or flattened central nuclei. The deeper cells of the epithelium had mucus vesicles pressing the elongated nuclei toward the basal part of the cells, which may vary according to region (Figure 4e). Purushothaman *et al.* (2016) and Mello *et al.* (2019) described the same nuclear positions and morphology of superficial epithelial cells in the gastric mucous membrane of Asian seabass (*L. calcarifer*) and catfish (Pimelodidae: *Lophiosilurus alexandri*), respectively.

It was found in hybrid tricross surubim that the pits (crypts) in the cardiac region of the stomach are shallow, and as they approach the pylorus and/or intestine, become thinner and deeper and increase in number (Figure 4a), similar to reports by Atenciogarcía *et al.* (2007) and Costa (2015). In the lamina propria of the mucous membrane examined here, it was possible to identify the presence of simple and branched gastric glands (Figure 4c), also described in other fish species such the large yellow corvina (Sciaenidae: *Larimichthys crocea*), oriental catfish (Sisoridae: *Glyptosternum maculatum*), yellowhead catfish (Bagridae: *Pelteobagrus fulvidraco*), dourada (“golden”) or gilthead seabream (Sparidae: *Sparus aurata*), and jiripoca (or jurupoca) or porthole shovelnose catfish (Pimelodidae: *Hemisorubim platyrhynchos*) (CATALDI *et al.*, 1987; CAO; WANG 2009; FACCIOLI *et al.*, 2014; KALHORO *et al.*, 2018; PAVELIN *et al.*, 2018). Mello *et al.* (2019) also observed in the own blade the presence of loose connective tissue and gastric glands only in the cardiac and stomach areas. These authors reported that gastric crypts communicate with gastric glands in the own blade.

In this study, the stomach submucosa of the cardiac region was thin, formed by loose connective tissue, with collagen connective fibers, blood vessels, and gastric glands (Figure 5a). The muscularis of the cardiac region, however, was shown to consist of two layers of smooth muscle fibers, the longitudinal inner and the outer circular (Figure 5f), similar to the findings of Santos *et al.* (2007) in the catfish (Pimelodidae: *Pimelodus maculatus*), and Vieira-Lopes *et al.* (2014) in *Oligosarcus hepsetus* (Characidae). With the aid of the histochemical techniques used here, the transition portion from the cardiac to the fundic region was recorded, and the change in tissue arrangements was noted. The posterior cardiac region, described here as fundic, was morphologically different from the cardiac and pyloric regions, which showed deepening of the folds and crypts (pits) in the cranio–caudal direction, and thin digitiform format, with the apex narrower than the base, occupying little lumen (observe the picture 6, below).

FIGURE 6. Photomicrographs of the transition region of the cardiac-fundic regions of the stomach of the hybrid tricross surubim. (a) Transition region of the cardiac-fundic regions. Fundic portion (pf), cardiac portion (pc), transition point (pt), fundic mucosa (tmf) and cardiac mucosa (tmc), gastric and fundic folds (pgf and pgc), gastric pit (fg), lumen (L); 10x PAS⁺. (b) Arrangement of the tunica of the funicular region 10x HE-AB. (c) Details of the fundic mucosa: tunica mucosa (tm) epithelium (ep), lamina propria (lp), gastric glands (gg), tunica muscularis (tmm), tunica serosa (tse) - objective 25x (TM). (d) Fundic region: tunica mucosa (tm) simple prismatic epithelium (epsp), lamina propria (lp), gastric glands (gg), gastric pit (fg) - objective 40x HE-AB. HE-AB and PAS



Source: Research Data.

In the hybrid tricross surubim of the present study, the occurrence of the lower lumen and a greater number of gastric glands in the cardiac region than in the funicular region (shows the Figure 6a, above), was also seen by Deshmukh *et al.* (2015). The division of the stomach into the three regions found here was based (i) on the distribution of the gastric glands, (ii) on the presence and thickness of the folds of the mucous membrane and (iii) on variations in the presence or absence of the lamina propria, submucosa and muscle tunic, equivalent to the reports of Canan *et al.* (2012) and Faccioli *et al.* (2014), in the dusky damselfish (*Stegastes fuscus*) and the jiripoca (Pimelodidae: *Hemisorubim platyrhynchos*), respectively. The tunica serosa of the hybrid tricross surubim was similar in all three regions studied, varying only in thickness in the outermost part (Figure 6b, above), as described by Atenciogarcía *et al.* (2007) in *Salminus affinis* and by Kalhoro *et al.* (2018) in the distal part of the stomach of *Larimichthys crocea*, high folds reducing the stomach lumen in the pyloric portion.

The three stomach regions observed in the present study demonstrated tunica submucosa formed by loose connective tissue rich in collagenous connective fibers and blood vessels and no glands. These glands were dispersed in the funicular region and most observed in the lamina propria (see the Figure 6b, above). It was

noted that there was a reduction in the number of gastric glands in the caudal direction, that is, cardiac to fundic (Figure 6a). Conversely, Faccioli *et al.* (2014) mentioned the absence of gastric glands in the pyloric region for *Silurus glanis*. Deshmukh *et al.* (2015) reported that the gastric pit of the Asian stinging catfish (Heteropneustidae: *Heteropneustes fossilis*) originated from invaginations in the mucous membrane through the lamina propria, with its basal part being the opening site of the gastric glands, allowing contact with the lumen.

In the present study, the mucous membrane of the stomach of the three regions (cardiac, fundic and pyloric) was formed in its entire length by a simple primitive epithelium reactive to PAS and HE-AB, coinciding with the findings in other fish by Faccioli *et al.* (2014), Moawad *et al.* (2017), Riddle *et al.* (2018), Alabssawy *et al.* (2019), and Gosavi *et al.* (2019). It was identified that the substances accumulated in the cells were neutral glycoproteins located at the apex of the cell, pushing the nucleus towards the base, as presented by Machado *et al.* (2013) in the digestive tract of the fat snook and common snook (Centropomidae: *Centropomus parallelus* and *C. undecimalis*). In fact, the PAS staining indicated the presence of neutral glycoproteins that serve to protect the mucosal surface against microorganisms and elevate the acidity of the stomach contents, as identified in the present study in the pyloric region (Figure 6a-b, above).

In the histochemical analyses with PAS and HE-AB staining, it was possible to identify the presence of two cell types: absorptive cells (enterocytes) and goblet cells in the epithelial tissue of the gastric mucosa, indicating the presence of acidic and neutral glycoproteins, which were also observed in the pyloric region of hybrid tricross surubim (Figure 6b, above). Similarly, Purushothaman *et al.* (2016) described these cells in the digestive tract of the Asian seabass (Latidae: *Lates calcarifer*), and Rodrigues and Cargnin-Ferreira (2017) in the pirarucu (Arapaimidae: *Arapaima gigas*). Kalhoro *et al.* (2018) reported the predominance of mucus-producing cells and gastric glands in the cardiac and fundic regions of *Larimichthys crocea*.

The epithelium of the stomach mucosa in the hybrid tricross surubim parietals was classified as a simple prismatic type, as also reported in *Silurus glanis*, by Köprücü and Yaman (2016). The finding of gastric glands between the epithelial mucous membrane and the lamina propria, arranged in parallel as described here was similar to the reports of Moawad *et al.* (2017). In the hybrid tricross surubim the presence of cells containing oval nuclei and acidophilic cytoplasm and prismatic epithelium in the stomach mucosa, was also viewed by Santos *et al.* (2015) in *Trachelyopterus striatulus* (Siluriformes: Auchenipteridae).

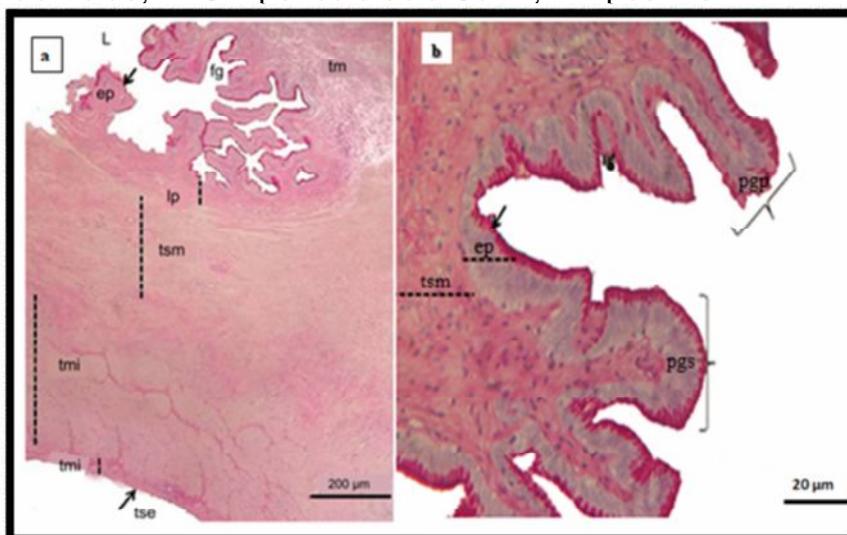
It is important to note that stomach folds in hybrid tricross surubim studied here were thick as in most omnivorous fish and, according to Menin and Mimura (1991), these structures are related to food storage. It was noticed that the mucous crypts of the hybrid tricross surubim were formed by a cell wall originating from the mucosal epithelium, and the gland ducts opened in the basal portion of the pit, corroborating the reports of Purushothaman *et al.* (2016). In the same way as described by Petrinc *et al.* (2005), the presence of stomach mucosal substances found in this study helped the transport of substances as larger particles of food, which has a protective function in the gastric mucosa of these fish.

The cells of the cardiac and fundic epithelial mucosa reacted positively to PAS and to HE-AB in the stomach epithelium of hybrid tricross surubim. This finding is comparable to ontogenic studies of species of cacharas (*Pseudoplatystoma punctifer*) carried out by Gisbert *et al.* (2014), who found during the embryonic

development of this species the predominance of gastric glands in the fundic region of the stomach and the formation of the pyloric sphincter at the junction of the pyloric stomach. As found here in hybrid tricross surubim, studies by Bo ina *et al.* (2016) demonstrated the presence of gastric glands in European hake (*Merluccius merluccius*), which, when stained with PAS and HE-AB, presented a slight reddish tone, characterized as neutral mucus by PAS suggesting the presence of glycosaminoglycans. A similar result was found for the pirarucu by Rodrigues and Cargnin-Ferreira (2017).

The pyloric portion of hybrid tricross surubim presented high, elongated, digitiform folds, very similar to those of the fundic region, but differing in height, as they were longer and thinner and simple or branched at the ends. The crypts (pits) were deeper but with fewer gastric glands. It was noticed that the pyloric region demonstrated a more developed muscular layer than the cardiac and fundic regions with variations in thickness (Figure 7a, as shown in the figure 2, below).

FIGURE 7. Photomicrographs of the pyloric region of the stomach of the hybrid tricross surubim, showing the (a) gastric pit (fg), lumen (l), tunica mucosa (tm) with epithelium (ep), lamina propria (lp), tunica submucosa (tsm), internal tunica muscularis (tmi), external tunica muscularis (tme), and tunica serosa (tse) and (b) primary and secondary gastric folds (pgp and pgs). Objectives: 5× (a) and 40× (b) PAS⁺. HE = hematoxylin and eosin; AB = Alcian blue; HE-AB = hematoxylin and eosin with Alcian blue; PAS = periodic-acid-Schiff; + = positive



Source: Research Data.

The serous tunic showed no differences in thickness in the cardiac and fundic regions (Figure 7b, above). Studies of the pyloric region of other fish demonstrated histological similarities with those identified here. For instance, Kalhoro *et al.* (2018) reported the presence of glands in the pyloric region and gastric glands secreting mucopolysaccharides, PAS positive, in Japanese gilt (*Sparidae: Acanthopagrus schlegelii*), with the same tunic found in the present study.

The findings of the present study, which indicated that gastric glands were scarce, sometimes absent in the pyloric region and more abundant in the fundic regions, contrast with the findings in jiripoca (*Pimelodidae: Hemisorubim platyrhynchos*) by Faccioli *et al.* (2014). These findings are in line with Ghosh and

Chakrabarti (2015) in Nile tilapia (Cichlidae: *Oreochromis niloticus*) and Indian catfish (Bagridae: *Mystus cavasius*). One other point that deserves attention is that the pits in the mucous layer of the pyloric region of the hybrid tricross surubim appeared irregular and branched, entered the lumen in several directions, and were well defined, which differed from the pyloric region of the stomach of tambaquis (*Colossoma macropomum*) as reported by Costa (2015). According to this previous study, the tambaquis pit showed a narrow lumen with a considerable amount of gastric glands, unlike that observed in the present study in which the pit in the pyloric region was deep with a distant inter-space and a wider lumen than in the other stomach regions, as well as a predominance of tubular gastric glands in the funicular region compared with the pyloric region (as already shown in the figures 5c and 6a, b previously).

It was possible to differentiate the internal and external tunicae muscularis of the pyloric region of hybrid tricross surubim from all other regions since the joints and the internal tunica muscularis were thicker than all other locations and the tunica serosa remained very thin as in other regions of the digestive tract. The results of the present study corroborate the findings reported in the literature regarding the histology of the gastrointestinal tract of teleost fish and other vertebrates. Conversely, Kalhoro *et al.* (2018) mentioned that there were significant histological differences in a number of vertebrate species, mainly in fish, varying according to body shape, sex, and eating habits. The histological study of the gastrointestinal tract of the hybrid tricross surubim demonstrated structures and tissue organization with patterns similar to vertebrates in general. However, there were differences in the number of overlapping layers in the esophagus (three) and stomach (four), with peculiar characteristics and morphology established by the invaginations and evaginations of the esophageal mucosa, forming crypts (pits), giving rise to the folds where the secretory glands discharge, and often occupying the entire extension of the lamina propria. The epithelium of the esophageal mucous membrane is formed by distinct layers of cells and tissues. In the stomach, a simple prismatic epithelium was observed, with gastric glands invading the lamina propria, varying in quantity, and decreasing in the craniocaudal direction. The submucosa was little seen in the esophageal and stomach portions, but when present, it was thin or fused to the lamina propria. Tunicae muscularis were irregular in thickness, appearing in one or two layers (internal and external). In the stomach pyloric region (Figure 7, above), the tunica muscularis was more developed than in other regions of the esophagus and stomach seen here.

The tunic appeared discontinuous and irregular, varying in shape, thickness, cell types, and substances.

CONCLUSION

The results obtained from the histopathological examination of hybrid tricross surubim, demonstrated that these fish exhibited characteristics and eating habits closer to carnivory, but with flexibility to partake in vegetable diets, that is, omnivory. Notably, there is a lack of studies examining Siluriformes of the family Pimelodidae, especially hybrids, since most studies have concentrated on native species. To the best of our knowledge, the present study reports the first histological and histochemical description of this novel Brazilian hybrid, highlighting the relevance of studying hybrids. Such studies could facilitate the formulation of better strategies of

managing fish feed resources based on the unique requirements under captive aquaculture systems.

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