



Development of the digestive system in larvae of the Neotropical fish *Prochilodus argenteus* (Characiformes, Prochilodontidae)

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ABSTRACT. The development of the digestive system in larvae of the Neotropical fish *P. argenteus* was analyzed histologically. On the 3rd day after hatching, the digestive system comprised oropharyngeal cavity, esophagus and simple undifferentiated tube. Since secreting cells, positive to Alcian Blue (AB), were found in the esophagus, digestive activity in the initial phase had occurred. On the 18 and 28th days after hatching, the esophagus was positive for AB and Periodic Acid Schiff (PAS) stain. The stomach was fully differentiated, with the cardiac, fundic and pyloric regions. Different regions of the epithelium were characterized by basic and acidic secreting cells (AB and PAS positive). On the 18 and 28th days after hatching, the intestine was long, coiled and divided into proximal, middle and distal segments with pyloric caeca. Secreting cells in different regions of the gut were either positive or negative for AB and PAS. Results showed that larvae of *P. argenteus* exhibited digestive activity on the third day after hatching, with fully differentiated stomach and intestines on the 18 and 28th days and their different regions featuring secreting cells.

Keywords: digestion capacity, larval development, *Prochilodus*, secreting cells.

Desenvolvimento do sistema digestório em larvas do peixe Neotropical *Prochilodus argenteus* (Characiformes, Prochilodontidae)

RESUMO. O presente estudo teve como objetivo acompanhar o desenvolvimento do sistema digestório em larvas do peixe Neotropical *Prochilodus argenteus* utilizando-se análises histológicas. No terceiro dia após a eclosão, o sistema digestório consistiu de cavidade orofaríngea, esôfago e um tubo simples indiferenciado. No entanto, as células do esôfago apresentavam marcação positivas para azul de Alcian (AB), indicando a possibilidade de ocorrência de atividade digestória nesta fase inicial. Nos dias 18 e 28 após a eclosão, as células do esôfago mostraram-se positivas para AB e ácido periódico de Schiff (PAS). O estômago se encontrava totalmente diferenciado, evidenciado pela presença da região cárdica, fúndica e pilórica. As células epiteliais dessas regiões apresentaram células secretoras ácidas e básicas (AB e PAS positivas). Nos dias 18 e 28 após a eclosão, o intestino da larva se mostrou longo, enrolado e dividido em segmentos proximal (com cecos pilóricos), médio e distal. As células epiteliais secretoras do intestino foram positivas ou negativas para a AB e PAS. Em conclusão, as larvas de *P. argenteus* exibiram atividade digestiva já no terceiro dia após a eclosão, e no 18 e 28^o dias já apresentavam estômago e intestino totalmente diferenciados, com células secretoras nas suas diferentes regiões.

Palavras-chave: capacidade de digestão, desenvolvimento larval, *Prochilodus*, células secretoras.

Introduction

Fish farms in Brazil have proved to be an important economic activity. Larval rearing is a critical period of development mainly due to the transition from endogenous to exogenous feeding (Santos & Luz, 2009). So that low growth and high mortality rates may be avoided, it is important to meet the nutritional requirements of fish larvae, particularly altricial larvae, characterized by an

undifferentiated digestive system and by dependence on live feed at the start of exogenous feeding to assist in the digestion process (Micale, Garaffo, Genovese, Spedicato, & Muglia, 2006).

The alimentary canal of fish larvae is morphologically and physiologically less developed than that of adults (Govoni, Boehlert, & Watanabe, 1986). Diets based on living organisms or inert feed are generally used on fish farms and depend on the stage of maturation of the digestive system of the

larvae (Lim, Dhert, & Sorgeloos, 2003). According to Papadakis et al. (2009), the rate of maturation and function of the digestive system is linked to the different types of food provided. Since the diet offered is not fully digested due to the non-production of enzymes by the digestive system (Chen, Qin, Kumar, Hutchinson, & Clarke, 2006; Moura et al., 2012), the success of different diets used in hatcheries is directly related to organogenesis (Önal, Langdon, & Çelik, 2008).

P. argenteus of the family Prochilodontidae, known in Brazil as curimatã-pacu, is native to the rivers of South America. It is a rheophilic species with high fertility rates reaching up to 15 kg of body weight (Arantes, Santos, Rizzo, & Bazolli, 2011). In the wild, adults are iliofagous fish and feed on particulate organic matter or on submerged vegetation. A balanced diet is required to meet the nutritional needs of this fish in confinement (Bomfim, Lanna, Serafini, Ribeiro, & Pena, 2005), whereas their larvae feed on plankton.

Current analysis investigates the development of the digestive system in *P. argenteus* larvae.

Material and methods

An experiment was conducted with *P. argenteus* larvae in January 2012 at the *Centro Integrado de Recursos Pesqueiros e Aquicultura of the Companhia do Desenvolvimento dos Vales do São Francisco e do Parnaíba* (Codevasf), in the state of Minas Gerais, Brazil. Larvae at the onset of exogenous feeding phase were distributed in tanks with constant aeration and a 12-hour light dark⁻¹ photoperiod and analyzed for a period of 28 days. The larvae were fed on newly hatched *Artemia* sp. nauplii between the 4 and 6th day after hatching. Seven hundred nauplii were offered twice a day to each larva. After this period, the larvae were fed *ad libitum* with a commercial diet containing 420 g kg⁻¹ crude protein (minimum), 100 g kg⁻¹ moisture (maximum), 67.5 g kg⁻¹ ether extract (minimum), 137.5 g kg⁻¹ mineral matter (maximum), 55 g kg⁻¹ crude fiber (maximum), 27 g kg⁻¹ calcium (maximum) and 9.25 g kg⁻¹ phosphorus (minimum).

Water temperature was taken at 6 am and 6 pm. Electrical conductivity ($\mu\text{S cm}^{-1}$), pH, turbidity and dissolved oxygen (mg L^{-1}) were evaluated at 7 am every five days with a Horiba W-22XDD device. Ammonium concentrations (mg L^{-1}) were determined at 7 am every five days by the Nessler method. The tanks were siphoned daily for the removal of waste and 60% of the water volume was renewed.

Specimens were sampled on the 3rd day after hatching (DAH) (total length: 6.40 mm; weight: 2.59 mg), 18th DAH (13.91 mm; 30.44 mg) and 28th DAH (25.67 mm; 148.64 mg) for the description of the development of the digestive system; anesthetized with benzocaine (0.2 g L^{-1}); fixed in Bouin's solution for 12h; and kept in 70% ethanol prior to histological analysis. The samples (six specimens at each age studied) were processed and stained for morphometric evaluation at the Cell Biology Laboratory of the Universidade Federal dos Vales do Jequitinhonha e Mucuri, using routine techniques. Dehydration was performed at increasing alcohol concentrations (70, 80, 90, 95 and 100%). Samples were then diaphanized in xylene (I, II, III) and embedded in paraffin. Further, 5- μm -thick sections were obtained with a microtome (LUPETEC MRP09) and the samples were placed on glass slides and stained with hematoxylin-eosin (HE), Alcian blue (AB) and periodic acid-Schiff (PAS). The morphological evaluation of the larval digestive system and photographic documentation were performed with a light microscope (Zeiss, Primo Star) coupled to a digital camera (AxioCam ERc5s). All procedures followed guidelines for the ethical treatment of animals (CEUA-015/2013).

Results and discussion

Table 1 displays the limnological variables during the experimental period.

Table 1. Mean pH, electrical conductivity, turbidity, dissolved oxygen and ammonium during the experimental period after the hatching of *Prochilodus argenteus* larvae.

Day	pH	Conductivity ($\mu\text{S cm}^{-1}$)	Turbidity (NTU)	Oxygen (mg L^{-1})	Ammonium (mg L^{-1})
8 th	6.36	295.80	2.87	5.96	0.50
12 th	6.87	308.49	3.08	5.12	0.69
16 th	6.58	244.89	5.72	5.71	0.88
20 th	6.88	290.96	5.48	6.19	1.95
24 th	7.09	349.23	5.09	6.23	1.91
28 th	6.94	403.23	9.00	5.64	1.50

Water temperature ranged between 26.0°C and 27.0°C, whilst electrical conductivity, pH, turbidity and dissolved oxygen remained within the comfort range for the species (Table 1), as reported for the genus *Prochilodus* (Bomfim et al., 2005; Makino et al., 2012). Ammonium levels were similar to or lower than those observed in a previous study when growth in length and weight was similar to that reported for other larvae of the genus *Prochilodus* (Santos & Luz, 2009), demonstrating appropriate larval cultivation conditions for *P. argenteus*.

Morphofunctional follow-up proved to be a valuable tool to assess the maturation of the developing digestive tract and evaluate changes in the ability to assimilate food throughout larval development. Such knowledge allows optimizing feeding management (Zaiss, Papadakis, Maingot, Divanach, & Mylonas, 2006). The supply of an inert diet in the early stages of fish development is highly desirable since it reduces hatchery costs. However, the acceptance of an inert diet depends on the maturation of the digestive system. The larvae of different species are fed on *Artemia* sp. nauplii to adapt or optimize the digestion process, which may involve a gradual transition period of co-feeding with live feed and inert feed or an abrupt transition between the two forms of feeding (Ballagh, Fielder & Pankhurst, 2010; Bonaldo, Parma, Badiani, Serratore, & Gatta, 2011; Chepkirui-Boit et al. 2011; Engrola et al., 2009; Mai et al., 2009).

The index of larvae survival in current experimental conditions was 55%, which suggests that the supply of *Artemia* sp. for three days was sufficient for the maturation of the digestive system in *P. argenteus* larvae, and facilitated the acceptance of inert feed and consequently aided species growth. This approach proved more efficient than the offer of inert feed as soon as mouth opening is observed in larvae, as reported for Neotropical species, such as *P. mesopotamicus* (Menossi et al., 2012), *P. argenteus* [Characiformes] and *Steindachneridion melanodermatum* (Feiden, Hayashi, & Boscolo, 2006).

On the 3rd DAH, the larvae measured 6.40 mm and 2.59 mg. They had pigmented eyes, an inflated swim bladder, digestive tube and notochord in formation (Figure 1A). Gill arches in the opercular region, neural tube formation and a flexed notochord were evident. The mouth was open and in the terminal position. Exogenous feeding was provided since the digestive tract was already open. However, yolk reserves remained (Figure 1B). The gastrointestinal tract consisted of the oropharyngeal cavity, esophagus and digestive tube in formation (Figure 1C). The esophagus was a straight tube connecting the oropharyngeal cavity and digestive tube, lined with stratified squamous epithelium. Mucous or secreting cells were detected in the epithelium, with positive staining for AB in the anterior esophageal region. Below the epithelium, the lamina propria consisted of loose connective tissues, followed by two muscle coats (Figure 1D, E). The forming digestive tube was lined with a simple, columnar epithelium with a striated border, similar to that found in the small intestine of mature

specimens. The hepatopancreas was also evident (Figure 1F).

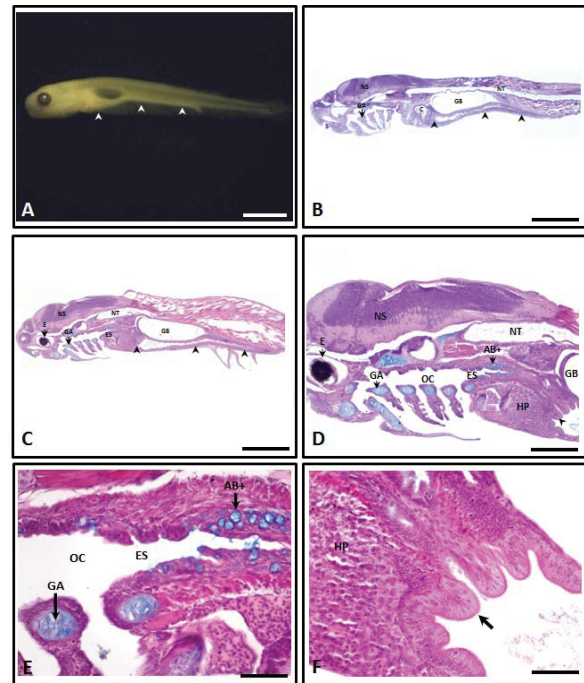


Figure 1. *Prochilodus argenteus* larvae on the 3rd day after hatching; A – photo obtained with the aid of a stereoscopic microscope. Arrowheads point to digestive tube in formation. B – micrograph of longitudinally sectioned larva. NS (nervous system), NT (notochord), GB (gas bladder), C (calf), GA (gill arches), arrowhead: primitive digestive tube (stain: HE). C – longitudinal section. E (eye), ES (esophagus). D – higher magnification of figure C. OC (oropharyngeal cavity), HP (hepatopancreas), AB+ (esophageal cells positive for Alcian Blue). E – details of figure D. F – photomicrograph of primitive digestive tube. Arrow: absorptive cells (AC) of primitive digestive tube; Bars: A = 1 mm; B and C = 500 μ m; D = 200 μ m; E and F = 50 μ m.

The morphological characteristics of the digestive system in *P. argenteus* larvae described herein were similar in several aspects to the larvae of other teleost fish. On the 3rd DAH, the eye with pigmented retina, gill arches, swim bladder, pectoral fin and caudal fin were observed and the mouth was open and in the sub-terminal position, similar to descriptions by Godinho and Godinho (2003).

Similar to *Prochilodus* (3rd DAH) (Figure 1A), the larvae of several species and orders, such as the Characiformes *Brycon orbignyanus* (Maciel et al., 2010), the Silurioformes *Hemisorubim platyrhynchos* (Faccioli et al., 2016), the Perciformes *Cichlasoma rophthalmus* (Cuenca-Soria et al., 2013) and *Symphysodon* sp. (Önal, Çelik, & Cirik, 2010) revealed an undifferentiated digestive tract and a yolk sac during the first days of life.

Yolk reserves were still visible although the digestive tract was apparently suitable for the onset of exogenous feeding. The above features have already been reported for other Neotropical species with different feeding habits, such as Brycon *orthotaenia* larvae (Gómez, Calcagno, & Fuentes., 2011) in which the mouth was open and complete absorption of the yolk sac occurred on the 3rd DAH, which indicated the onset of differentiation of the digestive system. In other species, yolk sac reserves have been observed at different stages, such as the larvae of *Z. jahu* (Nogueira et al., 2012), *R. quelen* (Rodrigues-Galdino et al., 2009) and *R. aspera* (Perini, Sato, Rizzo, & Bazolli, 2009), in which total absorption of the yolk sac has been reported to occur on the 2nd, 3rd and 5th DAH, respectively. After the opening of the mouth, larvae are generally able to ingest, digest and assimilate food, which depends on the diet provided and the development of the digestive system (Pedreira, Santos, Sampaio, Pereira, & Silva, 2008). *P. argenteus* larvae in the 3rd DAH ingested and assimilated *Artemia* sp. nauplii, which has also been reported for *M. sanctaefilomenae* larvae at the onset of exogenous feeding (Walter, 2013).

Maturation time of the digestive system is not similar among all fish species. The digestive tract of *M. platanus* (Galvão, Fenerich-Verani, Yamanaka, & Oliveira, 1997), a marine species with iliophagous eating habits, is characterized by an oropharyngeal cavity, esophagus and coiled intestine on the 3rd DAH. Further, *P. argenteus* is a freshwater species with iliophagous eating habits and a digestive system featuring an oropharyngeal cavity, esophagus and rectum digestive tube in formation on the 3rd DAH. The epithelium of the esophagus was positively stained for AB and revealed the rectum digestive tube lined with differentiated absorptive cells. Acid secretions in the esophagus evidence digestion activity and protection against microorganisms, as described for other species (Arellano, Dinis & Sarasquete, 1999, Sarasquete, Gisbert, Ribeiro, Vieira, & Dinis, 2001). All these features indicate the ability of the larvae to digest and absorb food. The same physiological behavior has been described for the larvae of *S. senegalensis* (Ribeiro, Sarasquete, & Dinis, 1999) and *P. fulvidraco* (Yang, Xie, Fan, Gao, & Fang, 2010), in which the digestive tract differentiates into an oropharyngeal cavity, esophagus, primary stomach and intestine on the 2nd DAH, demonstrating the ability to start exogenous feeding. The above structure structures are differentiated only on the 5th DAH in *P. maculatofasciatus* (Peña, Dumas, & Villalejo-Fuerte, 2003). Histochemical analysis by Faccioli et al. (2016) revealed that the esophagus of the

Silurioformes *H. platyrhynchos* had globet cells PAS and AB positive at 2nd DAH.

At this stage of development (3rd DAH) of the digestive system, *P. argenteus* larvae digested zooplankton even before the total consumption of yolk reserves, as observed for pacu *P. mesopotamicus* larvae, another Characiformes Neotropical fish (Portella et al., 2014), which were already capable of ingesting inert food.

Larvae measured 13.91 mm and 30.44 mg on the 18th DAH and the stomach was identifiable. Gastric glands were numerous in the cardiac and fundic regions, but absent from the pyloric region. The intestine was long and coiled. The wall of the esophagus was more pleated and lined with stratified squamous epithelium, followed by the lamina propria of connective tissue and muscle layers. The esophagus exhibited skeletal striated muscle tissues. The epithelial lining revealed glandular cells secreting neutral and acidic substances, identified by positive reactions to AB (Figure 2A-arrow) and PAS stains (Figure 2B-arrow).

The transition between the esophagus and the stomach was characterized by an abrupt change in the constitution of the tissues. The stomach was lined with simple prismatic epithelium and the lamina propria consisted of connective tissue. The cardiac and fundic regions exhibited glands (GS: glandular stomach) and the pyloric region showed muscle tissues (MS: muscular stomach) (Figure 2C and E). Epithelial cells lining the glandular (cardia and fundus) and muscular (pyloric) regions were positive for AB (Figure 2D: arrowheads) and PAS (Figure 2E: arrowhead), indicating the production of neutral and acidic secretions.

The pyloric region consisted of a thick muscle layer (MS: muscular stomach) responsible for the mechanical digestion required for iliophagous eating habits (Figure 2C-E). The intestine was long, coiled and divided into three segments: proximal, middle and distal. The epithelial lining in these regions was composed of simple columnar epithelium with a striated border. The initial region of the proximal segment was formed by pyloric ceca in continuity with the stomach muscles to allow the breakdown of food (Figure 2F). The region of the pyloric ceca and the proximal, middle and distal segments of the intestine were primarily composed of absorptive cells interspersed by a small number of cells positive for AB and PAS as well as by cells negative to AB and PAS (basic). The appearance is very similar to that observed in larvae with 28th DAH illustrated in Figure 3F (arrow and arrowhead).

Changes in the formation of the digestive tube were more prominent on the 28th DAH (Figure 3A)

when larvae measured 25.67 mm and 148.64 mg. The cells of the esophagus were positive for AB and PAS (Figure 3B and C). Positive reactions to AB and PAS were also detected in both the glandular (cardia and fundus) and muscular (pyloric) regions of the stomach, although photodocumentation was only performed for the pyloric region (Figure 3D and E). The intestine was highly coiled and occupied most of the coelomic cavity. The epithelial lining exhibited goblet cells of acidic (AB positive - arrow), neutral (PAS positive - not showed in Figure) and basic (AB and PAS negative - arrowhead) material (Figure 3F).

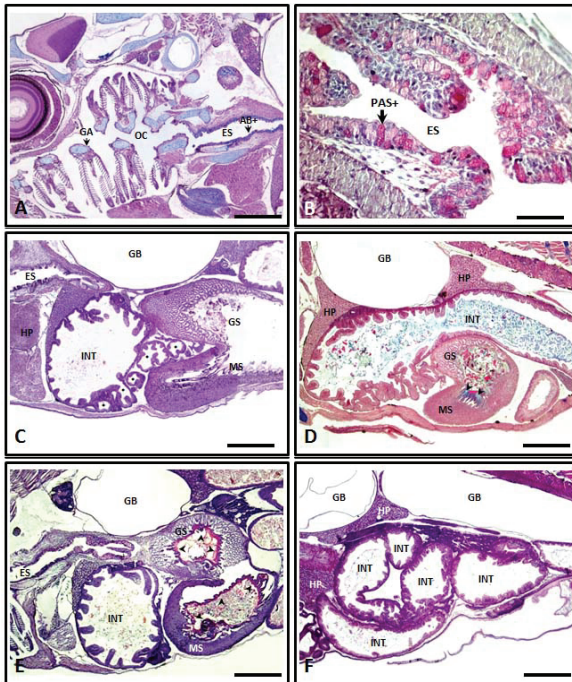


Figure 2. Photomicrographs of *Prochilodus argenteus* larvae split lengthwise on 18th day after hatching; A – GA (gill arches), OC (oropharyngeal cavity), ES (esophagus), esophageal region showing AB+ cells (arrow). B – esophageal region showing PAS+ cells (arrow). C – HP (hepatopancreas), GB (gas bladder), GS (glandular stomach), MS (muscular stomach), INT (intestine). D – glandular stomach showing AB+ cells (arrowheads). E – glandular stomach showing PAS+ cells (arrowheads). F – gut region showing goblet cell secretion (stain: HE). Bars = 50 μ m.

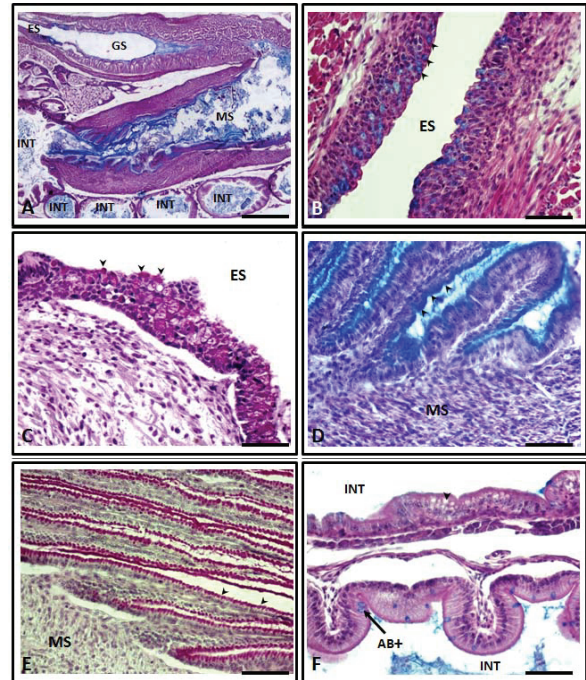


Figure 3. Photomicrographs of *Prochilodus argenteus* larvae split lengthwise on the 28th day after hatching; A – ES (esophagus), GS (glandular stomach), MS (muscular stomach), INT (intestine). B – esophageal region showing AB+ cells (arrowheads). C – esophageal region showing PAS+ cells (arrowheads). D – muscular stomach showing AB+ cells (arrowheads). E – muscular stomach showing PAS+ cells (arrowheads). F – gut region showing goblet cell secretion with AB+ (arrow) and secreting cells negative for AB and PAS (arrowhead). Bars = 50 μ m.

Although *P. argenteus* larvae accepted rations on the 4th DAH, more efficient digestion likely began when the stomach was able to produce secretions, as occurred on the 18th DAH. In the case of *Salminus brasiliensis* larvae, with a more accelerated development than *P. argenteus* larvae, the digestive system is reported to be physiologically able to digest on the 3rd DAH due to the production of acidic secretions in the stomach (Vega-Orellana, Fracalossi, & Sugai, 2006), although the authors provided live food after hatching and gradually replaced it with a formulated diet beginning on the 5th DAH to optimize the assimilation of the inert diet.

Stomach formation appears as a bulge at the end of the esophagus at the onset of exogenous feeding in other species, such as the larvae of *Seriola lalandi* (Chen et al., 2006). In *P. argenteus* larvae, the stomach was not evident in the early stage. In fact, the esophagus and the stomach were at a differentiation stage only on the 18th DAH, exhibiting epithelial lining composed of AB positive cells, which demonstrated the digestion process at this stage, similar to that reported in Silurioformes

H. platyrhynchos on the 21st DAH (Faccioli et al., 2016). Differentiation in *P. argenteus* was even more evident on the 28th DAH.

The stomach of *S. lalandi* larvae is reported to be differentiated into the cardiac, fundic and pyloric regions on the 18th DAH (Chen et al., 2006), similar to *P. argenteus* larvae in current investigation. However, *Seriola lalandi* larvae exhibited gastric cells in the cardia and fundic regions, whereas the three regions in *P. argenteus* were secreting acidic and neutral substances, identified by AB and PAS staining. The pyloric ceca, an important feature of the digestive system that assists in the process of nutrient absorption combined to other segments of the intestine, were formed in the two species by the 18th DAH.

Despite similar development in the above example, the maturation period of the digestive system in *P. argenteus* differs from that of other species. In *U. cirrosa* L. larvae, the stomach appears morphologically differentiated with abundant gastric glands in the cardiac region by the 9th DAH (Zaiss et al., 2006). Other fish species, such as *P. californicus* larvae in which the stomach is reported to be differentiated into different regions between the 27 and 30th DAH (Gisbert, Piedrahita, & Conklin, 2004), have a slower maturation of the digestive system when compared to that in *P. argenteus*. The intestine, which was coiled throughout the development of *P. argenteus* larvae, was quite evident by the 28th DAH, with mean total length 25.67 mm. Analyzing *G. morhua* L. larvae, Wold, Hoehne-Reitan, Rainuzzo and Kjørsvik (2008) reported that gut development was related to the size of the larvae rather than to age. The authors state that the supply of an inappropriate formulated diet, even with a co-feeding transition process, may impair larval growth. *P. argenteus* larvae exhibited AB and PAS positive cells interspersed with absorptive cells in pyloric ceca as well as different regions of the intestine on the 28th DAH, demonstrating the functionality of the intestinal region, which is similar to findings described for other teleosts (Gisbert et al., 2004; Ribeiro et al., 1999; Qu et al., 2012).

Differences in the development of the digestive system are related to different feeding habits among fish species, with functionality emerging at different stages of life, as reported for iliophagous species, such as the larvae of *P. argenteus* and juveniles of *R. aspera*, *H. regani*, *H. ternetzi*, *H. margaritifera*, *H. microstomus* and *M. aculeatus* (Delariva & Augustine, 2001), and of omnivorous species, such as *Leporinus friderici* juveniles (Albrecht & Caramaschi, 2003), which enhance a greater utilization of nutrients in foods.

Conclusion

The digestive system in *P. argenteus* larvae exhibits the production of acids in the esophagus and a primitive digestive tube with the capacity for nutrient absorption by the 3rd DAH. On the 18th DAH, the stomach is functional and the production of acidic material occurs in the esophagus and in the stomach. The intestine evidenced pyloric ceca evident, which increase the area of nutrient absorption. These morphological and physiological changes are more noticeable by the 28th DAH. The above results demonstrate the ability to absorb exogenous inert feed in the early stages of the life of the species.

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