

Ontogeny of the digestive tract of *Arapaima gigas* (Schinz, 1822) (Osteoglossiformes: Arapaimidae) larvae

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Early-life survival of *Arapaima gigas* is one of the main challenges of its farming. In this study, we described the morphological and histochemical development of the gastrointestinal tract of arapaima larvae. Larvae were collected from a pond when they started to swim to the water surface (initial day) and were housed in indoor tanks. Daily samplings ($n = 10$) were performed from 0 to the 11th day after the collection (DAC) and then on the 14th, 17th, and 20th DAC. On the initial day, arapaima larvae (0.05 ± 0.01 g; 2.21 ± 0.06 cm) had opened mouth and anus and no yolk sac. In addition, larvae presented well-developed digestive organs. Gastric glands were fully formed, with positive reactions to alcian blue (AB) pH 1.0 as well as to periodic acid-Schiff (PAS) in the simple columnar epithelium. There were folds throughout the intestine and brush border, with an AB pH of 2.5 + PAS positive mucins. From 1 to 11 DAC, larvae presented increasing concentrations of gastric glands and thickness of the stomach muscular layer. From 14 to 20 DAC, the intestine presented high-fold complexity. We suggest that arapaima larvae may be fed exogenous inert diets at a size of around 2 cm.

KEYWORDS

digestive system, histochemistry, larvae, mucins, paiche, pirarucu

1 | INTRODUCTION

Arapaima gigas (Schinz, 1822) (Osteoglossiformes: Arapaimidae) is a carnivorous fish that reaches sizes of 3 m in length and weigh up to 200 kg (Ferraris, 2003). In Brazil, its fishing is regulated and allowed only in community-based

sustainable management in protected areas during the dry season (Cavole, Arantes, & Castello, 2015). However, arapaima farming is legally permitted.

Because of its fast growth (around 10 kg per year), the interest in farming arapaima has grown in the last two decades (Farias et al., 2015; Imbiriba, 2001; Mattos, Nascimento Filho, Barreto, Braga, & Fortes-Silva, 2016). Arapaima has already been introduced in the United States (Lawson, Tuckett, Lawson, Watson, & Hill, 2015), China, Cuba, Mexico, Philippines, Singapore, and Thailand (Food and Agriculture Organizations of the United Nations, 2016). However, early-life production is one of the main challenges of the development of arapaima farming.

In fish life history, the larval stage is crucial because it involves morphological and functional changes that determine its viability (Govoni, Boechlert, & Watanabe, 1986). The successful development of the digestive system is crucial for the survival and growth in fish larvae because an efficient digestive system enables fish to capture, ingest, digest, and absorb feed (Kjorsvik, Pittman, & Pavlov, 2004). The basic mechanisms of organ and system development are similar in all teleosts, although there are considerable differences among species concerning the timing of differentiation, development, and functionality during early ontogeny (Pittman et al., 2013). Knowledge on digestive tract differentiation is essential for understanding the nutritional physiology of fish larvae and synchronizing the physiological stage of development with feeding practices and rearing protocols (Segner, Rösch, Verreth, & Witt, 1993).

Several studies on the ontogeny of the gastrointestinal tract in fish larvae have become available over the last 15 years, mainly focusing on nontropical marine species (e.g., Rønnestad et al., 2013; Yúfera & Darias, 2007; Zambonino-Infante & Cahu, 2001). Information on neotropical fish is limited to a few species, such as *Hoplias lacerdae* (Luz & Portella, 2005) and *Piaractus mesopotamicus* (Portella et al., 2014; Tesser, Carneiro, & Portella, 2005). In addition, there are no studies available on arapaima larvae.

The size and reproductive behavior of arapaima broodstock (Lima, Rodrigues, Varela, Torati, & Maciel, 2015) make the capture of the early offspring very difficult and dangerous, which results in fish farmers catching them several days after swim bladder inflation, when the larvae are swimming to the water surface together with the breeding male.

The weaning from live feed to artificial diets usually results in high mortality and is one of the most critical stages affecting the production effectiveness of many fish species (Fletcher et al., 2007). Information on the ontogeny, especially related to histological and histochemical development of the gastrointestinal tract, may support other studies that contribute to the formulation of more efficient feeding protocols for the species (Zaiss, Papadakis, Maingot, Divanach, & Mylonas, 2006). This work aims to describe the morphological events in the gastrointestinal tract of arapaima during early ontogeny.

2 | MATERIALS AND METHODS

This study has been approved by the Ethical Committee of Animal Experimentation and Research of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil (protocol number 016/2016). Arapaima larvae were obtained from natural spawning in a pond of wild broodfish in a commercial fish farm (Piscigranja Boa Esperança, Rondônia, Brazil). The larvae were collected when they started to swim to the water surface together with the breeding male (Day 0), corresponding to 5–7 days after hatch (DAH) (which is an estimate based on empirical experience of the fish farmer). The acronym DAC (days after collection) was used as nomenclature to identify subsequent collections. The larvae ($n = 702$) were equally distributed in two circular fiberglass tanks (1,000 L). Water volume was increased because of larval growth. The water volume of the tanks was 300 L prior to weaning and 500 L from weaning (corresponding to a 1.17 individuals/L stocking density and 0.70 individuals/L, respectively) in a flow-through system with a water exchange rate of 0.03 m³/s. Dissolved oxygen in water was maintained at 5.60 ± 0.52 mg/L, temperature at $27.9 \pm 0.8^\circ\text{C}$, pH at 7.0 ± 0.17 , and total ammonia at 0.46 ± 0.58 mg/L. A 12/12 h light/dark photoperiod cycle was adopted, and the light intensity was 900 lx at water surface. The larvae were fed every 2 h (from 6:00 a.m. to 6:00 p.m.), mostly with natural zooplankton (55%) and unenriched (45%)

Artemia sp. nauplii (SepArt cysts; INVE, Dendermonde, Belgium) in the mean proportion of 1:389 (389 individuals/larvae/meal). Natural zooplankton was a mixture of species, mostly Cladocera (78.7%), Copepoda (11.1%), and Ostracoda (10.2%), which were collected using a plankton net from a large earthen pond. A nominal sieve opening of 400 µm was used to classify the zooplankton before offering to the larvae. This live feed period continued until the 11th DAC (10 DAC; 0.39 ± 0.05 g and 4.28 ± 0.20 cm).

From the 11th DAC (0.50 ± 0.04 g and 4.50 ± 0.23 cm), cofeeding was initiated using microextruded commercial feed (Aquaxcel Starter WW 4512, 0.8–1.0 mm, 45% crude protein; Cargill Franklinton, LA) during the day. At night, the larvae were fed natural zooplankton in a ratio of 1:815 (815 individuals/larva/meal; Figure 1).

Daily sampling ($n = 10$) of each replicate was performed until 11 DAC. After that, the samples were taken on 14, 17, and 20 DAC. All larvae were sampled, euthanized by lethal dose of anesthetic, and fixed in 10% formaldehyde. After 24-h fixation, the 10 larvae were transferred to 70% alcohol solution and analyzed in the Thematic Laboratory of Optical and Electronic Microscopy of the INPA.

Larvae were dissected, and the gastrointestinal tract was included in paraffin. Longitudinal sections of 3 µm were obtained with a semiautomatic microtome LEICA RM 2245 (Leica Biosystems Inc., Buffalo Grove, IL), mounted on permanent slides, stained with hematoxylin and eosin for morphological analysis. Slides were also stained with periodic acid-Schiff (PAS) reactions, alcian blue (AB) pH 1.0 and pH 2.5, and a combination of AB pH 2.5 and PAS (Cao & Wang, 2009) for histochemical analysis. PAS stains for neutral mucins (MacManus, 1948); AB pH 1.0 and pH 2.5 stain for acidic mucins sulfated and carboxylated, respectively; and the combination of AB pH 2.5 with PAS stains for both (Scott, 1972).

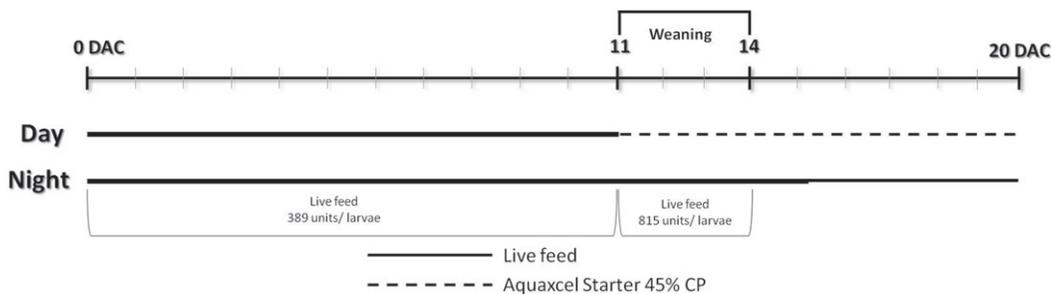


FIGURE 1 Schematic feeding of arapaima larvae during the sampling

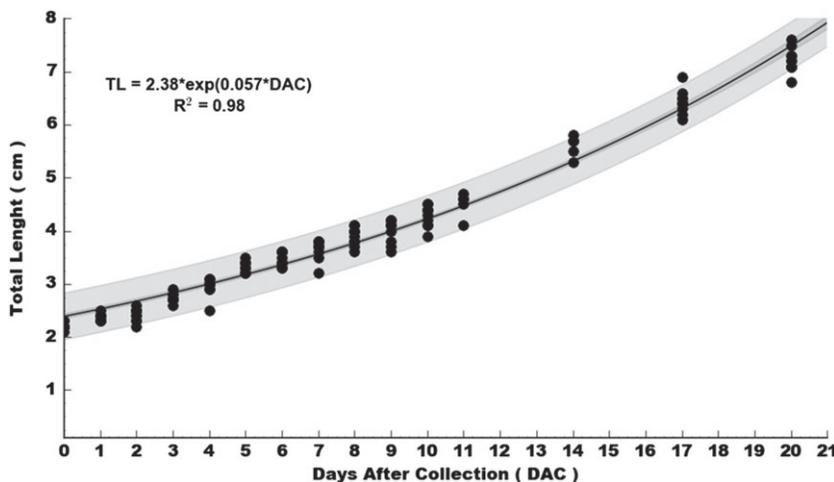


FIGURE 2 Exponential growth curve (total length) of arapaima larvae during ontogeny

To determine the larval growth curve, wet weight and total length were correlated separately against the collection period (DAC). To find the best fit among the regression models used, they were compared by the Akaike Information Criterion (Aho, Derryberry, & Peterson, 2014) and the *F* test (Motulsky & Christopoulos, 2004) using CurveExpert Professional Version 2.6.3 software (Hyams Development, Huntsville, AL).

3 | RESULTS

3.1 | Biometric data

Wet weight and total length fitted in an exponential equation (Figures 2 and 3).

Larval size (total length) increased by more than 200% from initial length during 21 days. Larvae of arapaima showed increasing weight gain equivalent to 38 times their initial weight, including the weaning period, with a survival rate of 99.3%.

3.2 | Macroscopic analysis

At the initial DAC (0 DAC 0.05 ± 0.01 g and 2.21 ± 0.06 cm), arapaima larvae presented an opened mouth and anus, pigmented eyes, and no yolk reserves. However, the fins were not fully formed, and there was no formation of scales (Figure 4A). At this time, the gastrointestinal tract presented three well-defined structures: esophagus, stomach, and intestine (Figures 4B and 5A). In some individuals, it was possible to observe the pyloric cecum develop from 0 DAC (0.05 ± 0.01 g and 2.21 ± 0.06 cm). In relation to the accessory glands, both liver and pancreas were formed from 0 DAC.

3.3 | Microscopic analysis

At 0 DAC, the esophagus goblet cells presented acid and neutral mucin activity (Table 1, Figure 5B). The saccular-shaped stomach presented gastric glands fully formed, housed in the lamina propria with positive reactions for AB pH 1.0 (Table 1, Figure 5C). In addition, the simple columnar epithelium showed a positive PAS reaction (Figure 5D). There were folds throughout the intestine and a brush border with mucins that were AB pH 2.5 + PAS positive (Table 2, Figure 5E,F).

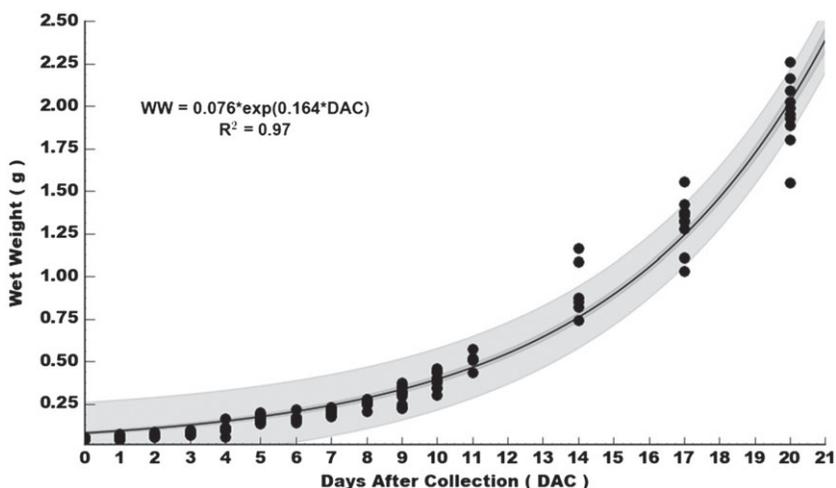


FIGURE 3 Exponential growth curve (wet weight) of arapaima larvae during ontogeny

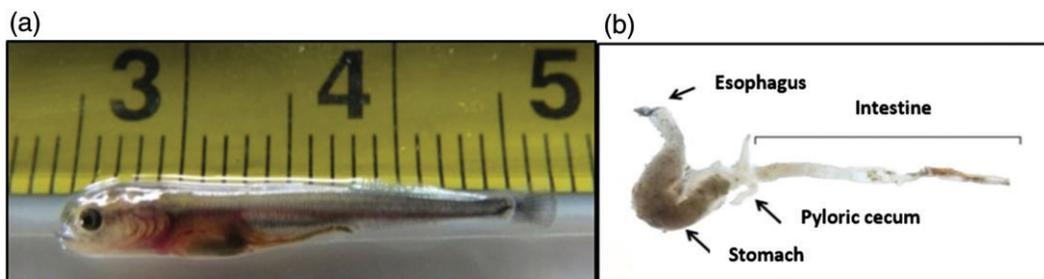


FIGURE 4 Macroscopic features of arapaima larvae. (A) Larvae at day of collection (0 day after collection [DAC]). (B) Larvae gastrointestinal tract at 0 DAC

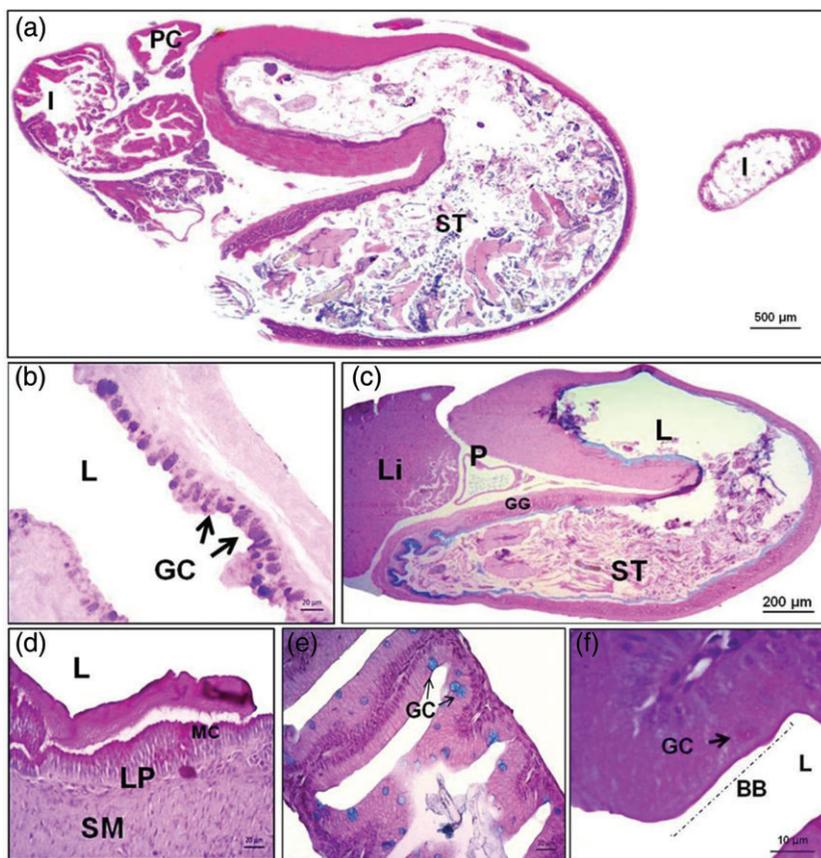


FIGURE 5 Histological and histochemical sections of the digestive system of *Arapaima gigas* larvae in 1 day after collection (5–7 days after hatch). (A) Histological section of digestive tract, showing the pyloric cecum. Hematoxylin and eosin. (B) Positive periodic acid-Schiff (PAS)-alcian blue (AB) pH 2.5 reaction in the goblet cells of the esophagus. PAS + AB pH 2.5. (C) Gastric glands secreting sulfated acid mucins. AB pH 1.0 and counter-staining hematoxylin. (D) Simple columnar epithelium with positive PAS reaction and connective tissue composing lamina propria and submucosa of the stomach. PAS. (E) Goblet cells of the posterior intestine with AB reaction pH 1.0 positive. AB pH 1.0 and counter-staining hematoxylin. (F) Intestinal epithelium showing brush border and goblet cells with neutral mucins. PAS. *Note.* BB = brush border; GC = goblet cells; GG, gastric glands; I, intestine; L, lumen; Li, liver; LP = lamina propria; MC = superficial mucous cells; P = pancreas; PC = pyloric cecum; SM = submucosa; ST = stomach

TABLE 1 Histochemical analysis in the digestive system of *Arapaima gigas* larvae for up to 20 days of collection (DAC)

DAC	Techniques employed	Regions/cells				
		Esophagus/ goblet	Stomach/ epithelial	Stomach (glands)/ Oxynticopeptic	Pyloric cecum/ goblet	Intestine/ goblet
0	PAS	+++	+++	+++	+++	+++
	AB pH 2.5	++	+++	+	+++	+++
	AB pH 1.0	++	++	+	++	+++
	AB pH 2.5 + PAS	+++	++	+++	+++	+++
3	PAS	+++	+++	+++	++	++
	AB pH 2.5	+++	++	++	+	+
	AB pH 1.0	++	++	+	+	+
	AB pH 2.5 + PAS	++	++	+++	+	+
7	PAS	+++	+++	+++	+	+
	AB pH 2.5	+++	+++	++	++	+++
	AB pH 1.0	++	+++	+	+++	++
	AB pH 2.5 + PAS	+++	+++	+++	++	++
11	PAS	+++	+++	+++	+++	+++
	AB pH 2.5	+++	+++	++	+++	+++
	AB pH 1.0	+++	+++	+	+++	+++
	AB pH 2.5 + PAS	+++	+++	+++	+++	+++
20	PAS	+++	+++	+++	++	+++
	AB pH 2.5	+++	+++	+++	++	+++
	AB pH 1.0	+++	+++	+	+++	+++
	AB pH 2.5 + PAS	+++	+++	+++	+++	+++

Note. Staining intensity: - = negative; + = weak; ++ = moderate; +++ = strong; PAS = periodic acid-Schiff; AB = alcian blue.

The intestine convolution (loops) started at 5 DAC (0.06 ± 0.01 g and 2.42 ± 0.12 cm) (Table 2, Figure 7A).

In general, there were no significant changes in the structures from the 2nd (0.06 ± 0.01 g and 2.36 ± 0.07 cm) to the 11th DAC (0.50 ± 0.04 g and 4.50 ± 0.23 cm), with the exception of the gastric glands. Apparently, these

TABLE 2 Summary of main morphological events during the development of digestive system in arapaima larvae for up to 20 days after collection

Events	Days After Collection			
	0	5	11	20
Esophagus goblet cells	+	++	++	++
Saccular-shaped stomach	++	++	++	++
Gastric glands	+	+	++	++
Stomach muscular layer	-	+	++	++
Presence of pyloric cecum	+/-	+	+	+
Folds in the intestine	+	++	++	++
Brush border	+	++	++	++
Intestine convolution (loops)	-	+	++	++
Complexity in intestine's folds	-	+	++	++
Liver	+	++	++	++
Pancreas	+	++	++	++

Note. +/- = present in some larvae; + = developed; ++ = highly developed; - = undeveloped.

increased in concentration (Figures 6A–C), as did the thickness of the stomach muscular layer (Table 2, Figure 6). Moreover, stomach epithelial cells were found to be positive for a combination of AB pH 2.5 + PAS (Table 1, Figures 7B–C), as well as the pyloric cecum that was shown to be positive to AB pH 2.5, and the intestine presented a positive reaction to AB pH 1.0 and AB pH 2.5 + PAS association (Figures 6D–F).

The post cofeeding period, mainly on the 17th DAC (1.32 ± 0.15 g and 6.43 ± 0.22 cm), was marked by thickening of the stomach muscular layer and relative increase of stomach size until the 20th DAC (1.97 ± 0.20 and 7.22 ± 0.23 cm) (Figures 7D–E). In addition, the intestine presented higher complexity in its folds (Table 2, Figure 4F), compared to earlier stages.

4 | DISCUSSION

The rapid early development of arapaima larvae is similar to that of most neotropical species, in which individuals may reach up to 250% of their body mass in 2 weeks after hatching, depending on the diet provided (Portella et al., 2014). The cofeeding period seems to be an efficient protocol in farming conditions because of the arapaima's continuous weight gain, including when larvae were fed only a microdiet. High growth rates in fish larvae appear to be associated with a low cost of growth and high food conversion efficiency, which can be enhanced if the fish is raised under optimum conditions of water quality and diet (Conceição, Dersjant-li, & Verreth, 1998).

In the present study, at 0 DAC (0.05 ± 0.01 g and 2.21 ± 0.06 cm), the larvae presented no endogenous reserves and a relatively well-developed digestive system. These two facts indicate that, at the time the arapaima comes to the surface, it is well equipped for exogenous feeding. This is relevant for proper early feeding management, once a suitable diet is available at the start of exogenous feeding, to avoid adverse effects on the larvae's ontogenetic development (Hamre et al., 2013; Segner et al., 1993), growth (Shan, Quan, & Dou, 2008), survival rate (Zhang et al., 2009), and health conditions (Gisbert, Conklin, & Piedrahita, 2004). The acid and neutral mucin

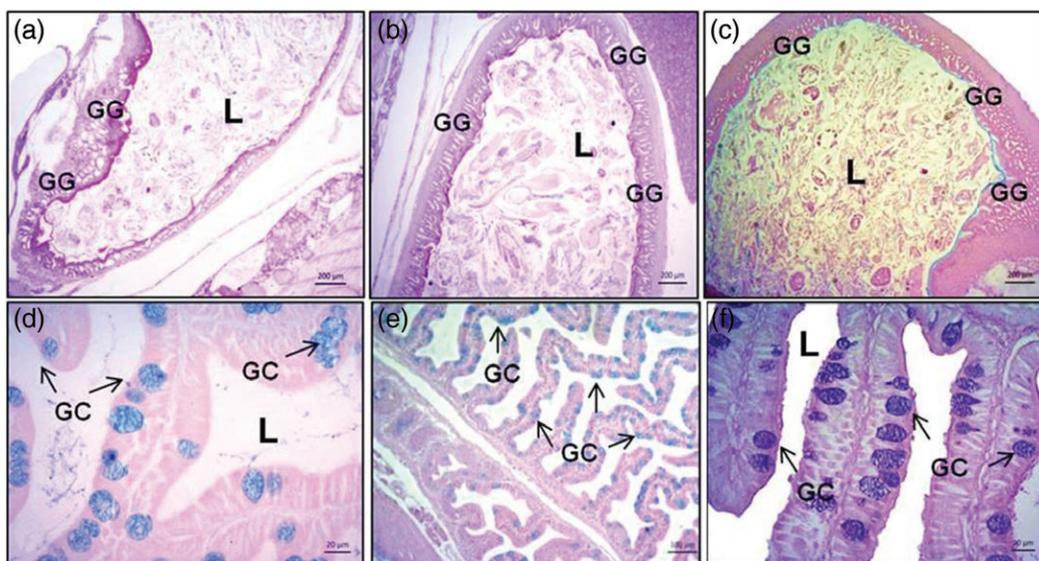


FIGURE 6 Histochemical sections of the digestive tract of *Arapaima gigas* larvae. (A) Gastric glands of larvae at 3 days after collection (DAC) (7–9 days after hatch [DAH]). Periodic acid-Schiff (PAS). (B) Gastric glands of larvae at 7 DAC (11–13 DAH). PAS. (C) Gastric glands of larvae at 11 DAC (15–17 DAH). Alcian blue (AB) pH 1.0. (D) Pyloric cecum of larvae at 11 DAC, with positive reaction for AB pH 2.5. (E) Intestine of larvae at 11 DAC, AB pH 1.0 positive. (F) Goblet cells of the intestine of larvae at 11 DAC, with positive reaction to combination of AB pH 2.5 + PAS. Note. GC = goblet cells; GG = gastric glands; L = lumen

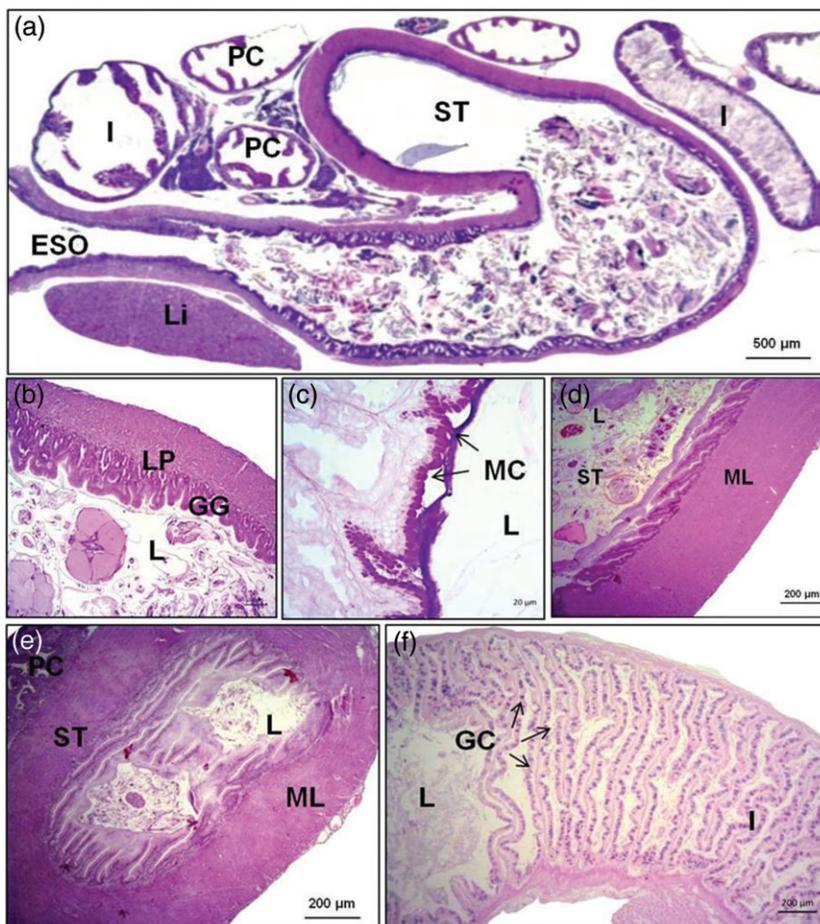


FIGURE 7 Histochemical sections of the digestive tract of *Arapaima gigas* larvae. (A) Histological section of the larvae intestine at 5 days after collection (DAC) with signs of folding, showing the pyloric cecum. Hematoxylin and eosin (HE). (B) Gastric glands of larvae at 11 DAC (16–18 days after hatch [DAH]). HE. (C) Epithelial cells of the stomach with periodic acid-Schiff (PAS) positive neutral mucins at 7 DAC (12–14 DAH). PAS + alcian blue (AB) pH 2.5. (D) Muscle layer of gastric mucosa of larvae at 17 DAC (21–24 DAH). HE. (E) Muscle layer of the gastric mucosa of larvae at 21 DAC (24–27 DAH). HE. (F) Histological section of the intestine, evidencing the complexity of intestinal folds, at 21 DAC (24–27 DAH) and goblet cells with positive reaction to AB association pH 2.5 + PAS. Note. Eso = esophagus; GC = goblet cells; GG = gastric glands; I = intestine; L = lumen; Li = liver; LP = lamina propria; MC = mucous cells; ML, muscle layer; PC = pyloric cecum; ST = stomach

secretions in the esophageal epithelium at 0 DAC in arapaima larvae confirm the results with *Sparus aurata*, *Solea senegalensis*, *Acipenser baeri* (Sarasquete, Gisbert, Ribeiro, Vieira, & Dinis, 2001), and *Umbrina cirrosa* larvae (Zaiss et al., 2006). These species present large amounts of neutral glycoconjugates in their goblet cells in the esophagus distal region, while large amounts of acid glycoconjugates were observed in the proximal region.

This condition suggests the occurrence of digestive activity that contributes to chyme formation and epithelium lubrication during digestion (Scocco, Accili, Menghi, & Ceccarelli, 1998). The acid mucins can increase the fluid viscosity (Díaz, García, & Goldemberg, 2008) to lubricate and protect the epithelium from pathogenic and mechanical damage (Fletcher & Grant, 1969; Sarasquete et al., 2001). Neutral mucins contribute by emulsifying the feed and initiate pregastric digestion (Grau, Crespo, Sarasquete, & Gonzales, 1992; Murray, Wright, & Goff, 1996).

In arapaima, the gastric glands were morphologically formed at 0 DAC (0.05 ± 0.01 g and 2.21 ± 0.06 cm). From this point on, they would only secrete more acid substances (pepsin and hydrochloric acid). This level of digestive

maturation is similar to that of *H. lacerdae* larvae at 5 DAH (Luz & Portella, 2005), as well as to that of *P. fasciatum* and *P. mesopotamicus* larvae at 10 DAH (Dabrowski & Portella, 2006; Tesser et al., 2005). Moreover, positive reactions for acid and neutral mucins, such as the ones observed in the present study (Figure 3C–D), have been suggested as indicators of the functionality of the digestive tract and the ability of the fish larvae to digest inert feed (Ma, Qin, Hutchinson, Chen, & Song, 2014). However, it contrasts with *S. aurata* larvae, which presented negative histochemical reactions to these dyes during the exotrophic phase (Elbal, García-Hernández, Lozano, & Agulleiro, 2004).

The pancreas and liver development in arapaima larvae were similar to those found in other neotropical freshwater species, such as *P. mesopotamicus*, which presented these accessory organs developed only at 4 and 2 DAH, respectively (Tesser et al., 2005), for *P. fasciatum* (both at 2 DAH; Dabrowski & Portella, 2006) and *H. lacerdae* (5 DAH for liver and 7 DAH for pancreas; Luz & Portella, 2005).

Two well-developed pyloric ceca were present in 20% of the arapaima larvae from 0 DAC, corresponding to 5–7 DAH, which is similar for *U. cirrosa* larvae (13 DAH; Zaiss et al., 2006) and *Brycon orbignyanus* larvae, in which the pyloric ceca were visible from the 7th DAH (Maciel et al., 2010). This organ is mainly related to the increase in the nutrient absorption surface (Hoar & Randal, 1969; Rønnestad et al., 2013; Rust, 2002). The neutral mucins activity in the pyloric ceca suggests that digestive enzymes are present and that nutrient absorption occurs in this organ (Hoar & Randal, 1969). These results should be later confirmed by digestive enzymes activity essays.

The intestinal convolution of arapaima larvae started at 5 DAC (0.16 ± 0.02 g and 3.29 ± 0.09 cm). The intestinal loop curvature and convection help in increasing the time of feed passage and, consequently, in nutrient absorption (Seixas-Filho, Brás, & Gomide, 2000), which positively affect fish growth and development.

The synchronization of the organism's physiology with feeding rearing practices can reduce or replace the use of live feed by inert diets (Lazo, Darias, & Gisbert, 2011; Srichanun, Tantikitti, Utarabhand, & Kortner, 2013). Therefore, arapaima larval digestive maturation at 0 DAC indicates that it may be possible to perform successful cofeeding, or even a full replacement with inert feed, starting at this early stage (around 2 cm).

5 | CONCLUSION

The gastrointestinal tracts in arapaima larvae are well developed from the first day of collection from breeding ponds. This suggests that arapaima larvae are capable of being fed on an exogenous inert diet at a size of around 2 cm. Further studies on the enzymatic apparatus are necessary to support the hypothesis of the mature digestive system functionality in these very early stages.

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