Immunohistochemical and Ultrastructural Evidence of Functional Organization Along the *Corydoras paleatus* Intestine

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KEY WORDS Neotropical catfish; Na^+K^+-ATPase; PCNA; air-breathing organ

ABSTRACT The Neotropical catfish, *Corydoras paleatus* (Callichthyidae) is a facultative air-breathing teleost that makes use of the caudal portion of the intestine as an accessory air-breathing organ. This portion is highly modified, being well vascularized with capillaries between epithelial cells, which makes it well suited for gas exchange. Instead, the cranial portion is a digestion and absorption site, as it has a typical intestinal epithelium with columnar cells arranged in a single row, villi and less vascularized tunica mucosa. Therefore, the intestine was studied by light and electron microscopy to assess differences between the cranial, middle and caudal portions. To characterize the potential for cell proliferation of this organ, we used anti-proliferating cell nuclear antigen antibody and anti-Na^+K^+-ATPase monoclonal antibody to detect the presence of Na^+/K^+ pump. In *C. paleatus* it was observed that cell dynamics showed a decreasing gradient of proliferation in cranio-caudal direction. Also, the intestine of this catfish is an important organ in ionoregulation: the basolateral Na^+/K^+ pump may have an active role, transporting Na^+ out of the cell while helping to maintain the repose potential and to regulate cellular volume. *Microsc. Res. Tech. 79:140–148, 2016.*

INTRODUCTION

Benthic fishes that inhabit freshwater environments where the probability of hypoxia is high have developed different systems for atmospheric oxygen uptake (Kramer, 1987). To be adapted to atmospheric breathing, a reduction in the thickness of the barrier membrane and an increase in both vasculature and surface for gas exchange are observed. In the Argentine ichthyofauna, there are many species with bimodal breathing (Ringuelet, 1975); one of them is the Neotropical catfish, *Corydoras paleatus* (Jenyns, 1842) (Siluriformes, Callichthyidae). The air-ventilation cycle of Siluriformes consists in an ascent to the surface, once there the fish opens its mouth, expands the oral cavity and breathes in air. As new air is inspired, the release of old air from the anus commences and, as the fish turns towards the bottom, it compresses its oral cavity, which facilitates the transmission of air into the intestine and forces more air out of the anus (Graham, 1997; Jucá-Chagas and Boccardo, 2006). The amount of breathing ascents depends on the depth, temperature and concentration of dissolved oxygen in water (Gómez, 1996).

In fresh water, the osmolarity of a fish's body fluids is higher than the external environment. Consequently, there is a tendency to lose ions from the organism and absorb water.To avoid this, freshwater fishes produce diluted urine, but take salt actively from their environment using the gills and the food intake, as well as reabsorbing salt from their urine before it is excreted (Sakamoto and McCormick, 2006; Hwang and Lee, 2007). It is known that the combined activity of the Na^+/K^+ ATPase (NKP) in the basolateral membranes and the Na^+/K^+ $2Cl^-$ cotransporter in the apical membranes are responsible for ion exchange. Absorption is driven osmotically in response to a gradient established by the activity of the NKP (Cutler and Cramb, 2002). Because of this, the intestine is one of the major osmoregulatory organs of teleosts together with gills, kidney and skin (Sakamoto and McCormick, 2006).

The interplay between cell proliferation, differentiation, and apoptosis is fundamental for the maintenance of the gastrointestinal system. Fish cells proliferate in the basal area of the intestinal fold and migrate towards the apex, where they are destroyed in the apoptosis process (Ortego et al., 1994; Sanden and Olsvik, 2009). To date, despite the commercial importance of *C. paleatus* as ornamental fish, few basic studies on the intestine of this species have been carried out. The purpose of this study was to

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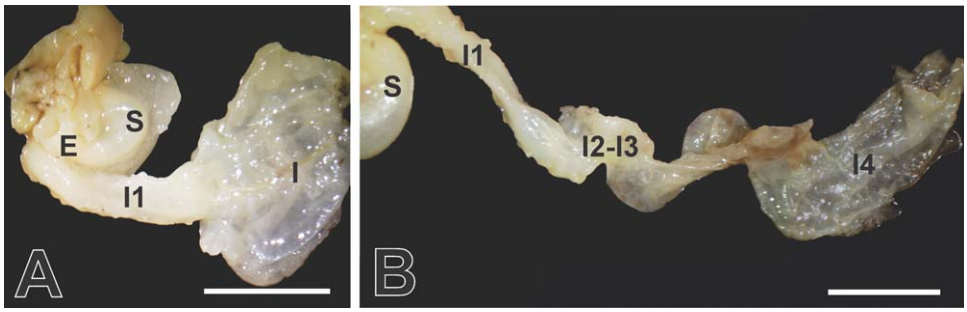

Fig. 1. General structure of the digestive tract of *Corydoras paleatus*. **A**. Gastrointestinal tract showing an esophagus, stomach, liver and cranial intestine. **B**. Middle and caudal intestine. E: esophagus, I: intestine, I1: cranial intestine, I2-3: middle intestine, I4: caudal intestine, S: stomach. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

describe by means of histological techniques, as well as TEM (transmission electron microscopy) and SEM (scanning electron microscopy) morphological analyses, the normal structure and ultrastructure of the intestine in this species. Furthermore, an immunohistochemical technique was used to locate and identify the NKP and the proliferating cell nuclear antigen (PCNA), two important proteins in osmotic equilibrium processes and cellular renewal, respectively. These techniques provide a deeper insight into the morphological and functional aspects of the intestine and supply more data for the culture of this species.

MATERIALS AND METHODS

Twenty-five healthy adult specimens (15 females and 10 males) of *C. paleatus* from La Plata city (Buenos Aires, Argentina) were used. The handling, collection and killing of all individuals followed the guidelines of the American Fisheries Society (AFS, 2004). Fish were transported alive in plastic bags, and were kept in an aquarium at 22°C and pH 7.0 (Gómez, 1996) for a minimum of 3 weeks, they were fed at sunset with food for bottom fish (Tetra min tropical tablet, Germany). The specimens were sacrificed by anesthesia overdose using a Eugenol (30 mg L^{-1}) (García-Gómez et al., 2002).

Samples of intestine were divided into three portions (cranial, middle, and caudal) considering the shape, transparency, thickness of the wall and the width of the lumen in transverse sections. The intestine samples were rapidly excised and fixed by immersion in 10% buffered formalin for light microscopic studies. Samples were routinely processed and embedded in paraffin wax. Histological sections were cut by sledge microtome, prepared according to standard protocol, and then stained using the following techniques: hematoxylin and eosin (H&E), PAS for differentiation of mucosubstances and Gomori's technique for reticulin fibers identification. Micrographs were taken with an Olympus microscope, CX 31 equipped with an Olympus camera U-CMAD3 (Tokyo, Japan).

Small samples of intestine were fixed in cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 4 h at 4°C, post-fixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer, and then dehydrated in a graded series of ethanol. Thereafter, intestine samples for SEM study were critical point-dried and sputter-coated with gold. Specimens were viewed and photographed using Philips SEM 505 and Soft Imaging System ADDA II (SIS) software which belongs to *CINDECA*, School of Exact Sciences, National University of La Plata (UNLP). For TEM, small pieces (0.5–1 mm^3) were embedded in Epon 812. Semithin sections, obtained from the epoxy resin blocks and stained with toluidine blue, were used to select areas for thin sectioning. Thin sections (60 nm) were stained with uranile acetate and lead citrate, and photographed with a JEOL JEM 1200-EXII belonging to School of Veterinary Sciences, UNLP.

For immunohistochemistry, sections (3 μm) were mounted on slides coated with g-methacryloxypropyltrimethoxy-silane (M6514, Sigma, St. Louis, MO), passed through a decreasing graded alcohol scale and incubated with 0.03% H2O2 in methanol (purum P99.0%) for 30 min at room temperature. Sections were then rinsed twice in PBS and exposed to microwave with a power of 750 W 10 min, to antigen retrieval was used a buffer citrate solution (pH 6.0). For PCNA localization, sections were then incubated with mouse monoclonal anti-PCNA antibody (clone PC 10, ascites fluid, Sigma Chemical, St. Louis, MO; 1:3,000) at room temperature for 1 h. The En Vision® 151 detection system + HRP system labeled anti-mouse polymer (Dako Cytomation) was applied for 30 min. For NKP determination, sections were then incubated with mouse monoclonal anti-NKP antibody DSHB (Developmental Studies Hybridoma Bank, clone SP2/0Ag8; 1:200) at room temperature for 1 h. The Zymed® Cat. N° 81-6540; 1:120, detection system was applied for 30 min, between each of the steps described were conducted three washes with PBS for 5 min. Liquid DAB (Dako® Cytomation) was used as chromogen and haematoxylin for counterstaining. Negative control sections were prepared by omitting primary antibody. Positive control sections for PCNA were samples of mammalian intestine (Zanuzzi et al., 2012) and for NKP were samples of chicken kidney (Pastor et al., 2008). To calculate the proliferation index in the four intestinal portions we counted at

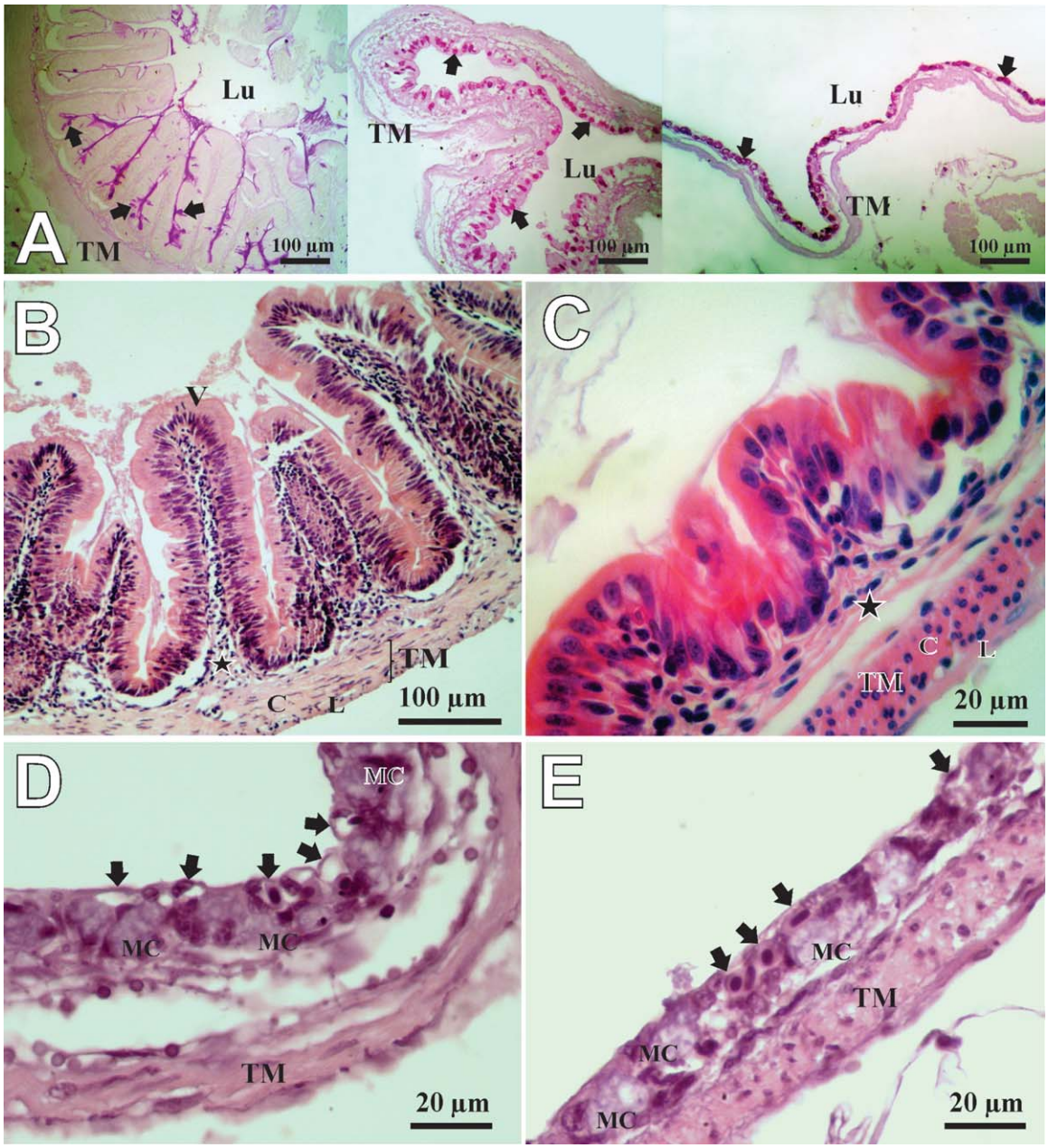

Fig. 2. Histology of the intestinal wall of *C. paleatus*. **A**. Panoramic views of the intestine wall thickness in cranial, middle and caudal portions. Note that goblet cells are highly PAS-positive (arrows). PAS technique. **B**. Cross-section of I1, showing development of villi. H&E. **C**. I2 portion with shorter and thicker villi and a higher development of lamina propria/submucosa than those of the I1 portion. **D** and **E**. I4 portion, enterocytes and goblet cells are observed. Epithelium and capillaries (black arrows) interspersed between them, reaching the mucosal lumen. C: circular (inner) layer of tunica muscularis, L: longitudinal (outer) layer of tunica muscularis, Lu: lumen, ★: lamina propria-submucosa, TM: tunica muscularis, V: villus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

least 700 enterocytes per slide using a 100× objective. The proliferation index was expressed as:

$$\text{Proliferation Index} = \text{number of enterocytes labeled} \times 100/\text{total number of enterocytes.}$$

For each intestinal portion, data are presented as means ± standard error. Means were compared using ANOVA, Tukey's test was used as ad hoc test to establish the signification on the differences.

RESULTS

Intestine of *C. paleatus* showed differences in diameter along its lenght; it was folded on itself and lacked pyloric caeca. Our observations showed that the cranial portion or intestine 1 (I1) had a typical tubular form and its wall was opaque (Figs. 1A and 1B); the middle area, which was subdivided for histological preparations into two sectors identified as I2 and I3, had a broader lumen and its wall became thinner and more translucent than I1 (Fig. 1B); in the caudal

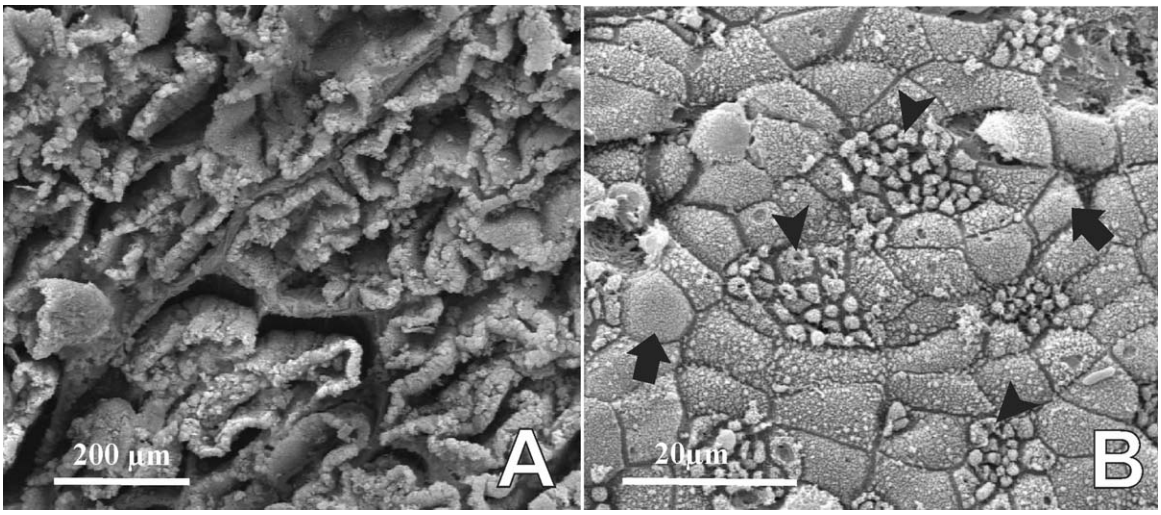

Fig. 3. Scanning electron photomicrographs of the intestinal mucosa of *C. paleatus*. **A**. Surface of I1 mucosa showing development of villi. **B**. I4 mucosa showing the two cell types, short MV enterocytes (arrows) and long MV enterocytes (arrowheads).

portion or intestine 4 (I4), the lumen diameter increased and the wall was even thinner (Fig. 1B). The average total length was 54.9 ± 2.01 mm for females and 45.9 ± 1.76 mm for males. Macroscopically, boundaries were not observed between different areas due to the fact that intestine sphincters were absent, but the presence of air bubbles in the caudal portion was observed. Microscopically, the typical four-layered structure of a vertebrate's intestine was present, although—due to the absence of muscularis mucosae—no boundaries were observed between the tunica mucosa and submucosa. Intestinal crypts or any other gland types were not observed.

Intestinal Mucosa

In I1, the tunica mucosa presented long villi (Figs. 2A and 2B), which in this area reached their maximum length and were more numerous; gradually decreasing in height toward the caudal region. By SEM, it was observed that the villi had a foliated appearance (Fig. 3A). When this area was observed with TEM, the intestinal epithelium was composed of columnar enterocytes and goblet cells. Enterocytes had long microvilli (MV) (Fig. 4A), a rounded or oval nucleus rich in euchromatin, and mitochondria located in the supranuclear cytoplasm. Goblet cells (Fig. 4B) in this portion were scarce and rounded.

The wall thickness of I2 decreased gradually toward the caudal portion (Figs. 2A and 2C). The villi also decreased in height until they disappeared in I3. Enterocytes from I2 to I3 were gradually transformed into cuboidal cells with numerous supranuclear mitochondria (Figs. 4C and 4D). When this sector was observed with TEM, the intestinal epithelium was characterized as being composed of two types of enterocytes that differed by their MV height (short MV enterocytes and long MV enterocytes) (Fig. 4C). The epithelial basal lamina was observed as being in intimate contact with continuous-type capillaries (Figs. 4C and 4D).

In the caudal portion (I4), an extremely thin wall was observed (Figs. 2A, 2D, and 2E). In this portion, the epithelium was cuboidal (Figs. 2D, 2E, and 4E) to squamous, with capillaries (Figs. 2D and 2E) that reached the lumen of the mucosa interspersed between the enterocytes and goblet cells. On the intestinal surface, SEM micrographs showed large sections with short MV cells and small areas with long MV cells. Some long MV cells showed a depression in the center (Fig. 3B); this depression would correspond with the apical surface of goblet cells. This interpretation was made according to TEM observations (Fig. 4C), which showed along the intestinal wall the presence of cuboidal short MV enterocytes and goblet cells, with each of the latter appearing to be surrounded by several long MV enterocytes.

Lamina Propria/Submucosa

As mentioned above, due to the difficulty differentiating with routine techniques the boundaries between lamina propria and submucosa tunica, we described them together. Although in sections stained with the Gomori's reticulin technique, dense connective tissue rich in reticular fibers was observed. This connective tissue was located on the boundary between tunica mucosa and submucosa, and between tunica submucosa and muscularis (Figs. 5A–5C). The photomicrographs taken with TEM confirmed the presence of a thin layer of reticular fibers separating the lamina propria of submucosa tunica (Fig. 4F).

Air–Blood Barrier

According to the interpretation made with optical microscope and TEM, portions I3 and I4 could be the gas exchange sites. The thinnest air-blood barrier consists of a short MV enterocytes and their basal lamina, and capillary endothelial cells and their basal lamina. It was observed that these two basal laminae were fused together (Figs. 4C and 4D).

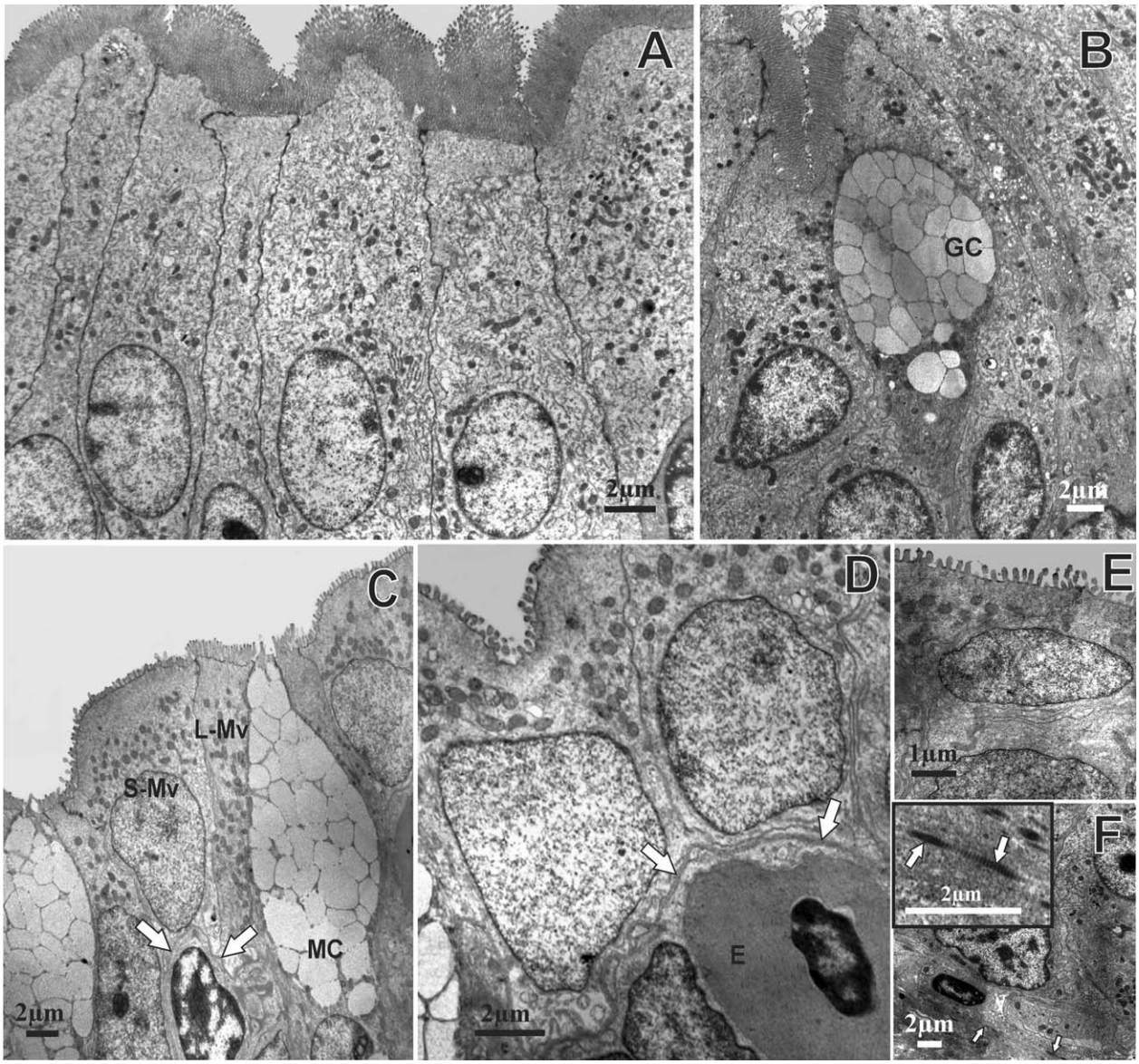

Fig. 4. Transmission electron photomicrographs of the intestinal mucosa of *C. paleatus*. **A**. I1 portion; cylindrical enterocytes can be observed. **B**. Goblet cell located in the cranial portion of the intestine among enterocytes. **C**–**E**. Photomicrographs showing enterocyte height gradually decreasing toward the caudal portion and the supranuclear abundance of mitochondria. Arrows show the air-blood barrier. Middle intestine (C and D), caudal intestine (E). **F**. Cranial intestine, arrows show a thin layer of reticular fibers separating the lamina propria from the submucosa tunica. Inset: detail of reticular fibers. E: erythrocyte, GC: goblet cells, L-Mv: long microvilli cell, S-Mv: short microvilli cell.

Tunica Muscularis

Tunica muscularis showed two layers of smooth muscle cells, an inner circular layer and an outer longitudinal one (Figs. 2B and 2C). Both layers were separated by connective tissue rich in reticular fibers and where the myenteric plexus was observed. Towards the caudal region, the outer longitudinal layer became thinner, with only two or three smooth muscle fibers in the middle portion of the intestine (Fig. 2C). In I4, the tunica muscularis was restricted to the circular layer (Figs. 2D and 2E). In the muscle layers, elastic fibers were observed.

Tunica Serosa

This tunica was a serous membrane that presented a simple squamous epithelium (mesothelium) and a small amount of underlying connective tissue; in some areas, highly capillarized.

Immunohistochemistry Techniques

Proliferating Cell Nuclear Antigen (PCNA). Immunostaining with anti-PCNA antibody in *C. paleatus* gut showed great immunoreactivity of this protein in the nuclei of enterocytes of I1 portion, leading us to assume that these were in proliferation

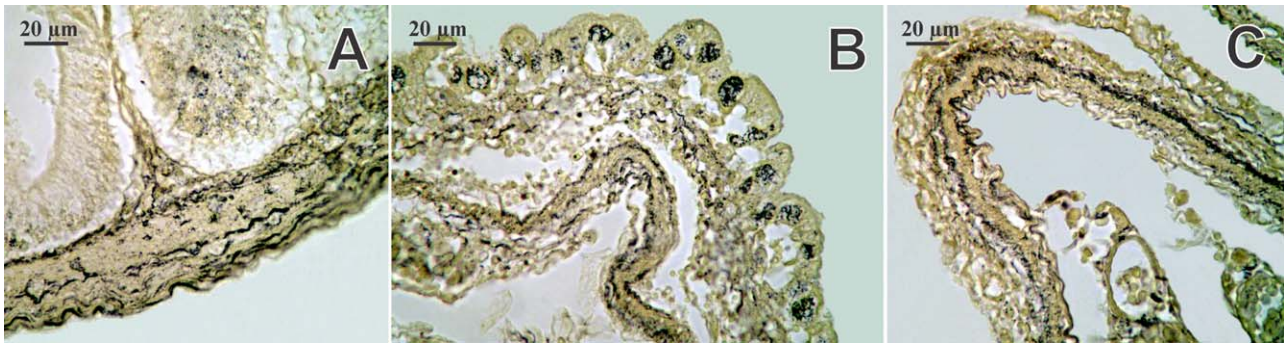

Fig. 5. Photomicrographs with Gomori's reticulin technique. Collagen and reticular fibers separate the different tunics of the intestinal wall of *C. paleatus*. **A**. Cranial intestine. **B**. Middle intestine. **C**. Caudal intestine. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

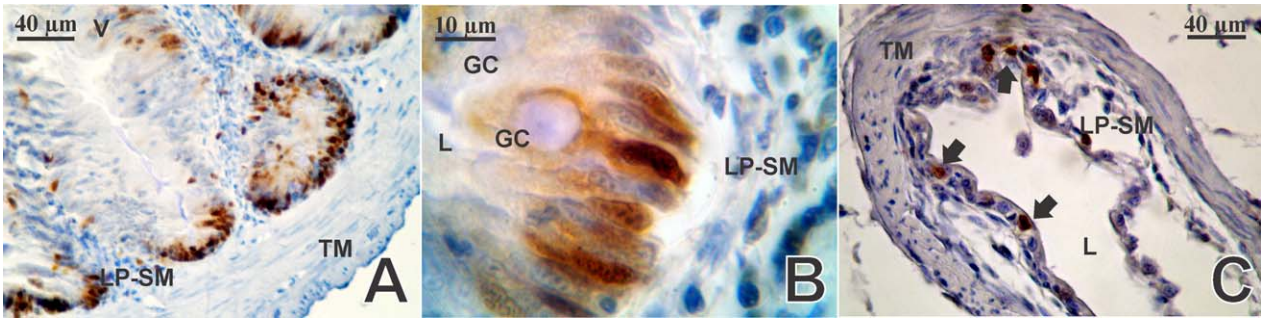

Fig. 6. Immunohistochemical characterization of PCNA labeled nuclei in the intestine of *C. paleatus*. **A**. Labeled pattern for PCNA-positive cells along the intestinal villi. **B**. Higher magnification of the basal region showing an intense labeling in the majority of the nuclei. **C**. I4 showing minor labeling of the nuclei (arrows). GC: goblet cells, L: lumen, LP-SM: lamina propria/submucosa, TM: tunica muscularis, V: villus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Table 1). The labeled pattern for PCNA-positive cells along the intestinal villus showed differences (Fig. 6A). On the basal surface of the villus, the majority of the nuclei showed a very intense immunoreactivity (Fig. 6B); the amount of labeled nuclei decreased in the lateral regions, being negative at the apex. However, scarce immunoreactivity was observed in the nuclei of the I2, I3, and I4 portions (Fig. 6C).

Na^+/K^+ATPase. Immunoreactivity to NKP in all of the enterocytes in the four examined portions was found. The NKP was located on the basolateral surface of the plasma membrane of enterocytes. In I1, intensity differences were observed in the immunostaining along the height of the villus. The basal surface of enterocytes from the base of the intestinal villi exhibited very intense labeling (Fig. 7A), becoming from intense to moderate on the lateral surfaces of the cells. Along the lateral regions of the villus, labeling was maintained with moderate intensity on the basolateral surfaces of the cells; whereas in the apical villus region, labeling intensity was slight to null (Fig. 7B). In the I2 portion, labeling was intense and homogeneous in all of the basolateral surfaces of the cells, but no differences were seen among the regions of the villus. In the I3 portion, the labeling was also intense in the basolateral enterocyte membrane, but only up to the height of the nucleus; however, in the lateral membrane of the apical region, this positivity was not observed (Fig. 7C). In the I4 portion, the immunostaining was again strong and homogeneous on the basolateral surfaces of all enterocytes (Fig. 7D).

DISCUSSION

The difficulty in obtaining oxygen from many aquatic environments has led to the evolution of air breathing among fishes. In several groups of fish, elements of the gastrointestinal tract have been adapted to extract oxygen from the air and have become air-breathing organs (Nelson and Dehn, 2011). The Neotropical catfish of the Callichthyidae family occupy a hypoxia-prone habitat. These fish use a section of their intestine as an air-breathing organ.

Although it is frequently considered that the intestinal wall is composed of four tunics—from the lumen to the exterior: mucosa, submucosa, muscularis, and serosa—(Caceci et al., 1996), several authors refer to the tunica submucosa not as a defined layer, but rather as lamina propria/submucosa or directly as mucosa, because it is difficult to differentiate it with routine techniques (Çinar and Şenol, 2006; Díaz et al., 2008; Park et al., 2003; Podkowa and Goiakowska-Witalinska, 2002). This difficulty in distinguishing the lamina propria from the tunica submucosa is associated with the absence of the muscularis mucosa layer (Xiong et al., 2011). However, in *C. paleatus*, a very thin layer of reticular fibers separating both tunics can be observed.

In *C. paleatus*, the histological organization of the intestinal tunics coincided with other teleosts studied, except in the I3 and I4, where the capillaries are located between and apically to the epithelial cells. This type of highly vascularized wall has been observed in other fish that use the intestine as an accessory air-breathing organ, for instance another species of the same genus, *Corydoras aeneus*, (Podkowa and Goiakowska-Witalinska, 2002), as well as in other genera of Callichthyidae, such as *Megalechis thoracata* (=*Hoplosternum thoracatum*) and *Hoplosternum littorale* (Huebner and Chee, 1978; Jucá-Chagas and Boccardo, 2006), and in some members of the Cobitidae family, such as *Misgurnus anguillicaudatus*, *Misgurnus fossilis*, *Misgurnus mizolepis* and *Lepidocephalichthys guntea* (=*Lepidocephalus guntea*) (Gonçalves et al., 2007; Jasinski, 1973; Koyama, 1958; McMahon and Burggren, 1987; Yadav and Singh, 1980). The origin of the reduction in the intestine wall towards the caudal portion varies in the different species: in *M. thoracata*, it is due to the absence of submucosa tunica and to the reduction of the muscularis tunica (Huebner and Chee, 1978), whereas in *L. guntea*, *C. aeneus*, *M. anguillicaudatus* (Park et al., 2003; Podkowa and Goiakowska-Witalinska, 2002; Yadav and Singh, 1980;), and in *C. paleatus*, it is due to a greater reduction in the width of the lamina propria/submucosa tunica and in the muscular tunica, the latter being restricted to the inner circular layer.

Podkowa and Goiakowska-Witalinska (2002) described two types of epithelial cells, differentiated by their shape and MV lengths. In the cranial portion of the *C. aeneus* intestine, these cells are in charge of the absorption, a function that decreases towards the caudal portion, where it is replaced by the breathing function; however, the presence of enterocytes MV and visceral type capillaries suggest that in this sector some nutrients are absorbed. The presence of goblet cells in the intestinal epithelium is common in teleosts (Çinar and Şenol, 2006; Hernández et al., 2009); the glycoconjugates produced by them, according to Díaz et al. (2008b), provide lubrication for the passage of ingested food and also to protect of the thin-walled intestine from desiccation and physical or chemical damage.

TABLE 1. Percentage of PCNA positive nuclei in the villi epithelium

	I1	I2	I3	I4
Mean	42.14 ± 5.00	3.41 ± 2.00	14.57 ± 4.16	4.10 ± 2.11

Intestine 1 $P < 0.001$ with respect to intestine 2, intestine 3 and intestine 4. Date expressed as Mean ± standard error.

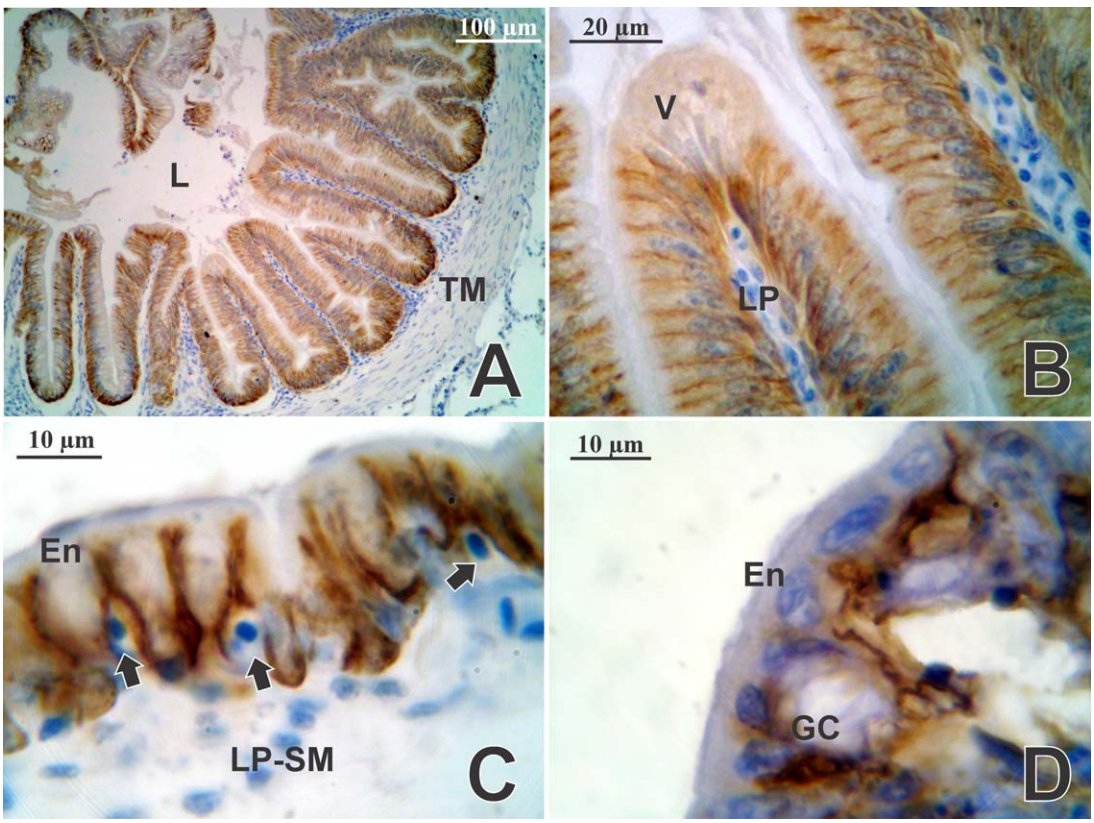

Fig. 7. Immunohistochemical characterization of NKP-labeled enterocytes in the intestine of *C. paleatus*. **A**. Panoramic view of the I1 intestinal wall, showing intense immunostaining in the enterocytes of the villi basal surface. **B**. Immunostaining along the villus showing intensity differences; note the negativity of the apical region. **C**. In the I2 portion, the labeling was intense and homogeneous in all basolateral surfaces to the cells; the arrows indicate the vascularization of the lamina propria. **D**. Intense labeling in basolateral enterocyte membrane in the I3 portion, only up to the height of the nuclei. En: enterocyte, GC: goblet cells, L: lumen, LP: lamina propria, LP-SM: lamina propria/submucosa, TM: tunica muscularis, V: villus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The intestinal mucosa provides an important site of exposure to chemical pollutants and metabolic diseases; however, there are few studies on the intestinal integrity of fish in relation to markers of cell turnover. Using an anti-PCNA antibody makes it possible to recognize cells in cycling phases, so it is important in renewable epithelia to determine the indices of cell proliferation, as their changes often precede pathological conditions and are the first sign of abnormality in the intestinal tract (Berntssen et al., 2004). Cell proliferation in the intestine depends on several factors, including diet, microbiota, and the position of the intestinal stem cells situated both along the craniocaudal axis and at the base of the intestinal folds (Bakke-McKellep et al., 2007; Wong and Wright, 1999). In *C. paleatus*, it was observed—as studies on *Pseudopleuronectes americanus*, *Oreochromis niloticus* and *Salmo salar* have already shown—(Mommsen et al., 2003; Sanden and Olsvik, 2009; Trier and Moxey, 1980) that cell dynamics in normal conditions presented a decreasing gradient of proliferation in craniocaudal direction. Locally, the maintenance of cellular homeostasis and the relationship between proliferation, differentiation and apoptosis have been associated with the expression of various molecules, such as transcription factors and inducer molecules, many of which are conserved among mammals and fish (Richmond and Breault, 2010). Variations have been found in the proliferation indices of the stem cells in the small and large intestine (Barbeito et al., 2003; Hughes et al., 2012), which is similar to what happens with the indices of apoptosis (González and Barbeito, 2010); both results indicate a lower turnover rate. However, there is also a regulation related to the intestinal flora that in mammals is at least partially mediated by the bacterial toxins that bind to the receptor guanylyl cyclase C, which changes along the craniocaudal axis of the intestine, implying an exogenous regulation (Li et al., 2007). In teleosts, it has been determined that the microbiota also regulates the proliferation and apoptosis processes (Bakke-McKellep et al., 2007). Moreover, the lower indices of proliferation of portions I3 and I4, which have a respiratory function, may be related to the scarce renewal of other epithelial populations of gas exchange, such as pneumocytes in mammals (Steele et al., 1992).

As mentioned above, the ultrastructural variation of enterocytes is related to the functional differences between these cells along the intestine. In the caudal portion, these cells have morphological characteristics, such as the abundance of mitochondria and the complexity of the basolateral folds, which would be associated with osmoregulation, recirculation of water, and ion transport (Abaurrea-Equisoaín and Ostos-Garrido, 1996). Observations using the anti-NKP antibody in *C. paleatus* coincide with Genz et al. (2008), who—apart from the accepted role of NaCl excretion by the gills and the recovery of these ions by intestinal absorption—demonstrated that these two organs work together to maintain acid-base homeostasis. In agreement with Grosell et al. (2011), the freshwater fish intestine is an important organ in ionoregulation, as it contributes to the uptake of Na^+ and Cl^- from the lumen to compensate for the diffusional loss of these ions across the gill. The NKP basolateral position is widely distributed among different species of vertebrates; in some fish, it is complemented by the existence of a Na/K/Cl cotransporter located in the apical portion of the enterocytes (Lorin-Nebel et al., 2006). Therefore, the basolateral NKP may have an active role, as Na^+ is transported out of the cell, helping to maintain the repose potential and to regulate cellular volume. The abundance of NKP in the caudal intestine differs from what is found in marine fish, as this enzyme is abundant only in the cranial intestine in such fish (Guffey et al., 2011), which may be related to the differences in osmoregulation.

In conclusion, this study shows that, along the phylogeny of some teleosts such as *C. paleatus*, the intestine has acquired the role of an accessory respiratory organ, probably as a response to unfavorable environmental conditions, while maintaining the morphological characteristics for perform the absorption process. The results of the histological and ultrastructural studies performed in *C. paleatus* confirm the suggestions that the cranial portion of the intestine is the site of digestion and absorption, since its structure is the one typical of all vertebrates. Portions I3 and I4 would perform the function of a respiratory organ because their mucosa becomes thinner, villi are not observed, gas bubbles are present and cuboidal or squamous enterocytes are in close contact with the capillary network, which may even reach the lumen of the organ. Finally, changes in the production of PCNA within intestinal epithelia showed a decreasing gradient of proliferation in cranio-caudal direction similar to other species without intestinal respiratory functions. The localization of NKP support the intestinal osmoregulation functions found in other fishes.

REFERENCES

Abaurrea-Equisoaín MA, Ostos-Garrido MV. 1996. Enterocytes in the anterior intestine of *Oncorhynchus mykiss*: Cytological characteristics related to osmoregulation. Aquaculture 139:109–116.

AFS. 2004. Guidelines for the use of fishes in research. Bethesda, MD: American Fisheries Society.

Bakke-McKellep AM, Penn MH, Salas PM, Refstie S, Sperstad S, Landsverk T, Ringø E, Krogdahl A. 2007. Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.). Br J Nutr 97:699–713.

Barbeito CG, Albarenque SM, Reyna JC, Flamini MA, Andrés Laube PF, Badrán AF. 2002. Mitotic activity of the duodenal crypts enterocytes in mice transplanted with EA21a mammary carcinoma. Cell Biol Int 26:123–125.

Barbeito CG, González NV, Badrán AF. 2003. Sex and age related temporal variations in intestinal epithelium proliferation in the suckling mouse. Chronobiol Int 20:37–47.

Berntssen MHG, Hylland K, Julshamn K, Lundebye AK, Waagbø R. 2004. Maximum limits of organic and inorganic mercury in fish feed. Aquacult Nutr 10:83–97.

Caceci TEL, Habback HA, Smith SA, Smith BJ. 1996. The stomach of *Oreochromis niloticus* has three regions. J Fish Biol 50:939–952.

Çinar K, Şenol N. 2006. Histological and histochemical characterization of the mucosa of the digestive tract in flower fish (*Pseudophoxinus antalyae*). Anat Histol Embryol 35:147–151.

Cutler CP, Cramb G. 2002. Two isoforms of the $Na^+/K^+/2Cl^-$ cotransporter are expressed in the European eel (*Anguilla anguilla*). Biochim Biophys Acta (BBA) Biomembr 1566:92–103.

Díaz AO, García AM, Figueroa DE, Goldemberg AL. 2008a. The mucosa of the digestive tract in *Micropogonias furnieri*: A light and electron microscope approach. Anat Histol Embryol 37:251–256.

Díaz AO, García AM, Goldemberg AL. 2008b. Glycoconjugates in the mucosa of the digestive tract of *Cynoscion guatucupa*: A histochemical study. Acta Histochem 110:76–85.

García-Gómez A, de la Gándara F, Raja T. 2002. Utilización del aceite de clavo, *Syzygiu aromaticum* L. (Merr. & Perry), como anestésico eficaz y económico para labores rutinarias de manipulación de peces marinos cultivados. Bol Inst Esp Oceanogr 18:21–23.

Genz J, Taylor JR, Grosell M. 2008. Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*. J Exp Biol 211:2327–2335.

Gómez SE. 1996. Resitenza alla temperatura e alla salinità in pescidella provincia di Buenos Aires (Argentina), con implicazioni zoogeografiche. *In* Distribuzione della fauna ittica italiana. Atti Congressuali IV. Convegno Nazionale A.I.I.A. Riva del Garda, Italia. pp. 171–192.

Gonçalves AF, Castro LFC, Pereira-Wilson C, Coimbra J, Wilson JM. 2007. Is there a compromise between nutrient uptake and gas exchange in the gut of *Misgurnus anguillicaudatus*, an intestinal air-breathing fish? Comp Biochem Physiol D2 345–355.

González NV, Barbeito CG. 2010. Colchicine-induced apoptosis daily rhythms in male and female suckling mice. Anal Biol J 2:10–20.

Graham J. 1997. Air-breathing fishes: Evolution, diversity, and adaptation. San Diego, California: Academic Press. pp. 299.

Grosell M. 2011. The role of the gastrointestinal tract in salt and water balance. In: Grosell M, Farrell AP, Brauner CJ. The multifunctional gut of fish: Fish physiology, Vol. 30. Amsterdam: Academic Press. pp. 135–164.

Guffey S, Esbaugh A, Grossell M. 2011. Regulation of apical H^+-ATPase activity intestinal HCO_3^- secretion in marine fish osmoregulation. Am J Physiol 301:R1682–R1691.

Hernández DR, Pérez Gianeselli M, Domitrovic HA. 2009. Morphology, histology and histochemistry of the digestive system of South American catfish (*Rhamdia quelen*). Int J Morphol 27:105–111.

Huebner E, Chee G. 1978. Histological and ultrastructural specialization of the digestive tract of the intestinal air breather *Hoplosternum thoracatum* (Teleost). J Morphol 157:301–328.

Hughes KR, Gândara RM, Javkar T, Sablitzky F, Hock H, Potten CS, Mahida YR. 2012. Heterogeneity in histone 2B-green fluorescent protein-retaining putative small intestinal stem cells at cell position 4 and their absence in the colon. Am J Physiol Gastrointest Liver Physiol 303:G1188–G1201.

Hwang PP, Lee TH. 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp Biochem Physiol A Mol Integr Physiol 148:479–497.

Jasinski A. 1973. Air–blood barrier in the respiratory intestine of the pond-loach, *Misgurnus fossilis*. L Acta Anat 86:376–393.

Jucá-Chagas R, Boccardo L. 2006. The air-breathing cycle of *Hoplosternum littorale* (Hancock, 1828) (Siluriformes: Callichthyidae). Neotrop Ichthyol 4:371–373.

Koyama T. 1958. A study on the mechanism of the ingestion by intestinal respiration in the loach. Japanese J Ichthyol 7:95–98.

Kramer DL. 1987. Dissolved oxygen and fish behavior. Env Biol Fish 18:81–92.

Li P, Schulz S, Bombonati A, Palazzo JP, Hyslop TM, Xu Y, Baran AA, Siracusa LD, Pitari GM, Waldman SA. 2007. Guanylyl cyclase C suppresses intestinal tumorigenesis by restricting proliferation and maintaining genomic integrity. Gastroenterology 133:599–607.

Lorin-Nebel C, Boulo V, Bodinier C, Charmantier G. 2006. The $Na^+/K^+/2Cl^-$ cotransporter in the sea bass *Dicentrarchus labrax* during ontogeny: Involvement in osmoregulation. J Exp Biol 209:4908–4922.

McMahon BR, Burggren WW. 1987. Respiratory physiology of intestinal air breathing in the teleost fish *Misgurnus anguillicaudatus*. J Exp Biol 33:371–393.

Mommsen TP, Osachoff HL, Elliott ME. 2003. Metabolic zonation in teleost gastrointestinal tract. Effects of fasting and cortisol in tilapia. J Comp Physiol B Biochem Syst Environ Physiol 173:409–418.

Nelson JA, Dehn AM. 2011. The gastrointestinal tract in air breathing. In: Grosell M, Farrell AP, Brauner CJ. The multifunctional gut of fish: Fish physiology, Vol. 30. Amsterdam: Academic Press. pp. 395–433.

Ortego LS, Hawkins WE, Walker WW, Krol RM, Benson WH. 1994. Detection of proliferating cell nuclear antigen in tissues of 3 small fish species. Biotech Histochem 69:317–323.

Park JY, Kim IS, Kim SY. 2003. Structure and mucous histochemistry of the intestinal respiratory tract of the mud loach, *Misgurnus anguillicaudatus* (Cantor). J Appl Ichthyol 19:215–219.

Pastor R, Sbodio O, Galván SM, Rossini M. 2008. Correlación entre la expresión de dos biomarcadores (PCNA y NA^+/K^+ ATPasa) en branquias del *Pymelodus albicans* de las cuencas del río Salado y Paraná. Redvet IX:1695–7504.

Podkowa D, Goiakowska-Witalinska L. 2002. Adaptations to the air breathing in the posterior intestine of the catfish (*Corydoras aeneus*). The histological and ultrastructural study. Folia Biol (Kraków) 50:69–82.

Richmond CA, Breault DT. 2010. Regulation of gene expression in the intestinal epithelium. Prog Mol Biol Transl Sci 96:207–229.

Ringuelet RA. 1975. Zoogeografía y ecología de los peces de aguas continentales de la Argentina y consideraciones sobre las áreas ictiológicas de América del Sur. Ecosur 2:1–122.

Sakamoto T, McCormick SD. 2006. Prolactin and growth hormone in fish osmoregulation. Gen Comp Endocrinol 147:24–30.

Sanden M, Olsvik PA. 2009. Intestinal cellular localization of PCNA protein and CYP1A mRNA in Atlantic salmon *Salmo salar* L. exposed to a model toxicant. BMC Physiol 9:3.

Steele MP, Levine RA, Joyce-Brady M, Brody JS. 1992. A rat alveolar type II cell line developed by adenovirus 12SE1A gene transfer. Am J Respir Cell Mol Biol 6:50–56.

Trier JS, Moxey PC. 1980. Epithelial-cell proliferation in the intestine of the winter flounder, *Pseudopleuronectes americanus*. Cell Tissue Res 206:379–385.

Wong WM, Wright NA. 1999. Cell proliferation in gastrointestinal mucosa. J Clin Pathol 52:321–333.

Xiong D, Zhang L, Yu H, Xie C, Kong Y, Zeng Y, Huo B, Liu Z. 2011. A study of morphology and histology of the alimentary tract of *Glyptosternum maculatum* (Sisoridae, Siluriformes). Acta Zool (Stockholm) 92:161–169.

Yadav AN, Singh BR. 1980. The gut of an intestinal air breathing fish, *Lepidocephalus guntea* (Ham). Arch Biol (Bruxelles) 91: 413–422.

Zanuzzi CN, Nishida F, Portiansky EL, Fontana PA, Gimeno EJ, Barbeito CG. 2012. Effects of *Solanum glaucophyllum* toxicity on cell proliferation and apoptosis in the small and large intestine of rabbits. Res Vet Sci 93:336–342.