ORIGINAL ARTICLE

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Using gill and liver biomarkers to monitor the health of two neotropical fish species in a lake zone in Maranhão state

Biomarcadores branquiais e hepáticos para monitoramento da sanidade de duas espécies de peixes neotropicais em área lacustre maranhense, Maranhão

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ABSTRACT: The aim of the current study is to histologically assess two Neotropical fish species (*Hoplerythrinus unitaeniatus* and *Cichlasoma bimaculatum*) to monitor the health of a lake zone in Maranhão State. Forty-two (42) adult fish specimens - 21 belonged to species H. unitaeniatus and 21 to species C. Bimaculatum - were captured, euthanized and had their second right gill arch and liver fragments removed for histological analysis. Histological changes in specimens' gill and liver were semi-quantitatively assessed by calculating the histological alterations index (HAI) based on the severity of each lesion established as stages I, II and III. Results recorded for both species were presented together; they evidenced that both assessed organs have shown changes associated with all three severity stages. Gill changes mostly observed through the analysis comprised total (85.71%) and partial (85.71%) fusion of several lamellae, epithelial lifting (80.95%), congested blood vessels (76.19%), disorganized lamellae, (76.19%) and lamellar epithelial hyperplasia (76.19%) - stage I alterations. The most common liver changes comprised vacuolization (90.48%), melanomacrophage centers (78.57%), cellular contour deformation (66.67%) and nucleus at cell periphery (52.38%), stage I alterations and hyperemia (45.24%) and cytoplasmic degeneration (40.48%) - stage II. Unlike the HAI indices recorded for gills, liver HAI values presented better distributed rates among severity classifications; therefore, it has evidenced a more severe condition. Thus, both organs analyzed in the investigated species have shown several histological changes that may have resulted from specimens interactions with stressors capable of affecting their health.

KEYWORDS: Aquatic animals; stress; histology; lesions.

RESUMO: Objetivou-se avaliar histologicamente duas espécies de peixes neotropicais (*Hoplerythrinus unitaeniatus* e *Cichlasoma bimaculatum*) para monitoramento da sanidade em uma área lacustre maranhense. 42 espécimes de peixes adultos - 21 pertencentes à espécie *H. unitaeniatus* e 21 à espécie *C. bimaculatum* - foram capturados, eutanasiados e tiveram o segundo arco branquial direito e fragmentos de fígado removidos para análise histológica. As alterações histológicas branquiais e hepáticas foram avaliadas de forma semiquantitativa, por meio do cálculo do índice de alteração histológica (IAH), fundamentado na severidade de cada lesão estabelecida nos estágios I, II e III. Os resultados foram apresentados de forma conjunta para ambas espécies e foi constatado que ambos os órgãos possuíram alterações dos três estágios. As alterações branquiais mais observadas foram fusão completa (85,71%) e incompleta (85,71%) de várias lamelas, levantamento do epitélio (80,95%), congestão de vasos sanguíneos (76,19%), desorganização das lamelas (76,19%) e hiperplasia do epitélio lamelar (76,19%), todas de estágio I. As alterações hepáticas mais encontradas foram vacuolização (90,48%), centro de melanomacrófagos (78,57%), deformação do contorno celular (66,67%) e núcleo na periferia da célula (52,38%), alterações de estágio I e hiperemia (45,24%) e degeneração citoplasmática (40,48%), alterações de estágio II. Diferente dos índices de IAH das brânquias, o IAH do fígado apresentou porcentagens melhor distribuídas entre as classificações, evidenciando um quadro mais grave. Sendo assim, foi possível observar diversas alterações histológicas em ambos os órgãos das espécies avaliadas que podem ser resultado de interações com agentes estressores que impactam na sanidade dos espécimes.

PALAVRAS-CHAVE: Animais aquáticos; estresse; histologia; lesões.

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INTRODUCTION

Aquatic biota has been increasingly threatened by several stressors, such as variations in water temperature, salinity and pH, as well as low dissolved oxygen concentrations and toxins produced by algae. Heavy metals, chemical residues and pesticides are the main stressors capable of harming fish, among other aquatic organisms, in lake environments (BOYD, 2017). These environments are vulnerable to contamination, since they can accumulate high concentrations of different chemicals that can be either directly applied to them or derive from land runoff coming from neighboring regions (DI GIULIO; HINTON, 2008).

Like other vertebrates, fish manage to maintain their integrity through their immune system, although its homeostasis and efficiency can be affected by stress and lead to immunosuppression issues (TORT, 2011). These animals are susceptible to several pathogens and non-infectious agents, with emphasis on stress, which plays key role in the emergence of different diseases. Water quality is essential to help maintaining these animals' health, since it affects their physiology, reproduction, growth and behavior. Therefore, it is possible saying that several diseases affecting fish result from inappropriate environmental management (NOGA, 2010).

These organisms can be used as environmental quality bioindicators in water contamination and sanitation assessments, since they provide evidence of such issues through both the accumulation of substances in their bodies and the systemic effects resulting from their exposure to contaminants (MANAHAN, 2017). These stressors have significant impact on fish development and may cause histological changes such as inflammation, hyperplasia and necrosis in organs such as the gills and liver, in addition to cause malformations, deformities and mortality (DI GIULIO; NEWMAN, 2012).

Histological biomarkers can be used as diagnostic tool to determine the health of fish populations living in impacted environments, since they reflect aquatic ecosystems' conditions as a whole (PEREIRA et al., 2014). Histological studies conducted with fish often focus on different organs, mainly on the ones accounting for their metabolism, such as the liver, which can undergo structural and metabolic changes due to fish exposure to pollutants, toxins, microorganisms and parasites, as well as to the intake of certain food types (BOMBONATO et al., 2007; ROCHA et al., 2010). Gills are one of the main organs affected by being exposed to impacted environments and may present alterations such as lamellar aneurysm, hyperplasia and lamellae fusion (DI GIULIO; NEWMAN, 2012).

According to DE JESUS et al. (2022), most Maranhão State regions experience several environmental impacts, such as deforestation and pollution resulting from domestic sewage discharge, among others, as well as from impacts caused by livestock farming based on pigs, buffaloes and cattle breeding under precarious sanitary conditions. The aforementioned researchers have also advocated that environmental impacts can, at some point, contribute to animal stress, which, in its turn, can compromise animals' immune system and predispose them to develop diseases of different etiologies; consequently, it can compromise the dietary basis of several traditional communities.

Therefore, aquatic animals' health assessments based on histopathological analysis are of great value and extremely useful. Thus, the aim of the current study was to histologically assess gill and liver tissues of two neotropical fish species (*Hoplerythrinus unitaeniatus* and *Cichlasoma bimaculatum*) to monitor the health of a lake zone in Maranhão State.

MATERIAL AND METHODS

Legal Authorization

All the herein performed procedures were in compliance with the ethical principles established by the Brazilian College of Animal Experimentation (COBEA - Colégio Brasileiro de Experimentação Animal -, http://www.cobea.org.br) and by the Ethics Committee on Animal Experimentation (CEEA - Comitê de Ética em Experimentação Animal) of Maranhão State University (UEMA - Universidade Estadual do Maranhão) - Protocol n. 08/2021.

Study Site

The study was carried out in a lake zone located in the rural area of Anajatuba County, Maranhão State. The aforementioned county belongs to Baixada Maranhense region and it is located between the following geographic coordinates: Latitude 03°15'50" S and Longitude 44°37'12" W.

Climate in the investigated region is of the AW[1] type (humid tropical climate with dry winter season), based on Köppen's (1948) classification. The investigated region presents six-month dry season (from August to January) - three to four of these months are extremely dry, since they record total rainfall rate lower than 8%. It also presents six-month rainy season (from February to July) - at least two of these months are extremely wet, since they record total rainfall rate higher than 30%.

Fish sample collection

Two sample collections were carried out, one in the rainy season (February 2021) and the other one, in the dry season (August 2021) - biannual interval was implemented between collections. Forty-two (42) adult fish specimens were captured with the aid of 4-mesh (20 mm) casting net and 4-mesh (20 mm) gillnet - 21 specimens belonged to species *H. unitaeniatus* (*jeju*) (18.7 ± 2.5 cm and 92.70 ± 25.7 g) and 21 to species *C. bimaculatum* (black acara) (12.45 ± 0.77 cm and 44.32 ± 9.80 g).

Captured specimens were transported alive in isothermal boxes - which were filled with water from the capture site and provided with water oxygenation equipment - to the Laboratory for the Reproduction of Aquatic Resources (LARAQUA) at Maranhão State University (UEMA). Once in the laboratory, they were placed in tank filled with water and provided with constant oxygenation, for 12 hours, until analysis time.

Laboratory Processing and Histological Analysis

Fish were euthanized in laboratory environment by perforation in the upper part of their heads; it was done with the aid of a scalpel blade. All procedures were performed in compliance with ethical principles.

The second right gill arch and liver fragments were removed from each specimen with the aid of forceps and scissors, and fixed in 10% formalin. Histological analyses were performed at the Microscopy Laboratory of UEMA's Post-graduation Multiuser Laboratory (LAMP - Laboratório Multiusuários da Pós-Graduação). Gill and liver fragments were initially cleaved to reduce their dimensions to 3-5 mm, in thickness. It was done to enable the penetration and diffusion of reagents used at the histological processing stages.

Subsequently, the decalcification, dehydration, diaphanization, paraffin embedding and microtomy stages were carried out, based on Caputo, Gitirana and Manso (2010). Only gill samples were decalcified in 10% nitric acid, after fixation, for approximately six hours, in order to remove mineral salts from gill arches. Cross sections, approximately 5 µm in thickness, were stained with hematoxylin and eosin (HE). Slides were read under optical microscope, at 10x and 40x magnification, and the observed lesions were photomicrographed.

Histological alterations in gill and liver samples were semi-quantitatively assessed by calculating the histological alterations index (HAI) adapted from POLEKSIC and MITROVIC-TUTUNDZIC (1994), depending on the severity of each lesion (Table 1), as follows: (i) stage I alterations – the ones that do not compromise organs' functioning; (ii) stage II - more severe lesions capable of impairing organs' normal functioning; and, (iii) stage III - extremely severe and irreversible lesions.

HAI value of each fish was calculated based on the following formula: $HAI = 1 \times \Sigma I + 10 \times \Sigma II + 100 \times \Sigma III$, wherein I, II and III correspond to stages I, II and III, respectively. The aforementioned value was based on five categories established by POLEKSIC and MITROVIC-TUTUNDZIC (1994), namely: 0-10 = normal tissue function; 11-20 = mild-to-moderate tissue damage; 21-50 = moderate-to-severe tissue alteration; 51-100 = severe tissue alteration; higher than 100 = irreparable tissue damage.

RESULTS AND DISCUSSIONS

The herein assessed 42 gill fragments presented stage I, II and III alterations (Table 2). Given the similarity observed in alterations recorded for both assessed species, the current study has made the option for jointly presenting and discussing the results.

The histological analysis allowed seeing several changes at all three severity stages; some specimens have shown changes

Table 1. Classification of histological alterations observed in gill and liver samples, based on lesions' severity stage.

Histological Alterations		
Gills	**Liver**	**Stage**
Congested blood vessels Respiratory epithelium lifting Disorganized lamellae Lamellar epithelial hyperplasia Partial fusion of several lamellae Total fusion of several lamellae Blood sinus dilation Respiratory epithelium hypertrophy	Nucleus at cell periphery Cell contour deformation Cell hypertrophy Nuclear hypertrophy Cell atrophy Nuclear atrophy Melanomacrophage centers Vacuolization	I
Lamellar epithelium bleeding and rupture Mucus cell hyperplasia and hypertrophy Chloride cell hyperplasia and hypertrophy Total lamellar fusion Uncontrolled proliferative tissue thickening Pillar cell rupture	Nuclear vacuolization Cytoplasmic degeneration Hyperemia Cell disruption Bile stagnation Nuclear degeneration	II
Lamellar aneurysm Lamellar telangiectasia Cell necrosis and degeneration	Necrosis	III

Source: Adapted from Poleksic and Mitrovic-Tutundzic (1994).

Table 2. Frequency of gill alterations observed in 21 *Hoplerythrinus unitaeniatus* and 21 *Cichlasoma bimaculatum* specimens from a lake zone in Maranhão State.

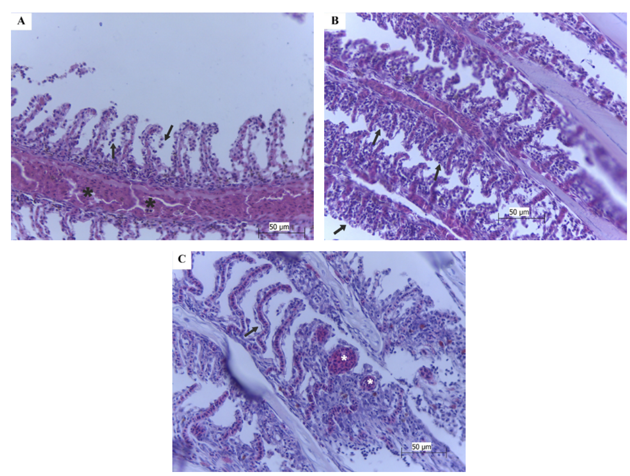
Stage	Histological Alterations in Gills	N	Frequency (%)
I	Partial fusion of several lamellae	36	85.71
	Total fusion of several lamellae	36	85.71
	Respiratory epithelium lifting	34	80.95
	Congested blood vessels	32	76.19
	Disorganized lamellae	32	76.19
	Lamellar epithelial hyperplasia	32	76.19
	Blood sinus dilation	28	66.67
	Respiratory epithelium hypertrophy	06	14.29
II	Lamellar epithelium bleeding and rupture	06	14.29
	Total lamellar fusion	04	9.52
	Uncontrolled proliferative tissue thickening	04	9.52
	Chloride cell hyperplasia and hypertrophy	02	4.76
	Mucus cell hyperplasia and hypertrophy	02	4.76
	Pillar cell rupture	02	4.76
III	Cell necrosis and degeneration	04	9.52
	Lamellar aneurysm	01	2.38

N= number of fish with gill alterations.

at two, or more, stages at the same time. The most observed Stage-I gill alteration comprised total (85.71%) and partial (85.71%) fusion of several lamellae, epithelial lifting (80.95%), congested blood vessels (76.19%), disorganized lamellae (76.19%) and lamellar epithelial hyperplasia (76.19%).

The most observed Stage-II alterations comprised lamellar epithelium bleeding and rupture (14.29%), total lamellar fusion (9.52%) and proliferative tissue thickening (9.52%). Although stage-II alterations are more severe than stage-I alterations, they can be reversed, unlike stage-III alterations, which are the most severe and irreversible ones (POLEKSIC; MITROVIC–TUTUNDZIC, 1994).

Cell necrosis and degeneration (9.52%), as well as lamellar aneurysm (2.38%), were the lesions found at stage III. Alterations typical of this stage pose greater risk to animal health; they can severely compromise organ functioning and gill breathing. Figure 1 shows some Stage-I, -II and -III gill lesions.



Source: Authors' collection.

Figure 1. Photomicrographs of gill alterations observed in *Hoplerythrinus unitaeniatus* and *Cichlasoma bimaculatum* specimens from a lake zone in Maranhão State. [A] Lamellar epithelium rupture (arrows), vasodilation and congestion (asterisks); [B] Secondary lamellae fusion and lamellar epithelial hyperplasia (arrows); [C] Lamellar aneurysm (asterisks) and epithelial lifting (arrow). HE staining. 40X objective lens.

Physiological responses to stress play key role in the survival of different organisms; they range from neurological reactions to increased plasma cortisol levels. In addition, repeated and chronic exposure to stressors has detrimental effects on many physiological aspects of fish (VIJAYAN; ALURU; LEATHERLAND, 2010). The first physiological reactions taking place in fish gills are observed in gill epithelium, which is an extensive area that enables high level of exposure to aquatic environments and makes fish vulnerable to several stressors (MOKHTAR, 2017).

Histological changes observed during the current study were compatible to lesions previously recorded by other scholars for fish deriving from contaminated environments (MACHADO, 1999; CASTRO et al., 2018). Gill alterations, such as lamellar fusion, edemas and aneurysms, can work as defensive responses by the body to stress caused by impacted environments (PEREIRA et al., 2014). The herein identified alterations can make breathing - which is the basic function of this organ - difficult.

PEREIRA et al. (2020) have defined gill histological analyses as very effective to assess environmental impacts and fish health. They analyzed gills of *Psectrogaster amazonica* specimens collected in Mearim River, Bacabal County, Maranhão State, and found stage-I, -II and -III alterations in the assessed samples, with emphasis on the incidence of parasites, which also work as stressor agents.

CANTANHÊDE et al. (2014) have histologically analyzed the gills of *Centropomus undecimalis* specimens from an impacted lake environment and observed that several samples had moderate-to-severe alterations in this organ, with emphasis on aneurysm, which was found in 30% of the total sample. The aforementioned authors have concluded that some alterations may be reversible if environmental conditions can be improved.

Based on the HAI values herein calculated for gills, 59.52% (n=25/42) of specimens presented normal tissue function; 23.80% (n=10/42) showed mild-to-moderate tissue damage; 4.77% (n=2/42) evidenced moderate-to-severe tissue changes; and 11.90% (n=5/42) irreparable tissue damage.

Gills are water quality indicators; therefore, changes observed in them may suggest reaction to stress caused by changes in environments fish live in. However, the fact that most specimens presented normal tissue functioning may indicate that environmental stressors were not high to the point of causing severe lesions and more severely compromising the organs in most analyzed fish.

Similar to what was observed for gills, histological liver analyses have shown Stage-I, -II and -III alterations in both *H. unitaeniatus* and *C. bimaculatum* specimens (Table 3). Given the similarity of alterations observed in both species, the current study has made the option for presenting and discussing the results, altogether.

Some species showed liver alterations in two or more stages at the same time. Analyses have confirmed several Stage-I alterations, with emphasis on vacuolization, which was observed in 90.48% of the assessed specimens. Melanomacrophages center (78.57%), cell contour deformation (66.67%) and nucleus at cell periphery (52.38%) were the other alterations mostly observed during the analyses.

Hyperemia (45.24%), cytoplasmic degeneration (40.48%) and nuclear vacuolization (21.43%) were the main alterations observed at Stage II. Necrosis (9.52%), which is the only stage-III alteration, was also identified throughout the current study. Similar to what was observed for gills, Stage-III liver alteration is the one posing the greatest risk to animal health, since it can severely compromise the liver function.

Other changes that were not described in the severity stages established by POLEKSIC and MITROVIC-TUTUNDZIC (1994), such as fatty degeneration (n=6/42; 14.29%), inflammatory process (n=6/42; 14.29%), bleeding (n=2/42; 4.76%), abscess (n=2/42; 4.76%) and evident nucleolus (n=2/42; 4.76%), were also observed in the herein analyzed samples. Some of the observed liver lesions are shown in Figure 2.

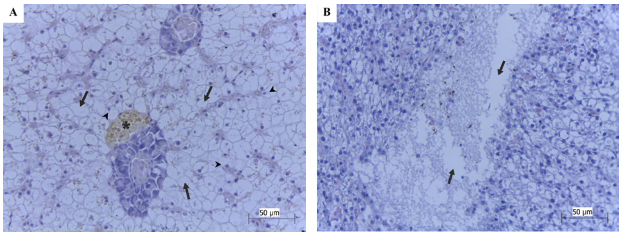
Stage II alterations in the liver were found in greater quantity when compared to Stage II gill alterations. This can be explained by the physiology of the liver, which has multiple functions such as bile production, nutrient assimilation, plasma protein synthesis, vitamin and glycogen storage, as well as toxic substances' biotransformation (GENTEN; TERWINGHE; DANGUY, 2009).

Liver shows high trend to accumulate chemical substances, such as heavy metals (LATIF et al., 2021). Changes in the liver, such as vacuolization and increased incidence of

Table 3. Frequency of liver lesions observed in *Hoplerythrinus unitaeniatus* and *Cichlasoma bimaculatum* specimens from a lake zone in Maranhão State.

Stage	Histological Alterations in Liver	N	Frequency (%)
I	Vacuolization	38	90.48
	Melanomacrophage center	33	78.57
	Cell contour deformation	28	66.67
	Nucleus at cell periphery	22	52.38
	Cell hypertrophy	03	7.14
	Nuclear hypertrophy	01	2.38
	Cell atrophy	01	2.38
	Nuclear atrophy	01	2.38
II	Hyperemia	19	45.24
	Cytoplasmic degeneration	17	40.48
	Nuclear vacuolization	09	21.43
	Nuclear degeneration	06	14.29
	Cell disruption	05	11.90
III	Necrosis	04	9.52

N= number of fish with liver alterations.



Source: Authors' collection.

Figure 2. Photomicrographs of liver alterations observed in *Hoplerythrinus unitaeniatus* and *Cichlasoma bimaculatum* specimens from a lake zone in Maranhão State. [A] Melanomacrophage center (asterisk), vacuolization (arrows) and nucleus at cell periphery (arrowheads); [B] Necrotic areas in the liver parenchyma (arrows). HE staining. 40X objective lens.

melanomacrophages centers, may suggest that the investigated animal may have been exposed to contamination (RICHARDSON et al., 2010). Therefore, this organ is susceptible to alterations resulting from stressors' action.

Histological analysis of *Oreochromis niloticus* liver carried out by OLIVEIRA et al. (2016), who also analyzed gills, has evidenced changes, such as necrosis and melanomacrophage center, similar to the ones observed in the present study. The aforementioned authors have also emphasized that the liver was the organ presenting the most severe lesions - their samples were also collected in lake environment. LIKEWISE, LEONE, VALDECANTOS and MARTÍNEZ (2018) have analyzed histopathological markers of environmental stress in *Odontesthes bonariensis* specimens living in impacted environments and observed that the liver was the most affected organ. It presented changes similar to the ones observed in the current study, such as necrosis and melanomacrophages' accumulation.

Based on the calculated HAI values, 26.19% (n = 11/42) of the analyzed specimens presented normal liver functioning; 26.19% (n = 11/42) presented mild-to-moderate liver damage; 38.09% (n = 16/42) showed moderate-to-severe liver damage; and 9.52% (n = 4/42) presented irreparable liver damage. Unlike HAI values recorded for gills, HAI values recorded for liver have shown better distributed rates among severity classifications, with emphasis on the highest rate (38.09%), which corresponded to moderate-to-severe tissue alterations. This outcome has suggested that the liver was more affected by the action of stressors than the gills.

Results have also shown alterations in both organs in 40 fish specimens. SOARES et al. (2020) have concluded that fish's gills and liver presented different responses to stress. On the other hand, they stated that gills were the most affected organ, since they presented the most significant alterations. According to SAGER et al. (2021), gills were the organs mostly sensitive to short-term exposure to the investigated contaminant; they also observed that liver was the most sensitive organ in the long run.

CONCLUSIONS

Histological analyses applied to the gills and liver of both herein investigated neotropical fish species enabled observing several histological alterations that may have resulted from animals' interactions with stressful agents. Some samples presented severe lesions capable of compromising the functioning of the assessed organs. Overall, lesions observed in the liver were more severe than the ones observed in gills. This outcome can be explained by liver's function of accumulating substances and by the time animals remained exposed to stressors.

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