

## Intestinal histomorphology in *Pseudoplatystoma fasciatum* fed bovine colostrum as source of protein and bioactive peptides

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**ABSTRACT:** Histological responses of the intestine are key for evaluating nutritional value of feed ingredients, since the organ is not only the chief site of feed digestion and nutrient absorption but also plays an important immunological function. Histomorphological alterations were evaluated in the intestine of juvenile striped catfish, *Pseudoplatystoma fasciatum*, fed diets containing 0 (control), 10 or 20% inclusion of lyophilized bovine colostrum (LBC), as source of protein or bioactive peptides, for either 30 or 60 days. Fish fed 20LBC presented at 60d a distinct pattern of macrophages and, some of them, higher number of vacuoles in rectum mucosa. The thickness of the muscle layer (TML) in fish fed diets with LBC was higher in the first portion of medium intestine than fish fed 0LBC. All fish presented significant increase of TML in the second portion of medium intestine along feeding period, but fish fed 20LBC had smaller values of TML than those of fish fed 0 and 10LBC which might be related to the higher intestinal coefficient found for this group. The TML of rectum was higher just for fish fed 10LBC. Dietary LBC altered morphometrical features of juvenile striped catfish intestine and possibly induced inflammatory reaction in the rectal mucosa, as a function of level of inclusion, feeding period and segment of intestine analyzed.

**Key words:** gut absorption, histology, mammal protein, lyophilized bovine colostrum, striped catfish

## Histomorfologia intestinal de *Pseudoplatystoma fasciatum* alimentado com colostro bovino como fonte de proteína e peptídeos bioativos

**RESUMO:** Respostas histológicas do intestino são fundamentais para avaliar o valor nutritivo de ingredientes alimentares, uma vez que o órgão não é só o principal local de digestão e absorção dos nutrientes, mas também exerce uma importante função imunológica. Alterações histomorfológicas foram avaliadas no intestino de juvenis de cachara, *Pseudoplatystoma fasciatum*, alimentado com dietas contendo 0 (controle), 10 e 20% de inclusão de colostro bovino liofilizado (CBL) como fonte de proteína e peptídeos bioativos, aos 30 e 60 dias. Aos 60 dias, peixes alimentados com 20CBL apresentaram macrófagos de aspecto distinto e alguns, uma grande concentração de vacúolos na mucosa retal. A espessura da camada muscular (ECM) em peixes alimentados com CBL foi maior na primeira porção do intestino médio em relação àqueles alimentados com 0CBL. Ao longo do período experimental, todos os peixes mostraram aumento significativo na ECM na segunda porção do intestino médio, entretanto peixes do grupo 20CBL tiveram menores valores para a ECM do que os demais, o que pode estar relacionado com o maior coeficiente intestinal encontrado para este grupo. A ECM no reto foi maior apenas para os peixes alimentados com 10CBL. A inclusão de CBL na dieta do cachara alterou características morfológicas do intestino e, possivelmente, induziu uma reação inflamatória na mucosa retal em função do nível de inclusão, período de alimentação e porção do intestino analisada.

**Palavras-chave:** absorção intestinal, histologia, proteína láctea, colostro bovino liofilizado, cachara

### Introduction

Knowledge on anatomical and morphophysiological features of fish digestive tract set bases for the study of fish nutrition (Datta Munshi and Dutta, 1996; Evans et al., 2005; Pezzato et al., 2004). Aslaksen et al. (2007), Romarheim et al. (2008), Urán et al. (2008) and Øverland et al. (2009), have regarded histological responses of the intestine as a supplementary tool to evaluate the nutritional value of an ingredient, since the organ is not only

the chief site of feed digestion and absorption of nutrients but also plays an important immunological function (Buddington et al., 1997).

The bovine colostrum is a complex food source, with high contents of proteins and a diversity of biologically active molecules, such as growth factors, especially insulin-like growth factors (IGFs) (Blum and Hammon, 2000; Foley and Otterby, 1978; Pakkanen and Aalto, 1997). The IGFs are peptides bearing mytogenic and antiapoptotic properties that can modulate the cellular ki-

netics through both autocrine and paracrine routes, thus modulating growth, development and differentiation of several body tissues (Baumrucker and Blum, 1993; Butler et al., 1998; Howarth, 2003; Pakkanen and Aalto, 1997). High cost and low availability of bovine colostrum may hamper its broad use in animal feeds. However, studying its inclusion in fish diets would provide information about the intestinal mechanisms of absorption by the concomitant evaluation of the effects of mammal protein and growth factors orally administered to fish.

The South American striped catfish *Pseudoplatystoma fasciatum* Linnaeus 1776 (Siluriforms: Pimelodidae) is a prized carnivorous fish with farming potential for sport fisheries, food-fish or ornamental fish trade, which has been explored for fish culture in Brazil since late 1980s (Campos, 2005). Therefore, research on farmed striped catfish performance, behavior and physiology is yet understandably recent and relatively scarce (Leonardo et al., 2004, 2006; Romagosa et al., 2003 a, b). Although intestinal anatomy and histology of striped catfish had already been described by Rodrigues et al. (2009), there is a lack on studies on the species' intestinal absorptive mechanisms. This study evaluates the occurrence of histomorphological alterations in the intestine of striped catfish, fed diets containing varying levels of bovine colostrum as protein and bioactive peptides source.

### Material and Methods

Farm-raised, feed-conditioned juvenile striped catfish were stocked in 60-L, covered plastic cages housed in

concrete tanks within a greenhouse, in a closed, continuous water circulation and aeration system ( $28 \pm 2^\circ\text{C}$ ). Fish were acclimated to experimental routine feeding to apparent satiety twice a day (7h00 and 16h00) for four weeks on a commercial carnivorous fish feed (45% crude protein - CP; 3,000 kcal  $\text{kg}^{-1}$  gross energy - GE). Upon the end of the acclimatization period, fish [ $35.35 \pm 2.11$  g;  $L_s = 14.39 \pm 0.53$  cm] were assigned to experimental groups in a totally randomized design,  $3 \times 2$  factorial scheme with three diets - 0 (control) , 10 and 20% inclusion lyophilized bovine colostrum (LBC) - and two feeding periods - 30 and 60 days ( $n=3$ ; 12 fish per cage). The bovine colostrum pool was obtained from multiparous dairy cows in Piracicaba, state of São Paulo, Brazil. The material was centrifuged twice for partial extraction of fat fraction (10 min.; 12,000 g,  $4^\circ\text{C}$ ), and subsequently homogenized and frozen for the lyophilization procedure. Formulation of experimental, approximately isonitrogenous, pelleted diets and their centesimal composition are presented on Table 1. Experimental diets and LBC were submitted to chemical analysis according to AOAC (2000) methods (Tables 1 and 2). Concentration of insulin-like growth factor - I (IGF-I) in the LBC was quantified through post-extraction immunoradiometric assay kit (Diagnostic Systems Laboratories Inc., Webster, TX, USA). Lyophilized colostrum was reconstituted with milk and centrifuged for 30 min. at 13,000 x g (Vega et al., 1991). Peptides were extracted from sampled colostrum aliquots under acid ethanol treatment and quantified in a gamma counter. The estimated concentrations of IGF-I in the experimental diets are presented in Table 2.

Table 1 – Chemical composition of the experimental diets.

Ingredients	Percent in dieta		
	Control	10LBC	20LBC
Colostrum (68.52%)	-	10	20
Fish meal (60% CP)	25	25	20
Corn gluten meal (60% CP)	22	22	17.7
Soybean meal (45% CP)	15	5	2.3
Wheat meal	14	14	14
Poultry by-product meal (57.8% CP)	10	10	10
Soybean oil	7.6	12	14
Corn (whole grain)	4.4	-	-
Mineral and vitamin mix <sup>b</sup>	2	2	2
Nutrient contents <sup>c</sup>	Control	10LBC	20LBC
Moisture	4.15	4.18	11.51
Crude protein (CP)	48.77	51.44	49.72
Lipids	16.31	18.91	20.57
Ash	8.37	8.16	7.49

<sup>a</sup>Control - no lyophilized bovine colostrum (LBC) included; 10LBC - 10% LBC included; 20LBC - 20% LBC included. <sup>b</sup>Mineral and vitamin mix (mg  $\text{kg}^{-1}$  unless otherwise noted). antioxidant 0.60 g; vitamin A 1,000,000 IU; vitamin D<sub>3</sub> 500,000 IU; vitamin E 20,000 IU; vitamin K<sub>3</sub> 500; thiamine 1,250; riboflavin 2,500 mg; pyridoxine 2,485; pantothenic acid 5,000; niacin 5,000; biotin 125; folic acid 250 mg; vitamin B<sub>12</sub> 3,750; ascorbic acid 28,000; Co 25; Cu 2,000; I 100 mg; Fe 13,820; Mn 3,750; Zn 150; Se 75. <sup>c</sup>Dry matter basis (AOAC, 2000).

Fish fasted for 24 h were randomly sampled (seven fish per treatment), euthanized by anesthetic overdoses (benzocaine; 5.0 g L<sup>-1</sup>), measured, weighted, and laparotomized. Intestinal tracts (IT, pyloric sphincter to anus) were then isolated, measured (intestinal length, L<sub>IT</sub>), separated into medium intestine and rectum by identification of the ileum-rectal valve (Bértin, 1958), and the former divided into two segments: S1 (anterior section; rectilinear) and S2 (posterior section; convolute).

Intestines regions were fixed in buffered (0.1 M, pH 7.2), 4% p-formaldehyde solution, and sub-divided into 5-mm sections, which were washed in phosphate buffer solution (0.1M, pH 7.2) four consecutive times. Washed material was dehydrated by immersion in increasing ethanol concentrations: 30, 50, 70, 90, and 100% (3x in the last one), and submitted to pre-infiltration with glycol-methacrylate resin diluted in ethanol 100% (1:1). The material was then embedded in pure glycol-methacrylate resin, and transversal, non-sequential, 5-µm sections were processed for staining with 0.05% toluidine-blue O (Sigma Chemical Company, St. Louis, MO, USA) solution, buffered with citric acid and anhydrate bi-phosphate solution (pH 4.5).

Histological analysis - thickness of the muscle layer (TML; 10 fields per segment at 100x magnification); number of goblet cells in the rectum (NGC; 20 fields at 200x magnification); observation of abnormal features in the rectal mucosa - was performed and images taken in light microscope coupled to Axio-Vision 2.05 image analysis system. Intestinal coefficients (IC = L<sub>IT</sub> / L<sub>e</sub>) were also estimated for each sampled fish (Bértin, 1958). Histomorphometrical data and IC were submitted to two-way ANOVA by PROC MIXED procedures (SAS, 1991); Tukey test (α = 0.05) was used for detection of statistical differences of means (Steel and Torrie, 1980). All numerical results are given by media ± standard deviation.

Table 2 - Chemical composition of lyophilized bovine colostrum.

Nutrient	Percent contents <sup>a</sup>
Dry matter	95.49
Crude protein	68.52
Lipids	15.00
Ash	3.69
IGF-I	µg kg <sup>-1</sup> of diet <sup>c</sup>
Control <sup>b</sup>	0
10LBC <sup>b</sup>	97.2
20LBC <sup>b</sup>	194.4

<sup>a</sup>Dry matter basis (AOAC, 2000). <sup>b</sup>Control - no lyophilized bovine colostrum (LBC) included; 10LBC - 10% LBC included; 20LBC - 20% LBC included. <sup>c</sup>Estimated values after IGF-I quantification in lyophilized bovine colostrum by immunoradiometric assay. IGF-I - insulin-like growth factor - I.

## Results and Discussion

At day 30 and 60, final weight and standard length were similar between fish fed the diets ( $p > 0.05$ ): 61.16 ± 4.81 g; 17.78 ± 0.76 cm, and 102.44 ± 13.35 g; 19.81 ± 1.54 cm, respectively. Diets or extension of feeding period did not influence NGC in the rectum (Table 3). However, fish fed diet 20LBC presented at day 60 a distinct pattern of macrophages, which predominate in the apical portion of intestinal folds, exhibited a distinct staining pattern, and were apparently bigger than those found in fish fed the control or 10LBC diets (Figure 1). Some fish fed the 20LBC diet also presented at day 60 higher incidence of vacuoles in the intestinal mucosa (Figure 2).

Fish fed diet 20LBC presented higher IC ( $p \leq 0.05$ ) than fish fed diets control or 10LBC (Table 4). At day 60, fish had smaller IC than at day 30 ( $p \leq 0.05$ ). Fish fed diets containing LBC presented higher values of TML in the S1 portion of medium intestine ( $p \leq 0.05$ ) in comparison to fish fed the control diet (Table 5). All fish presented significant increase of TML in S2 along the experimental period, but fish fed diet 20LBC presented values of TML smaller than those of fish fed diets control and 10LBC ( $p \leq 0.05$ ). The TML of rectum was affected only by diets; and was higher for fish fed diet 10LBC ( $p \leq 0.05$ ) (Table 5).

Proximal portions of fishes' intestines are credited with higher nutrient digestibility and absorption, and overall digestive capacity; the distal portions of fishes' intestines are important for absorption of intact proteins and peptides molecules (Bakke-McKellep et al., 2000; Ezeasor and Stokoe, 1981; Krogdahl et al., 2003; Ledger et al., 2002; Ostaszewska et al., 2005; van den Ingh et al., 1991). This ability of distal intestine may be related to its attributed immunological function (Georgopoulou and Vernier, 1986; Jenkins et al., 1994; Rombout et al., 1985; Rombout and van den Berg, 1989).

Fish fed diets 10LBC and 20LBC presented increase of TML in S1, possibly as a result of increased activity of absorption processes in this part of the intestine. On the other hand, the reduced TML observed for fish fed diet 20LBC in S2 might be related to the higher IC induced by ingestion of LBC, since the increasing of the intestinal length could have narrowed the TML in S2. In the rectum, probably because of its naturally increased permeability and immunological sensitivity, increased TML was registered only for fish fed diet 10LBC, deemed adequate for growth promotion, seeing

Table 3 - Average number of goblet cells in the rectum.

Feeding period	Experimental group <sup>a</sup>		
	Control	10LBC	20LBC
30 days	28.53 ± 6.98	31.85 ± 7.88	33.46 ± 7.60
60 days	31.53 ± 7.16	30.59 ± 7.58	32.24 ± 7.47

<sup>a</sup>Control - no lyophilized bovine colostrum (LBC) included; 10LBC - 10% LBC included; 20LBC - 20% LBC included.

Table 4 – Intestinal coefficient (IC) of juvenile striped catfish *Pseudoplatystoma fasciatum* fed diets containing lyophilized bovine colostrum for 30 or 60 days.

Feeding period	Experimental group <sup>a</sup>				Probability <sup>b</sup>		
	Control	10LBC	20LBC	Mean	Diet	Period	Diet X period
	IC			Mean			
30 days	0.84 ± 0.05	0.82 ± 0.08	0.92 ± 0.03	0.86 ± 0.07 <sup>x</sup>	≤ 0.05	≤ 0.05	ns*
60 days	0.68 ± 0.08	0.75 ± 0.07	0.76 ± 0.06	0.73 ± 0.07 <sup>y</sup>			
Mean	0.76 ± 0.10 <sup>x</sup>	0.78 ± 0.08 <sup>x</sup>	0.84 ± 0.10 <sup>y</sup>				

<sup>a</sup>Control – no lyophilized bovine colostrum (LBC) included; 10LBC – 10% LBC included; 20LBC – 20% LBC included. <sup>b</sup>Probability: diet X feeding period – interaction diet x period. \*ns: not significant. <sup>x,y</sup>Values followed by the same capital superscript in the same column do not differ (Tukey test;  $p \leq 0.05$ ). <sup>x,y</sup>Values followed by the same lower case superscript in the same line do not differ (Tukey test;  $p \leq 0.05$ ).

Table 5 – Thickness of the muscle layer (TML) of the intestine of juvenile striped catfish *Pseudoplatystoma fasciatum* fed diets containing lyophilized bovine colostrum for 30 or 60 days.

Feeding period	Experimental group <sup>a</sup>				Probability <sup>b</sup>		
	Control	10LBC	20LBC	Mean	Diet	Period	Diet X period
	TML on S1 <sup>c</sup> (µm)			Mean			
30 days	124.12 ± 30.36	140.85 ± 24.34	150.32 ± 27.31	138.43 ± 29.39	≤ 0.05	ns*	ns*
60 days	134.23 ± 27.56	154.03 ± 26.02	149.94 ± 34.46	146.07 ± 30.65			
Mean	129.18 ± 29.33 <sup>x</sup>	147.44 ± 25.96 <sup>y</sup>	150.13 ± 30.98 <sup>y</sup>				
	TML on S2 <sup>d</sup> (µm)			Mean			
30 days	141.65 ± 29.00	125.84 ± 19.96	114.05 ± 14.56	127.18 ± 24.65 <sup>x</sup>	≤ 0.05	≤ 0.05	ns*
60 days	142.53 ± 29.35	144.97 ± 17.82	131.95 ± 16.80	139.81 ± 22.68 <sup>y</sup>			
Mean	142.09 ± 29.07 <sup>x</sup>	135.41 ± 21.16 <sup>x</sup>	123.00 ± 30.98 <sup>y</sup>				
	TML on rectum (µm)			Mean			
30 days	201.63 ± 33.65	235.88 ± 45.71	196.25 ± 40.86	211.25 ± 3.87	≤ 0.05	ns*	ns*
60 days	189.23 ± 32.94	218.96 ± 47.01	197.65 ± 34.63	201.94 ± 40.52			
Mean	195.43 ± 33.76 <sup>x</sup>	227.42 ± 46.98 <sup>y</sup>	196.95 ± 37.74 <sup>x</sup>				

<sup>a</sup>Control – no lyophilized bovine colostrum (LBC) included; 10LBC – 10% LBC included; 20LBC – 20% LBC included. <sup>b</sup>Probability: Diet X Period – interaction diet x period. <sup>c</sup>S1 – cranial segment; <sup>d</sup>S2 – medium segment; \*ns: not significant. <sup>x,y</sup>Values followed by the same capital superscript in the same column do not differ (Tukey test;  $p \leq 0.05$ ). <sup>x,y</sup>Values followed by the same lower case superscript in the same line do not differ (Tukey test;  $p \leq 0.05$ ).

that the rectal mucosa of fish fed diet 20LBC presented a pathohistological reaction at day 60, which may have reduced the capacity of absorption of nutrients. Peterson et al. (1996) studied the effect of IGF-I supply in rats under total parenteral nutrition found augment jejunal mucosal weight, and increased villus height and crypt depth. Bühler et al. (1998) and Roffler et al. (2003) found an increase in intestinal villus size from neonatal calves fed bovine colostrum.

At day 60, fish fed diet 20LBC presented a pattern of macrophages (Figure 1) distinct from that observed at day 30 for fish fed any of the experimental diets, which can be have resulted from the complex composition of LBC and, therefore, its potential antigenic function for fishes. Rombout and van den Berg (1989) administered antigens via anal to carp, and registered increased number of macrophages in the distal intestine; the authors also considered the observed, enlarged intraepithelial macrophages morphologically similar to

melanomacrophages, thus able to drive local immune response. Melanomacrophages are pigmented macrophages aggregates, ordinarily found in the kidney, liver and spleen of bony fish (Agius and Roberts, 2003; Haugarvoll et al., 2006; Wolke, 1992), or adventitiously to inflamed regions (Koppang et al., 2005; Rodríguez et al., 2005); functions of melanomacrophages are multiple, complex and not yet totally understood. Although the distinct staining pattern, form and size of macrophages observed at day 60 in the intestines of fish fed diet 20LBC are similar to that of melanomacrophages, it is not possible to confirm this hypothesis by the histological techniques utilized.

Large and numerous vacuoles detected in the distal intestine of some fish fed diet 20LBC at day 60 (Figure 2) can be correlated with the higher inclusion of dietary lipids sources in diets 10 and 20LBC (Table 1), as well as with a pathohistological condition. According to Burrells et al. (1999), same as for livers, lipid vacuoles



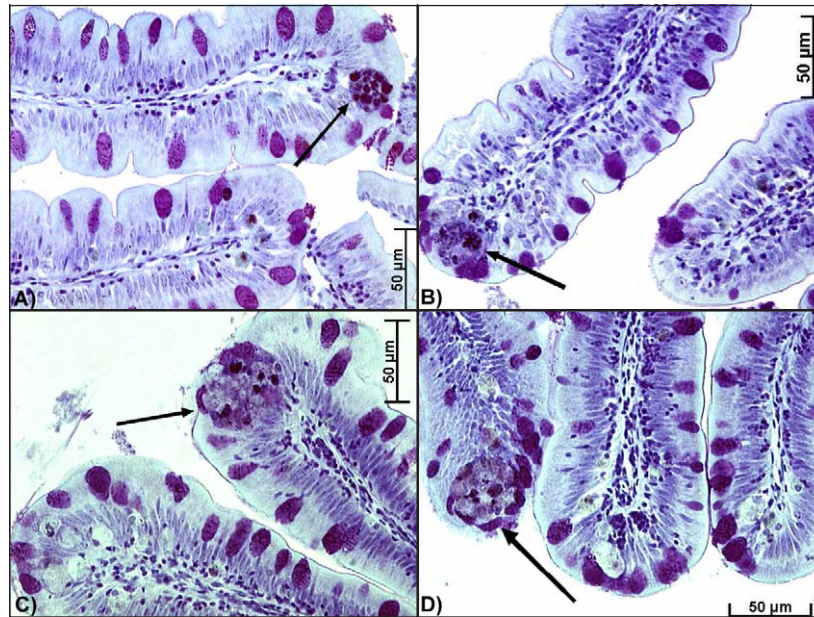


Figure 1 – Macrophages (arrows) in the rectal mucosa at 60 days: (A) fish fed control diet; (B) fish fed diet containing 10% lyophilized bovine colostrum; (C) and (D) fish fed diet containing 20% lyophilized bovine colostrum (400x).

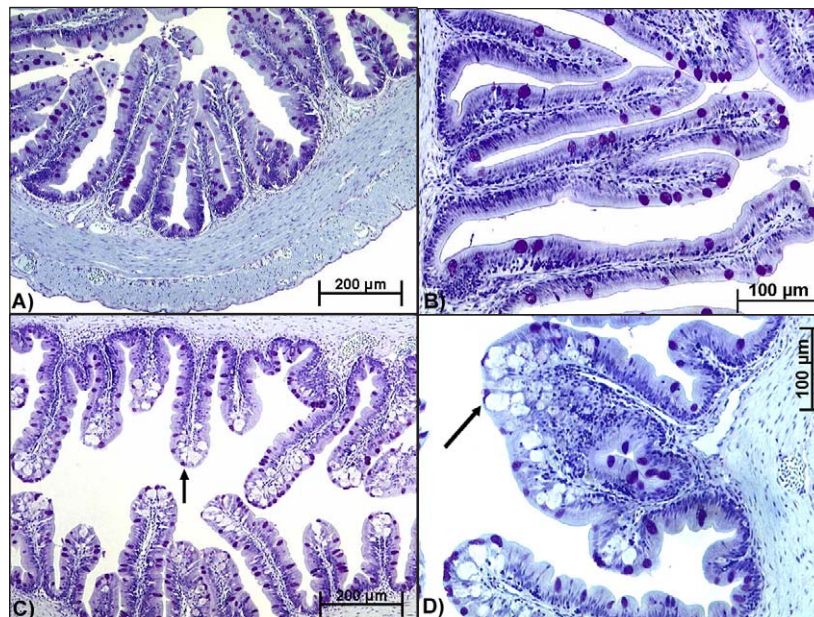


Figure 2 – Vacuoles (arrows) in the rectal mucosa at 60 days: (A) fish fed control diet (100x); (B) fish fed diet containing 10 % lyophilized bovine colostrum (200x); (C) and (D) fish fed diet containing 20% lyophilized bovine colostrum (100x and 200x, respectively).

in the intestine are studied because they are generally considered temporary storage site of lipids, particularly when the absorption level exceeds the lipoprotein synthesis capacity. On the other hand, Ostaszewaska et al. (2005) reported the presence of large vacuoles in the digestive tract of rainbow trout *Oncorhynchus mykiss* and pacu *Piaractus mesopotamicus*, considered those findings a pathological reaction to the replacement of casein with soybean protein concentrate in the diet of these species, and suggested that these vacuoles can lessen intestinal

nutrient absorption. Burrells et al. (1999) and Sitjá-Bobadilla et al. (2003) also consider that the presence of vacuoles in fishes' intestinal epithelium results in a loss of the mucosa integrity, which reduces fish specific growth rate and increase susceptibility to bacterial infection.

In conclusion, the inclusion of LBC in diets of striped catfish altered morphometrical features of juvenile striped catfish intestine and induced inflammatory reaction in the distal intestinal mucosa, depending on level

of inclusion, feeding period and segment of intestine analyzed. This information may elicit better understanding of fishes' intestinal mechanisms of absorption and foster future researches in this area.

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