



## COMPARATIVE HISTOLOGICAL ANALYSIS OF INTESTINES OF LOACH, GRASS CARP AND CATFISH PROVIDE INSIGHTS INTO ADAPTIVE CHARACTERISTICS IN AIR-BREATHING FISH

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### ABSTRACT

Accessory respiratory is generally accepted to have evolved independently on numerous occasions in adaption to aquatic hypoxia in freshwater habitats. In general, the air-breathing organ in fish is believed to be structurally modified to supplement respiration. In this study, intuitive evidence for elaborate structural modifications of the intestine, an air-breathing organ in mud loach (*Misgurnus anguillicaudatus*), compared with two other obligate aquatic breathers, grass carp (*Ctenopharyngodon idellus*) and yellow catfish (*Pelteobagrus fulvidraco*), were directly provided by histological and morphometric methods. As a result, a sharply decreasing height of mucosal folds and thickness of muscularis were manifested in loach intestine from its anterior to posterior region. Compared with grass carp and yellow catfish, loach had the smallest ratios of mucosal fold height/muscularis thickness to intestinal lumen radius in the posterior intestine. These suggested that the posterior intestine is the air-breathing location for the loach. Furthermore, length density of capillary ( $0.46 \pm 0.05 \mu\text{m}^{-2}$ ) in the posterior intestine of the loach was significantly higher than those of grass carp and yellow catfish. Meanwhile, diffusion distance of air-blood barrier ( $1.34 \pm 0.04 \mu\text{m}$ ) in the posterior intestine of the loach was significantly smaller than those of the other two fish species. In summary, the characteristics of highly vascularized, short diffusion distance of air-blood barrier, thinned and flattened made the posterior intestine a perfect air-breathing location for the loach.

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## INTRODUCTION

It is universally acknowledged that oxygen performs an exceptionally vital role for survival, growth, development and reproduction of bony fishes. The majority of fish are completely capable of satisfying continuously their aerobic demands from aquatic respiration with branchia. However, in response to some environmental or systemic factors, accessory respiration is stimulated in some fish species (Luiz et al., 2018; Giacomo et al., 2018). There are many different accessory respirational organs/tissues in fish, such as cutaneous layer, modified swim bladder, diverticula of the buccal, opercular or pharyngeal cavity, or alimentary tracts (Graham, 1997). Zhang et al. (2000) reported the specializations for terrestrial life in four species of oxudercine gobies mainly include the presence of dermal bulges, a thick middle cell layer and the vascularized epidermis. In addition, the pharynx of Asian swamp eel (*Monopterus albus*) was described short but complicated in structure, reflecting wrinkled pharyngeal wall and rather uneven surface interspersed holes, grooves and particles in different shapes among cells (Cao, 2008), which increased the contact area and improved the gas exchange. Although different bimodal breathers possess their respective air-breathing organs, they are believed to be structurally modified to supplement respiration.

Mud loach (*Misgurnus anguillicaudatus*), of the family Cobitidae, is a widespread, adaptable and weather-sensitive demersal fish readily available in fresh market. As a representative facultative air-breather, mud loach is notable for its respiratory posterior intestine, which is described as histologically modified to suit its role of respiration for the deficient oxygen supply within the environment (Park et al., 2003; Goncalves et al., 2007; Nelson, 2014). Based on histological examination, the posterior intestine of loach is remarkably less suitable to alimentary absorption on account of the absence of typical characteristics of an absorptive intestine, containing the columnar epithelial cells with brush borders, thick muscular walls and an increased absorptive surface area through longitudinal folding (Jasiński, 1973; McMahon and Burggren, 1987).

Since the physiology of air-breathing in fishes is a fascinating topic (Graham, 1997), a considerable part of previous researches on fishes with bimodal respiration have been carried out, however, there were scarce issues concerned with histological comparison between bimodal breathers and aquatic breathers, offering intuitive prominences of the respiratory adaptations. As a novel perspective in this study, mud loach was specially chosen as a bimodal breather, compared with two aquatic breathers, namely grass carp (*Ctenopharyngodon idellus*) and yellow catfish (*Pelteobagrus fulvidraco*), in terms of intestinal histology, and its dedicated characteristics of intestine modified for accessory respiration were discussed simultaneously.

## MATERIALS AND METHODS

### **Ethics statement**

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong Agricultural University. All handling procedures were made to minimize suffering of the animals. Tissue samples were collected after euthanasia with an overdose of MS 222.

### **Sampling and paraffin slicing**

Mud loach, grass carp and yellow catfish were all captured in Yezhi lake located in Wuhan City, China. After anaesthesia, the fish (n=15, 5 for each kind of fish species) were weighed and their body lengths were measured (Table 1). And then, intestines of the three fish species were dissected. Delicate morphology of the intestines was investigated. In terms of the attributes of the thickness of the wall, caliber of the cavity, and transparency, the intestine of each fish species was divided into three parts (anterior intestine (AI), middle intestine (MI) and posterior intestine (PI)). The anterior, middle and posterior intestines of the three fish species were fixed in 10% neutral buffered formaldehyde at 4°C for 24 h. The tissues were then dehydrated in increasing concentrations of ethanol series from 30% to 100%, cleared in xylene, immersed and embedded in paraffin with an embedding machine (YD-6L, Jinhua, China). After cooling into gelosis on the freezing stage (YD-6L), the tissue blocks were sectioned at 7 µm thickness by using a rotary microtome (YD-315). Subsequently, rather sound sections were undergoing a process of stretching and stoving with slices taken advantage of the Water Bath-Slide Drier (YD-AB).

### **Staining**

The drying sections were treated with xylene, graded ethanol in decreasing concentration and dionized water, then stained with Servicebio's Haematoxylin and counterstained with Eosin Y (HE), followed by dehydrating through hierarchical ethanol, clearing in xylene and mounting under coverslips in neutral resin. Alkaline phosphatase (AP) staining was performed by reference to Shao et al. (2004).

### **Measurements**

Photographic images were captured by using an Olympus microscope SZX7 accompanied with a camera Olympus DP74 (Tokyo, Japan) and a computer equipped with a measuring application (cellSens standard software). Here, mucosal fold height and muscular thickness of intestine were measured firstly. Then the radii of posterior intestine in the three fish species were estimated with aid of graphical fitting. The ratio of mucosal fold height

**Table 1.** Anatomical measurement of three fish species (n=15)

Species	Body length/mm	Wet Weight/g	Intestine length/mm	Intestinal ratio*
Mud loach	86.77 ± 1.81	5.40 ± 0.51	49.03 ± 3.93	0.56 ± 0.03
Grass carp	141.72 ± 13.6	62.16 ± 5.65	151.55 ± 9.07	1.07 ± 0.04
Yellow catfish	173.95 ± 11.67	86.75 ± 12.85	111.53 ± 14.94	0.64 ± 0.05

\* Intestinal ratio signifies the ratio between intestinal length and body length

to posterior intestine radius and the ratio of muscular thickness to posterior intestine radius were further calculated. The four histological parameters above were measured based on transverse sections staining HE. While, calculations of two air-breathing indicators, namely the air-blood diffusion distance (Zhang, et al., 2003) and capillary length densities (Shao, et al., 2004), were performed on sections dyed with AP. In details, the minimum distance from the capillary to intestinal epithelium was defined as the air-blood diffusion distance, and capillary length densities referred to capillary quantity per planar distance, of which computation was the ratio of double numbers of intestinal mucosa capillary endothelial cells to the area of a given view. Notably, all direct measurement stemmed from nine diverse microscopic visions chosen randomly from each sample section under three parallels.

#### Statistical analysis

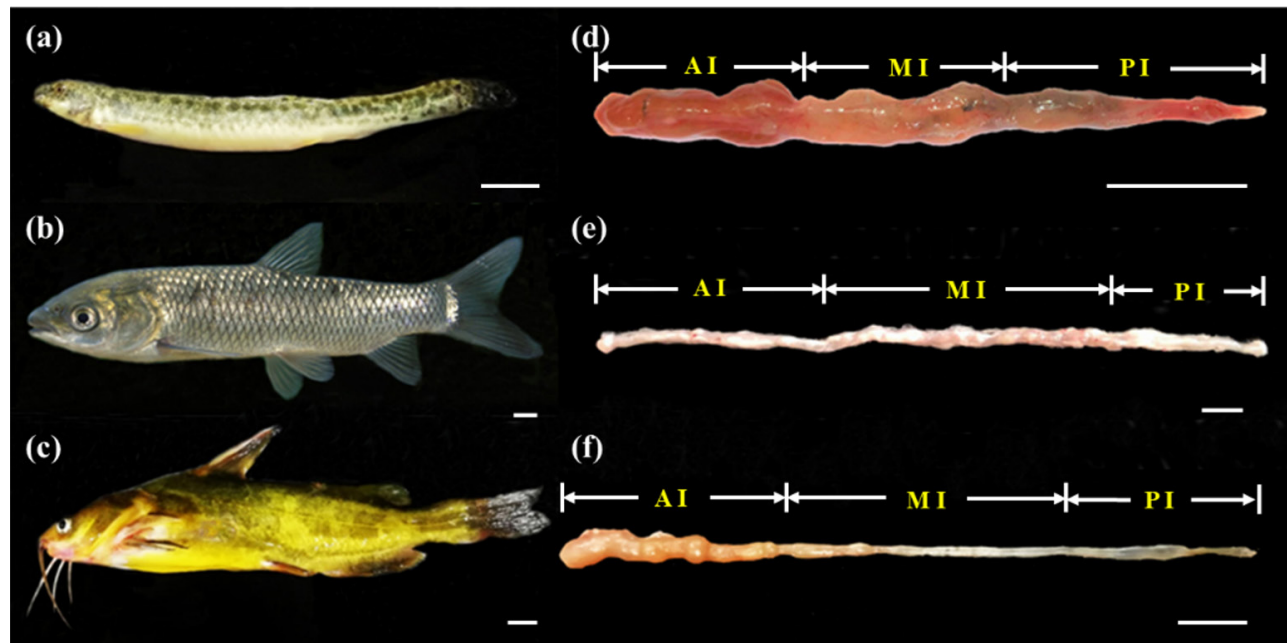
Data were expressed as mean ± SEM. All data were subjected to one-way ANOVA with LSD post hoc test using SPSS 23.0 (SPSS 23.0, Michigan Avenue, Chicago, IL, USA).  $p < 0.05$  was considered statistically significant.

## RESULTS

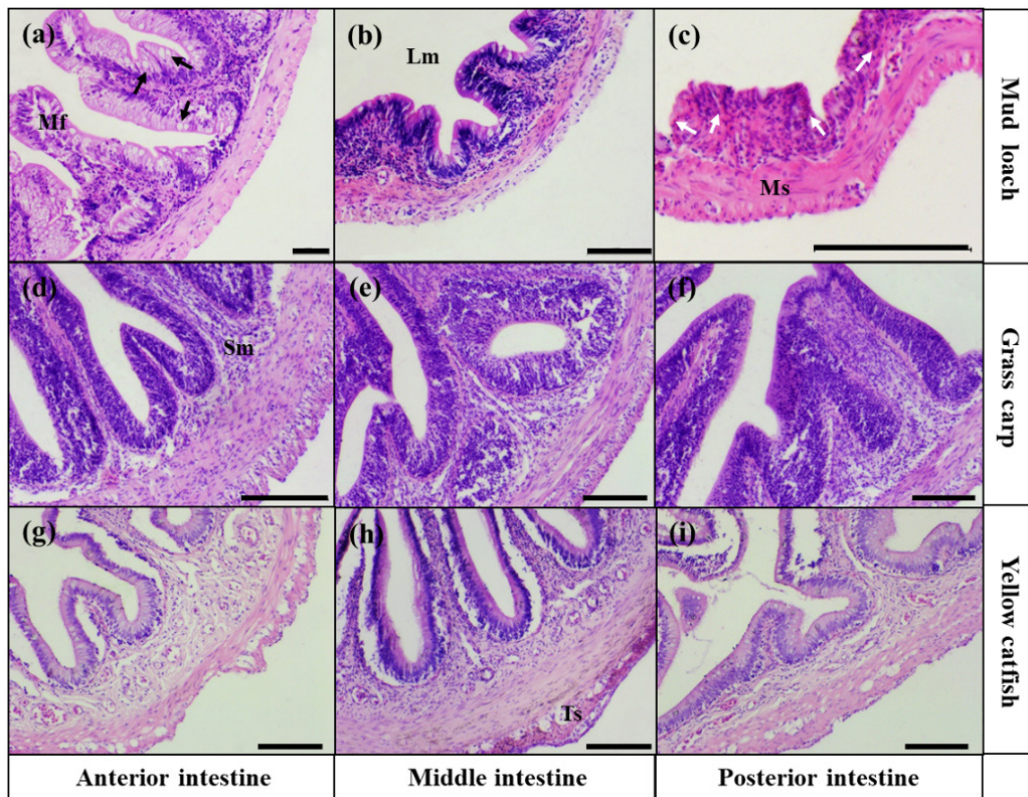
### The comparisons of intestinal anatomy and morphology of the three fish species

Fig. 1 shows mud loach, grass carp, yellow catfish and their intestine, including the AI, MI and PI. It was vividly depicted that in mud loach (Fig. 1d) the diameter of PI was much smaller than that of AI and MI, and the peritubular capillary was very abundant in the PI. There was a trend towards thinness and translucence along the intestine in the three fish species.

Fig. 2 shows the histological structures of intestinal tracts in the three fish species. Obviously, there was distinct differentiation between mucosal fold transition among the three intestinal portions of mud loach at first examination and other two fish species. Epithelia of the AI and MI in mud loach comprised a single layer of columnar cells with numerous globe cells in a marginal arrangement, as pointed by black arrows (Fig. 2a,b), while less globe cells but considerable capillaries filled with erythrocytes, as indicated by white arrows, were observed in the squamous epithelium of PI (Fig. 2c).



**Fig 1.** Morphology of mud loach, grass carp and yellow catfish, and the anatomical observation and their intestinal tracts. (a-c) The lateral views of mud loach, grass carp and yellow catfish; (d-f) the entire intestinal tracts of the loach, carp and catfish. The intestines are all partitioned into three different regions, namely anterior intestine (AI), middle intestine (MI) and posterior intestine (PI). Bar = 1 cm



**Fig 2.** Histological observations of intestinal transverse sections stained by HE of the three fish species. (a-c) Anterior-middle- and posterior- intestine of mud loach. Black arrows illustrate globe cells bordered in the epithelium and white arrows indicate capillaries with erythrocytes. (d-f) Anterior- middle- and posterior- intestine of grass carp. (g-i) Anterior-middle- and posterior- intestine of yellow catfish. Mf, mucosal folds; Lm, lumen; Ms, muscularis; Sm, submucosa; Se, tunica serosa. Bar = 10  $\mu\text{m}$

**Comparisons of histological parameters among the three fish species**

In the three fish species, significant declines of mucosal fold height were found along the intestinal tract from AI to PI (Fig. 3a), while the muscular thickness became significantly thinner from AI to PI in mud loach and yellow catfish (Fig. 3b). Fig. 3c and 3d respectively show that the ratio of mucosal fold height to posterior intestine radius and the ratio of muscular thickness to posterior intestine radius in mud loach were obviously smaller than those of the other two aquatic breathers, grass carp and yellow catfish.

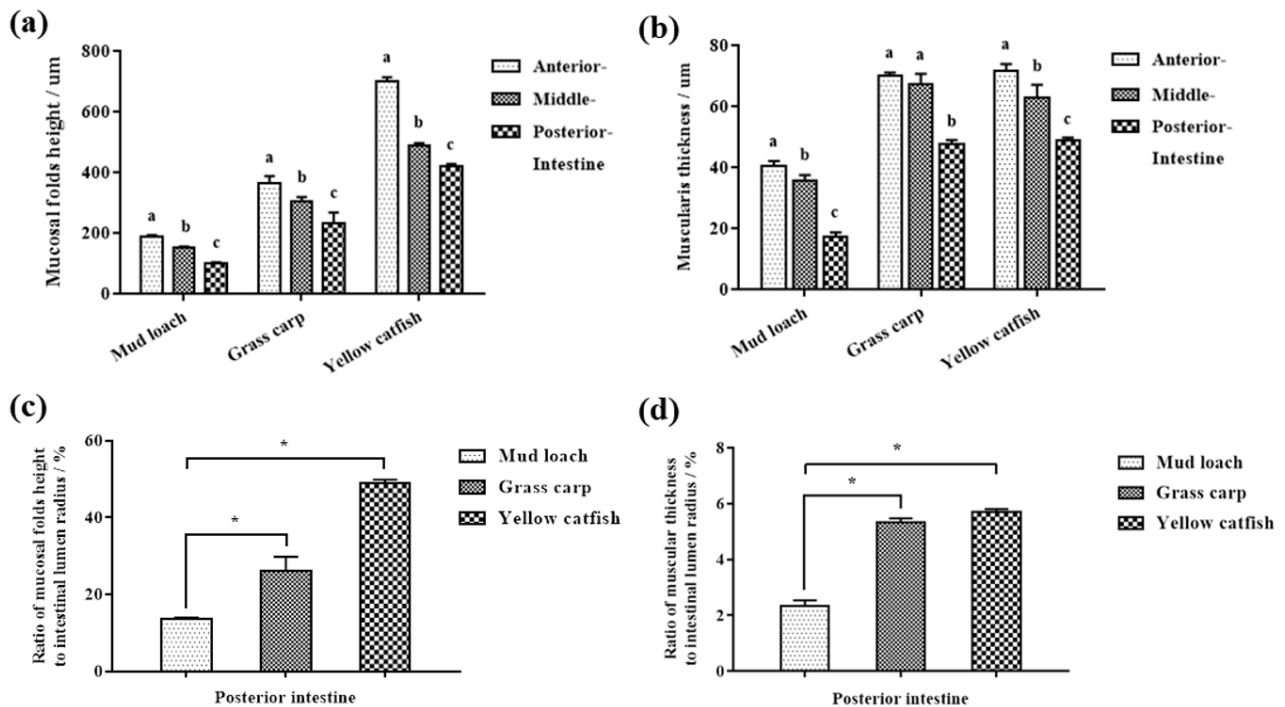
**Comparison of two air-breathing indicators of three species**

It is generally acknowledged that the air-breathing organs in fish are highly vascularized. Fig. 4 shows capillary length densities and air-blood diffusion distances in three intestinal portions of the three fish species. There was no significant difference in capillary length density in AI between mud loach and grass carp (Fig. 4a). Yellow catfish presented the lowest air-blood diffusion distance in AI.

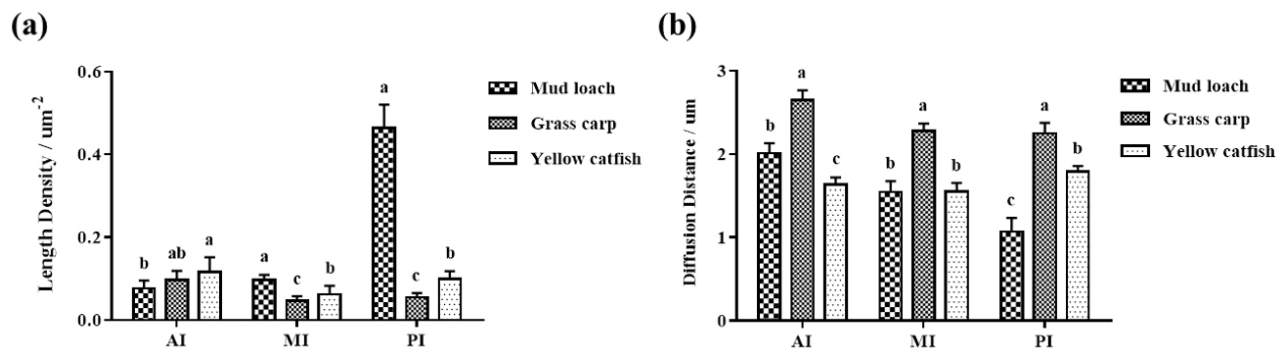
There was no significant difference in the diffusion distance in MI between mud loach and yellow catfish. When it came to the two indicators for accessory respiration, the length density of capillary vessels and diffusion distance of air-blood barrier of PI in mud loach, respectively highest of  $0.46 \pm 0.05 \mu\text{m}^{-2}$  and lowest of  $1.34 \pm 0.04 \mu\text{m}$ , both signified prominent difference from those of grass carp and yellow catfish (Fig. 4a,b).

**DISCUSSION**

A paralogous comparison of intestinal structures was firstly performed between a facultative air-breather (mud loach) and two aquatic breathers (grass carp and yellow catfish) here, to provide intuitive evidences for elaborate structural modifications of air-breathing organs. Even these three fish species share little in taxonomy and feeding habit, they have similar habitat settings, namely middle and demersal layer, and their intestines are all reported to partition into three portions (McMahon and Burggren, 1987; Ye et al., 2003; Cao and Wang, 2009).



**Fig 3.** Comparisons of the height of mucosal folds and the thickness of the muscular layer in intestine tracts among mud loach, grass carp and yellow catfish. (a) Comparisons of mucosal fold height of three intestinal portions among the three species. (b) Comparisons of muscular thickness of three intestinal portions among the three species. (c) Comparison of the ratios of mucosal fold height to posterior intestine radius among the three species. (d) Comparison of the ratios of muscular thickness to posterior intestine radius among the three species. Different letters and asterisks upon the column are representing a significant difference between species ( $p < 0.05$ )



**Fig 4.** Comparisons of capillary length densities and air-blood diffusion distances of intestinal tracts among the three fish species. (a) Comparisons of capillary length densities of anterior intestine (AI), middle intestine (MI) and posterior intestine (PI) among the three fish species. (b) Comparisons of air-blood diffusion distances of AI, MI and PI among the three fish species. Different letters upon the column are representing a significant difference between species ( $p < 0.05$ )

Therefore, intestines of sympatric grass carp and yellow carp were used to compare with the intestine (an air-breathing organ) of mud loach in this study.

It had been examined that more flattened mucosal folds and thinner intestinal muscle layer in PI of the facultative air-breather mud loach, significantly different from the other two aquatic breathers, implied its specific alternative function other than digestion and absorption. Moreover, finger-like mucosal folds stretched out of submucosa were emerged both in AI and MI, but replaced

by squamous epithelium in PI of loach, in agreement with the respiratory intestine of bronze *Corydoras corydoras aeneus* (Leszek et al., 2017). Goncalves et al. (2007) also reported that mucosa of PI in loach was embedded with intraepithelial capillaries, as well as erythrocytes in an elaborate arrangement, rather beneficial for gas exchange, which was authenticated in our results. All these dedicate alteration of PI in mud loach above mentioned and provided an opportune adaption for air-breathing.

Indeed, plentiful air-breathing fish species share some histological characteristics of accessory respiration organs in common. For instance, the PI of Neotropical catfish, *C. paleatus*, is described well vascularized with capillaries between epithelial cells (Plaul et al., 2016). Likewise, the penetration of an elaborate capillary bed into the epithelium was the primary transformation appeared in the respiratory intestine of *Hoplosternum thoracatum*, accompanied by a sharp decrease in mucosa epithelial thickness (Huebner and Chee 1978; Yadav, 1980). In addition, the organization of the stomach corpus was presented especial, and the epithelial cells of which located in the capillary mesh while flattened extensions covered the adjacent capillaries, according to the report from Satora in 1998. Thus, the air-breathing function of fish air-breathing organ primarily lies in its vascularized surface based on histological structure, and the degree of capillarity is mainly manifested in two indicators: capillary density and diffusion distance (Graham, 1997). Capillary vessels of air-breathing fish are abundantly distributed in the epithelium of the air-breathing organ, which reduces the diffusion distance of the air-blood barrier and enhances the function of gas exchange (Jiang and Pan, 2012). The length density of capillary vessels of PI in mud loach was significantly larger than that of grass carp and yellow catfish, while the diffusion distance of air-blood barrier in mud loach showed prominent smaller, supporting a further proof for the respiratory function of loach PI. Interestingly, the diffusion distance of PI in mud loach approached that of the air-breathing

opercular chamber in *Boleophthalmus boddarti* (Niva et al., 1981, Table 2). More amazingly, Hughes and Singh (1970) discovered that the diffusion distance of the air-blood barrier was as tiny as 1  $\mu\text{m}$  in *Anabas testudineus*, gigantically satisfying the demand of air-breathing. In summary, it is considered that epithelium of air-breathing organ in fish tends to be specialized in flattening and deep vascularization, implying short diffusion distance of air-blood barrier and rich capillary density, which immensely accommodates to accessory respiration in turn. The formation of abundant capillaries in the accessory respiratory organs of fish plays a vital role in guaranteeing the normal performance of air-breathing. In future, the molecular regulation mechanism of fish air-breathing remains to be examined systemically.

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## SAŽETAK

## USPOREDBA HISTOLOŠKE ANALIZE CRIJEVA VIJUNA, AMURA I SOMA, PRUŽA UVID U ADAPTIVNE KARAKTERISTIKE RIBA KOJE UDIŠU ZRAK

**Table 2.** Selected diffusion distances of air–blood barrier and capillary densities in the purported respiratory epithelium of some air-breathing fishes with different air-breathing organs (complement for Nelson, 2014)

Species (Latin name)	Air-breathing Organs (ABO)	Diffusion distance/ $\mu\text{m}$	Capillary density/ $\mu\text{m}^{-2}$	Source cited
<i>Boleophthalmus boddarti</i>	Opercular chamber	1.22		Niva et al., 1981
<i>Heteropneustes fossilis</i>	Air-sac	1.6		Munshi et al., 1986
<i>Anabas testudineus</i>	Supra-branchial chamber	0.21		Hughes and Singh, 1970
<i>Channa punctata</i>	Supra-branchial chamber	0.78		Hakim et al., 1978
<i>Periophthalmus cantonensis</i>	Skin	9		Tamura et al., 1976
<i>Boleophthalmus pectinirostris</i>	Skin	5.5 ~ 44.3		Zhang et al., 2000
<i>Dallia pectoralis</i>	Oesophagus	< 1		Crawford, 1974
<i>Ancistrus multispinis</i>	Stomach	0.6	0.07	Satora, 1998
<i>Pterygoplichthys anisitsi</i>	Stomach	0.40 ~ 0.74		Cruz et al., 2009
<i>Hoplosternum thoracatum</i>	Intestine	1 ~ 2		Huebner and Chee, 1978
<i>Lepidocephalichthys gun-tea</i>	Intestine	0.86 ~ 1.08	0.15	Yadav and Singh, 1980
<i>Paramisgurnus dabryanus</i>	Intestine	1.95±0.34		Liu and Wang, 2017
<i>Misgurnus anguillicaudatus</i>	Intestine	1.34±0.04	0.46±0.05	This study

Općenito je prihvaćeno da su pomoćni dišni organi riba u više navrata samostalno evoluirali pri prilagođavanju slatkovodnim staništima i vodenoj hipoksiji. Vjeruje se da je organ za udisanje zraka kod riba strukturno modificiran kako bi nadopunio respiraciju. U ovom istraživanju prezentirani su intuitivni dokazi detaljne strukturne modifikacije crijeva i organa za udisanje zraka kod muljnog vijuna (*Misgurnus anguillicaudatus*), uspoređujući ih amurom (*Ctenopharyngodon idellus*) i žutim somom (*Pelteobagrus fulvidraco*). Izravno dobiveni histološkom i morfometrijskom metodom. Kod muljnog vijuna očitovale se naglo smanjena visina nabora sluznice i debljina mišićnog sloja od prednjeg do stražnjeg dijela crijeva. U usporedbi sa amurom i žutim somom, vijun je imao i najmanji omjer visine nabora sluznice/debljine mišićnog sloja i polumjera crijeva u stražnjem dijelu crijevu. To sugerira da je moguće stražnji dio crijeva lokacija na kojoj vijun udiše zrak. Nadalje, gustoća kapilara ( $0,46 \pm 0,05 \mu\text{m}^{-2}$ ) u zadnjem crijevu vijuna bila je značajno viša od amura i žutog soma. Također, difuzijska udaljenost zračno-krvne barijere ( $1,34 \pm 0,04 \mu\text{m}$ ) u stražnjem crijevu vijuna bila je značajno manja od one u druge dvije vrste riba. Ukratko, karakteristike poput visoke prokrvljenosti, kratke udaljenosti difuzije zračno-krvne barijere, prorijeđenosti i spljoštenosti, učinile su stražnji dio crijeva savršenom lokacijom za udisanje zraka istraživane vrste vijuna.

**Cljučne riječi:** Udisanje zraka, crijevne strukture, *Misgurnus anguillicaudatus*, *Ctenopharyngodon idellus*, *Pelteobagrus fulvidraco*

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