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Evaluation of the Environmental Monitoring of the Cachoeira river Through Histopathological Alterations of *Astyanax fasciatus*

Santos Matheus L., Silva Gabriela L., Oliveira Cristina L. C. and Flores-Lopes Fábio*

Department of Biological Sciences, State University of Santa Cruz, Department of Biological Sciences, Rodovia Ilhéus – Itabuna, km 16, Salobrinho, CEP - 45662-000 - Ilhéus, BA - Brazil

*Corresponding Author

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Abstract: The analysis of histopathological changes in organs is considered an important parameter in environmental quality monitoring studies through various tissues that are essential organs to the organism. The objective of this work was to investigate the histological alterations severity in the gills and liver of *Astyanax fasciatus* of the Cachoeira river between the municipalities of Itapé and Ilhéus, Brazil. Seasonal collections were carried out in five points of the river between the cities of Itapé and Ilhéus. The specimens collected were fixed in 10% formalin and the gills and liver were removed and fixed. The routine histological technique was used sections were cut at 5 – 7 µm. The slides were stained with Hematoxylin and Eosin (HE). Light lesions were observed in the liver, such as cytoplasmic vacuolization, nuclear and cytoplasmic hypertrophy, deformation of the cellular and nuclear contour, alterations such as biliary stagnation, nuclear vacuolization and focal necrosis. In the gills, hypertrophy and epithelial cell hyperplasia, epithelial displacement and elevation, presence of parasite cyst, total fusion and lamellar disorganization, telangiectasis and aneurysm were observed. The results of the Histopathological Alteration Index (HAI) showed statistically significant differences among points 1, 2 and 3, demonstrating a potentially greater presence of a stressor agent. The presence of histological changes with different severity degrees in the gills and liver of *A. fasciatus* demonstrated that the Cachoeira river is in a progressive process of environmental degradation.

Keywords: *Astyanax*, Aquatic environment, Histopathology, Gills, Liver

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Introduction

The aquatic environment is fundamental in the composition of environments and has

been considered an important target for pollution and contamination due to the

disposal of organic, inorganic, heavy metal and other xenobiotic effluents associated with the development of agricultural activities, mining and exploitation of fossil resources. These types of discards can happen intentionally or not and their consequences accumulate along the trophic chain (Silva *et al.*, 2004).

Fish are often exposed to highly contaminated water, which according to Bernet *et al.* (1999) leads to different changes that can vary from biochemical alterations in single cells up to changes in the whole population. Al-Sabti and Metcalfe (1995) stated that species of these animals are important vehicles for the transferal of contaminants to human populations and may indicate a potential exposure to pollutants. The use of fish in environmental monitoring studies, according to Jenkins (2004), has several advantages, as they are directly associated with the sediment and water column, have poor mobility and exhibit some physiological mechanisms of biological response. Flores-Lopes *et al.* (2019) stressed that fish are important as an indicator of exposure to contaminants and histology is a great tool to assess the degree of pollution, particularly chronic and sublethal effects.

Flores-Lopes and Thomas (2011) carried out studies using different biological methods, which allowed to evaluate the response of some organisms to changes in habitats. Studies on fish biomarker are of great significance as they can help to evaluate organ alterations, enzymatic responses, and histological changes when exposed to polluted aquatic environment (Bassey, 2019). The technique of bioindicators has been used as a tool to evaluate the water resource quality

and has as experimental subjects sentinel species.

According to Maschio *et al.* (2004), fish indicate the first signs of environmental stress caused by contaminants at high levels of biological organization. Within the biodiversity found in the aquatic ecosystem, these animals exhibit great morphological diversity, besides populating different ecological niches (Santos-Filho *et al.*, 2014).

The species *Astyanax fasciatus* (Cuvier, 1819) is considered non-migratory, present from Central America to the delta of the river Plate in Argentina. This species has been used as a biomarker due to its abundant presence and environmental tolerance, which allows the usefulness of these specimens (Schulz and Martins-Junior, 2001; Carrasco-Letelier, 2006).

Histological analysis of several fish organs allows the evaluation of the direct and indirect toxic effects on tissues and organs, constituting an excellent mechanism in order to verify the action of the xenobiotics on the biomarkers (Lins *et al.*, 2010). This type of analysis can mark the physiological response to the stressor agent demonstrating the occurrence of changes caused by the action of the stressor. The gills, liver and kidney are the most used in the processes of environmental assessment, being in most cases, responsible for the metabolism and/or excretion of xenobiotics.

According to Cerqueira and Fernandes (2002), the gills are fundamental organs for performing various vital functions (breathing, osmoregulation and excretion) in fish, as they exhibit a large surface of permanent contact with the external environment and are responsible for performing the gas and ionic

exchanges with the same. Mallatt (1985), Mazon *et al.* (1999) and Cerqueira and Fernandes (2002) reported that the gills are sensitive to chemical and physical changes in the aquatic environment, becoming the target organ in fish for waterborne pollutants and the liver is the largest organ of biotransformation and an excellent organ to be used to evaluate the environmental toxicity (Santos-Filho *et al.*, 2014).

The Cachoeira river Basin covers one of the major socioeconomic regions of Bahia (Nacif, 2000) and it has undergone an intense process of degradation, mainly caused by the discharge of untreated municipal sewage into its waters, the use of fertilizers and pesticides on cocoa plantations and the illegal presence of tanneries (Paulo *et al.*, 2012).

The objective of this study was to evaluate the quality of the Cachoeira river environment quality through *A. fasciatus* gills and liver by evaluating histological alterations in order to compare the environmental quality of the river points sampled.

Materials and Methods

Four sampling points were selected in the Cachoeira river, between the municipalities of Itapé and Ilhéus. The samplings were performed in September and December of 2014, and January and April of 2015 at the following points: point 1 – Estiva (14° 56' 935" S and 39° 28' 469" W); point 2 – Vila de Itapé (14° 53' 934" S and 39° 25' 781" W); point 3 – Farm Sta. Amélia - Itapé (14° 52' 719" S and 39° 24' 772" W) and point 4 – near Pitú Point (14° 47' 788" S and 39° 12' 107" W) (Fig. 1).

Collections were carried out with five trawls (15 m x 1.5 m x 0.5 cm each)

(Malabarba and Reis, 1987). The specimens were fixed in the field in 10% formaldehyde. Samples were taken to the Laboratório de Histologia Animal (Laboratory of Animal Histology) at the Universidade Estadual de Santa Cruz (UESC), Bahia, Brazil, where a sorting and identification of the collected species was carried out. The specimens were stored in 70% alcohol and the measurements of standard length (mm) and total weight (g) were taken. Samples were selected and deposited in the Coleção Científica de Ictiologia (Ichthyology Scientific Collection) at UESC.

The gills and liver were removed from the samples and preserved in 70% alcohol. Gill arches were decalcified with 20% EDTA (Tetrasodium Ethylenediaminetetracetate Acid) for four days to one week. The tissues were dehydrated and embedded in paraffin by the routine histological technique (Michalany, 1980). Sections were cut at 5-7 µm. The slides were stained with Hematoxylin and Eosin (HE) according to Behmer *et al.* (1976) for general visualization of the affected tissues and organs.

Histopathological changes were classified from 0 to 3, where 0 = no change, 1 = slight change, 2 = moderate change and 3 = severe change (Hose *et al.*, 1996). The definitions of mild, moderate and severe anomalies have been modified from Poleksic and Mitrovic-Tutundzic (1994) and are characterized as follows: slight change (1) changes that do not damage organ tissues in such a way that an improvement in environmental conditions allows the restructuring and resumption of its function. Moderate (2) more severe changes lead to effects on tissues associated with organ function; are repairable injuries, but if large areas of the organ are affected or

Fig. 1: Bahia state map. In detail the Cachoeira river and the sampling points. 1 – Estiva; 2 – Itapé; 3 – Sta. Amelia and 4 – Point doPitú.

maintained in situations of chronic pollution, they can lead to severe changes; occur in practically the whole organ. Severe change (3) recovery of organ structure is not possible, even with improved water quality or end of exposure to a toxic stimulus (Tables 1, 2).

The occurrence of histopathological changes was assessed semi-quantitatively by the degree of tissue change (Histopathological Alteration Index - HAI) that is based on lesion severity. To calculate HAI, changes in each organ were classified into progressive stages of tissue damage (Table 1). HAI value was calculated for each animal by the formula: $HAI = (1X SI) + (10X SII) + (100X SIII)$, where I, II and III correspond to the number of alterations of stages 1, 2 and 3, respectively, and S represents the sum of the number of changes of a given stage. HAI indicates the conditions in which the specimen was found (Table 2). HAI values between 0 and 10 indicate normal organ function; values

between 11 and 20 indicate slight damage to the organ; between 21 and 50 indicate moderate changes in the organ; values between 50 and 100 indicate severe lesions and values above 100 indicate irreparable organ damage (Poleksic and Mitrovic-Tutundzic, 1994). Some histopathological changes were selected and photographed through a photomicroscope.

Statistical analysis was performed using non-parametric ANOVA variance analysis to compare the means of HAI at each sampling point. Then the Turkey test was used to evaluate the variations of environmental conditions throughout the study period.

Results

A total of 54 individuals of *Astyanax fasciatus* were collected. The individuals' standard length ranged from 20.7 mm to 62.3 mm and the total weight ranged from 0.25 g to 6.0 g.

Table 1: List of the histopathologic alterations observed in the gills in stages I, II and III stages of severity of the alterations (modified from Poleksic and Mitrovic-Tutundzic, 1994).

Internship	Changes in Gills
I	Hyperplasia and hypertrophy of epithelial cells Cell Aggregation Displacement of lamellar epithelium Elevation of lamellar epithelium Lamellar melt and disorganization Secondary lamellar shortening
II	Hypertrophy and hyperplasia of mucous cells Vacuolization of primary lamellae Parasitic cyst Hypertrophy and hyperplasia of chloride cells
III	Lamellar aneurysm Cellular degeneration Lamellar telangiectasis

Table 2: Histopathological changes observed in the liver of *Astyanax fasciatus* collected in the Cachoeira river hydrographic basin. I, II and III - Stages of severity of changes

Internship	Changes
I	Nucleus at the cell periphery Cellular contour deformation Nuclear Contour deformation Cellular hypertrophy Nuclear hypertrophy Cellular atrophy Nuclear atrophy Centres of melanomacrophages Cytoplasmic vacuolization
II	Nuclear vacuolization Cytoplasmic degeneration Nuclear degeneration Biliary stagnation Hyperemia
III	Focal necrosis Tumor Alteration of exocrine pancreas

Histologically the gill arcs and their lamellas had structures with the same format as in the other teleosts. Through histological analysis it was possible to verify the tissue composition of each region: the primary lamella, consisting of epithelial cells, chloride cells, endothelium, mucosa and melanocytes, which form the lamellar epithelial tissue and the secondary lamella, which had its walls covered by squamous epithelium (Fig. 2A). Histological analysis allowed to verify the presence of protozoan parasite cysts (Fig. 2B).

Mild deformations were observed in the individuals analyzed at all collection points. At point 1, no severe alteration was observed in any sample, and 100% of the individuals presented some mild alteration, such as epithelial cell hypertrophy and hyperplasia, lamellar fusion and disorganization, epithelial elevation, cell aggregation and displacement of the epithelium, and primary lamellae vacuolization. At points 2, 3 and 4, changes such as hypertrophy and hyperplasia of chloride and mucosal cells, lamellar aneurysm and lamellar telangiectasis were observed (Figs. 2C, D).

The histopathological analysis of the liver presented the same structure as that of the other teleosts (Fig. 3A), and demonstrated light alteration (Fig. 3B) such as hepatocytes with nuclear contour deformation and nuclear hypertrophy (Fig. 3C) and hepatocytes with cytoplasmic vacuolization (Fig. 3D). A greater predominance of moderate (Figs. 4E, F) and severe changes (Figs. 4G, H), such as cytoplasmic vacuolization, biliary stagnation and focal necrosis were observed at points 1 and 2. At points 3 and 4, a predominance of the mild type was observed, with cytoplasmic vacuolization being the most frequent. These were the points where the occurrence of

hyperemia was observed, a type of moderate alteration.

HAI results for gills showed that point 1 had the lowest mean (5.125), which implies that morphological changes did not compromise organ function. Points 2, 3 and 4 presented high HAI values, 84, 120.75 and 86.5, respectively. This probably indicates an occurrence of severe changes in fish at points 2 and 4, and irreparable lesions in individuals at point 3 (Fig. 5A).

The HAI analysis for liver showed that points 1 and 2 presented the highest mean values, 59.6 and 70.42, respectively. Points 3 and 4 present mean values 42,18 and 22,13, respectively. The one-way ANOVA indicated a statistically significant difference among the points collected and the HAI for gills [$F(3,50) = 5,205$; $p < 0.05$]. The Tukey Post-Hoc test indicated a difference between the HAI at point 1 and HAI at points 3 and 4. Regarding the results of the liver analysis, one-way ANOVA indicated that there was no statistically significant difference between the changes in the different points analyzed [$F(3, 27) = 1.413$; $p = 0.2606$] (Fig. 5B). The Student's t test showed that, when compared, the means of the alterations of the two organs in the different points did not show statistical difference [$t(3) = 0.8423$, $p > 0.05$].

Discussion

The use of fish in environmental monitoring studies has several advantages, as they are directly related with the sediment and water column, have poor mobility and exhibit some physiological mechanisms of biological responses (Jenkins, 2004). According to Schmidt *et al.* (1999), the set of effluents and discharge consists of a complex mixture of toxic elements that significantly increase the

Fig. 2: Histological sections of *Astyanax fasciatus* gills. (A)- Normal Gill, HE. (B to D) - Gills with histopathological changes (Arrows: 1 - Primary filament, 2 - Secondary filament, 3 - Protozoan cyst, 4 - Lamellar disorganization, 5 - Hypertrophy and epithelial cell hyperplasia, 6 - Cell degeneration traits). HE.

Fig. 3: Liver cells of the *Astyanax fasciatus* with normal structure, HE. (A) Normal, (B) Epithelial parenchyma with slight alteration. Cytoplasmic and normal exocrine pancreas. (C) Hepatocytes with nuclear contour deformation and nuclear hypertrophy (arrows), HE. (D) Hepatocytes with cytoplasmic vacuolization. P - Exocrine pancreas. HE.

Fig. 4: Histological slides of *Astyanax fasciatus* liver. Observation of the moderate stage (E – F). Severe stage (G – H). (E) – Nuclear vacuolation, HE. (F) – Bile stagnation, HE. (G) – Alteration of the exocrine pancreas, HE. (H) – Focal necrosis, HE.

Fig. 5: Result of HAI calculation for histopathological changes in the gills and liver. A) Changes in gills at collection points. B) Changes in the liver at the collection points. Data are reported as mean $\pm$ SEM. # - indicates $p < 0.05$ with respect to point 1.

impact on aquatic ecosystems, providing an unfavorable condition for the survival and maintenance of species diversity.

In this study various significant results were observed, that demonstrated the occurrence of a high number of histopathological changes present in the gills and liver of *A. fasciatus* species and the implication that these pathological changes are probably caused by changes in the environment quality. As a result of direct contact with water, histological analysis showed that the gills represent an important organ used as a biomarker and allow the evaluation of the pollutant action dissolved in water through changes that these substances cause in the tissue. Bassey (2019) stated that histology is a very useful technique towards the assessment of toxic substances in aquatic organisms from polluted ecosystems.

A greater presence of stressors was observed, compromising the survival of the several existing species, since the highest degree of lesion severity in the gills was verified in this place. In all the sampling points, it was observed that the individuals presented morphological changes in the gills, which caused the occurrence of deformity in the epithelial structure (elevation and displacement of the epithelium), hypertrophy and hyperplasia of the secretory cells and secondary lamella fusion. Several studies carried out by Silva *et al.* (2004), Camargo and Martinez (2007), Santos (2010) and Paulo *et al.* (2012) reported the occurrence of several types of alterations used in histological evaluation studies as a parameter for environmental monitoring.

The analysis demonstrated the deterioration of the Cachoeira river basin in

recent years, mainly due to pollution from the human population, industries and agriculture (Pinho, 2001). In the analysis, the parameters demonstrated that the phosphorus concentration was 25 times higher than that quoted in the CONAMA 357 resolution for class 2 rivers (Brazil, 2005) and that the biological oxygen demand (BOD) is also below the CONAMA, suggesting an increase in the organic load.

Klump *et al.* (2002) found similar results using eutrophication indicators, noting that the concentration of metals (aluminum, copper) increased along the river course, a phenomenon mainly related to the agricultural practices used in the cocoa plantations. When studying water quality, authors like Paulo *et al.* (2020) and Flores-Lopes *et al.* (2020) observed a high concentration of metals in several organs. Histological analysis allowed the observation of different types of changes. Local fauna also presented environmental degradation marks when evaluating histopathological changes in the liver of *Poecilia viviparous* (Paulo *et al.*, 2012). The results noticed in this study are similar to those observed in other studies and show that little has been done to improve the quality of the environment.

These modifications were considered an adaptive strategies of fish to the exposure of the toxic agents present in the aquatic environment. The elevation of the epithelium was observed as one of the first defense adaptations, reflecting the increase in the diffusion distance between the water and the blood, damaging the consumption of oxygen (Camargo and Martinez, 2007). As gills are constantly exchanging elements with the environment, any elevation or reduction of this exchange can lead to respiratory

disturbances and electrolyte imbalance (Cerqueira and Fernandes, 2002). The chloride cell hypertrophy observed is probably a physiological compensation of the fish due to an ionic imbalance caused by the increase in ion concentration in the river (Santos, 2008).

According to Silva *et al.* (2004), hepatocyte atrophy or hypertrophy is an organism's compensatory response, making the hepatocytes hypo- or hyperfunctional to maintain homeostasis, even under the action of cellular insults. The observed hyperemia is a compensation of the organism in the attempt to increase blood flow to the liver parenchyma to increase tissue oxygenation, transport macrophages to injured regions or accelerate the detoxification process and contour deformation is a dysfunction caused by toxic agents that affect the metabolic functions of the cell and can compromise the entire organ (Silva *et al.*, 2004).

The presence of lamellar aneurysms in most of the collected specimen was observed by Flores-Lopes *et al.* (2001) and Devi and Mishra (2013). Heath (1995) demonstrated that this type of lesion causes rupture of the pillar cells and, consequently, disarrangement of the lamellar structure, besides being considered an irreversible lesion. This change was observed closer to the urban zone; it is likely that as the course of the river approaches cities, human aggression to the ecosystem increases. It was verified that the recovery of individuals who presented moderate injuries was still possible, as long as the stressor's action was ended (Flores-Lopes *et al.*, 2011; Devi and Mishra, 2013). Takashima and Hibiya (1995) pointed out that some of the more severe lesions that are

related to blood supply accumulate at the extremities of the lamellae that could rupture.

Devi and Mishra (2013) verified the presence of lamellar telangiectasis, a type of lesion similar to the aneurysm, but of a lower degree and without cellular disruption and cellular necrosis/degeneration. Mallat (1985) observed a consequence of chronic exposure to toxic agents such as heavy metals. Thomas (1990) and The *et al.* (1997) demonstrated that changes as hepatocyte vacuolization, glycogen depletion, inflammation, alteration in the shape of sinusoid vessels and neoplasias can be interpreted as responses to environmental stress, being considered good histopathological indicators of environmental quality. Among the pathologies observed, cytoplasmic vacuolization, present in almost all points, was the most frequent.

Dyk *et al.* (2007) observed that the cytoplasmic vacuolization is caused by the accumulation of heavy metals or due to the accumulation of organochlorine pesticides. Its effects are the inhibition of proteins, which when affected, compromise the metabolic pathway of energy reserves that accumulate within the cytoplasm, as there is also a disruption of the microtubules which modifies the use of the cellular substrate. Silva *et al.* (2004) observed that modifications such as nuclear contour deformation and nuclear vacuolization can be interpreted as nonspecific response to pesticides.

Santos (2010) and Devi and Mishra (2013) have suggested that the occurrence of several types of histopathological changes are a biological response to pesticides released in rivers and focal necrosis are partial death of the tissue due to the action of a stressor agent. Santos (2010) suggests that this partial death

would be due to the inability to metabolize contamination of the aquatic environment by domestic sewage and/or pesticides.

Flores-Lopes and Malabarba (2007) observed that Melanomacrophage Centers (MMCs) are a type of alteration characterized by its diminutive size close to large blood vessels. This type of change may be related to a response of the humoral immune system to a chemical agent that causes inflammation, especially when associated with hyperemia. According to Silva *et al.* (2004), the nucleus in the periphery of the cell, the cytoplasm and nucleus degeneration are nonspecific biological responses to environmental stressors of both organic and inorganic nature. According to Flores-Lopes *et al.* (2019), histological analysis has revealed that such changes usually occur along with other modifications, such as rectilineation of sinusoidal vessels and cells with cytoplasmic degeneration.

The results obtained in this study demonstrated a constant presence of lymphocytes, macrophages and congestion of sinusoidal vessels. In addition, different types of histopathological changes observed showed a degradation of the structure of the individuals of *A. fasciatus* along the river course. It was possible to verify that the alterations were being caused by several substances present in the water, normally polluting, which caused a change in the quality of the environments. Severe lesions were more frequent in the gills, while mild to moderate changes were more constant, demonstrating that the water quality of the environments is influencing this organ more.

In some specimens, the presence of parasites involved in a cyst coated with

epithelial tissue was observed. This is probably due to the increase of the organic load in the river, favoring the proliferation of microorganisms. Flores-Lopes *et al.* (2001) verified the existence of a type of parasitism in the fish musculature, mainly of the species *Astyanax alburnus*, that caused several lesions. According to Flores-Lopes and Thomaz (2011) and Flores-Lopes (2014) the changes caused by the presence of these parasites in the gills of *A. fasciatus* were called black spot diseases and it is similar to the histological result observed in the present study. These authors suggested that the high frequency of the parasite causing the black spot disease can indicate less degraded environments, and as such, it potentially indicates better environmental quality. Histological analysis performed by Flores-Lopes *et al.* (2002) observe a high intensity of digenetic trematodes, probably belonging to the order Strigeatoidea, in individuals with kypholordosis, with more than half of these organisms located in the spine.

Conclusion

The results obtained showed that the Cachoeira river is undergoing a process of environmental degradation, due to the action of several stressors. The contact of these substances with the different organs of *A. fasciatus* has caused the occurrence of different modifications. Several types of histopathological changes serve as a warning for the increase in environmental degradation. Thus, it indicates the need for further studies to provide data on the action of various types of stressors on species and environments.

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