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ZOOLOGY

Histopathological analysis of liver of the catfish *Pimelodus maculatus* in a tropical eutrophic reservoir from Southeastern Brazil

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ABSTRACT. We described liver tissues of the long-whiskered catfish *Pimelodus maculatus* using histological techniques through structures stained with hematoxylin and eosin. A total of 21 adult individuals were collected using gill nets during the winter of 2012, in a eutrophic reservoir in Southeastern Brazil. The main aim was to characterize the tissues and eventual changes, since this organ has a notable sensitivity to changes in altered aquatic systems. The liver contains the exocrine pancreas (hepatopancreas) that have pancreatic cells arranged around a branch of the portal vein. The hepatocytes are diffuse and arranged in cords**.** Normal liver of *P. maculatus* have a continuous compact field of weakly eosinophilic, rounded hepatocytes with scattered islands of connective tissue that enclose the bile ducts and blood vessels. The organization of exocrine pancreatic tissues is similar to the acinar morphology of many teleosts. Histopathological alterations in liver were evaluated semi-quantitatively and based on the severity of the lesions. The alterations in each organ were classified in progressive stages of damage to the tissue. Six types of liver alterations were found: 1) cytoplasmic vacuolization; 2) congestion; 3) inflammatory infiltrated; 4) infiltration of adipocytes; 5) steatosis; 6) granuloma. Incidence of melano-macrophage centers were observed in the liver. However, we cannot directly associate such changes with the eutrophic conditions of the reservoir, because we have no available reference area to compare. These findings are a baseline contribution, which enables comparisons with similar fish species in other tropical aquatic systems in further studies.

Keywords: liver; freshwater fish; hepatic alterations; reservoirs.

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Introduction

The liver is one of the most important organs of fish, because it plays a key role in the metabolic and biochemical processes associated with feeding ingestion or dealing with pollutants in the waters (Liebel, Tomotake, & Oliveira-Ribeiro, 2013; Ostaszewska, Chojnacki, Kamaszewski, & Sawosz-Chwalibóg, 2016). Several functions are attributed to the liver, such as the metabolism of proteins, lipids and carbohydrates, the storage and distribution of compound reserves, besides its fundamental role in xenobiotic biotransformation, detoxification and excretion (Brito et al., 2012). The liver is notable for its sensitivity to a great variety of environmental factors (Chavan, & Muley, 2014), being used for assessing fish diseases or as biomarker of aquatic contaminants (Liebel et al., 2013; Passantino et al., 2014). As widely accept in studies of biomarkers of environmental condition, organs such as liver and gills are the main target used to evaluate water quality, because their histopathological alteration can be associated to pollutants or other sources of water alterations (Marcon et al., 2016).

The growth in anthropogenic activities associated to industry and agriculture has increased pollution of aquatic ecosystems. This issue has raised concerns worldwide over the last decades. As fish are continuously exposed to chemicals in polluted waters, they could be used as biological markers of pollutants in aquatic systems (Milošković & Simić, 2015). In this sense, eutrophication is a potential environmental stressor, which alters the fundamental ecological processes that structure freshwater communities (Jacobson, Hansen, Bethke, & Cross, 2017). Disentangling the effects of these multiple stressors is required in order to fully understand the consequences eutrophication on freshwater ecosystems (Mantyka-Pringle, Martin, Moffatt, Linke, & Rhodes, 2014; Jacobson et al., 2017).

Histological studies are important for describing and comparing tissues in key organs, especially in those associated with changes in environmental conditions, such as the liver. The liver is regarded as the central organ of xenobiotic metabolism in fish, and alterations in this organ may be useful as biomarkers that indicate exposure to environmental stressors (Van Dyk, Cochrane, & Wagenaar, 2012; Dane & Sisman, 2017). Liver alterations are very useful tool to evaluate different levels of pollution, particularly those related to sub-lethal and chronic effects. Such alterations provide a rapid method for detecting effects of contaminants because the liver is a key organ for the initial detection of environmental changes (Brito et al., 2012; Paulino et al., 2014). In this sense, the melano-macrophage centers (MMC) are useful health biomarkers since they play an important role in defense and inflammatory responses, and they are responsible for the removal of foreign particles, dead cells and products of phagocytosis (Leknes, 2001; Steinel & Bolnick, 2017).

The first step to detect histological anomalies in the liver is the knowledge of the normal structure (without impairment), and the studies describing both normal and altered organs in order to assess the level of deleterious influence of the system on the individuals. Several anatomical and histochemical studies have been performed on livers of freshwater species from temperate areas. Among the species that have been studied in this subject, we can highlight the histology of the liver of the channel catfish *Ictalurus punctatus* (Rafinesque, 1818), the tigerfish *Hydrocynus forskahlii* (Cuvier, 1819), the trout *Oncorhynchus mykiss* (Walbaum, 1792), the goldfish *Carassius auratus* (Linnaeus 1758) and the Siberian sturgeon *Acipenser baerii* (Brandt, 1869) (Ostaszewska et al., 2016). However, information on liver tissues in tropical freshwater systems are scarce (e.g., Bombonato, Rochel, Vicentini, & Vicentini, 2007; Faccioli, Chedid, Bombonato, Vicentini, & Vicentini, 2014; Marcon, Bazzoli, Mounteer, & Benjamin, 2015). As most of Brazilian freshwater systems are undergoing increasing pollution, especially eutrophication, there is a need for studies on this subject on eutrophic systems.

The Funil Reservoir blocks the middle reaches of the Paraíba do Sul River, one of the most used riverine systems in Brazil that is used for many purposes, among them, hydro-power generation, flow regulation and water supply. The Paraíba do Sul River (PSR) basin drains a major industrialized area in Brazil and suffers several impacts along its extension, with the heaviest alteration occurring in the middle reaches, where generalized pollution from organic and industrial effluents are carried into the river, therefore, decreasing water quality due to poorly planned sewage treatment of the nearby municipalities (Linde-Arias, Inácio, Novo, Albuquerque, & Moreira, 2008b). This reservoir receives waste disposal of many industries (metallurgical, steel, paper, rubber and chemical), agricultural activities and domestic sewage discharges (Linde-Arias, Inácio, De Albuquerque, Freire, & Moreira, 2008a). According to Branco, Rocha, Pinto, Gomara, and Filippo (2002), Funil Reservoir is increasingly developing eutrophic conditions due to anthropogenic influences. The area has gone through an accelerated increase in industrial activity and urbanization, with intensification of environmental problems (Lima, Ribeiro, Barbosa, & Rotunno-Filho, 2016). As about 80% of the sewage is untreated (Teixeira & Oliveira, 2014), the PSR receives a large portion of the sewage that exerts a large influence on river water quality. As a result, the Funil Reservoir has experienced large eutrophication in recent decades, resulting in frequent and intense cyanobacterial blooms (Branco et al., 2002; Rocha, Branco, Sampaio, Gomara, & Filippo, 2002; Rangel, Silva, Rosa, Roland, & Huszar, 2012; Pacheco et al., 2017). Despite this situation, the reservoir supports a rich fish community (Duarte, Bemquerer, & Araújo, 2015). Among the fish species, the long-whiskered catfish *Pimelodus maculatus* Lacepède, 1803, is one of the most abundant and widely distributed fish that are found not only in this reservoir, but also through the entire Paraiba do Sul River extent (Araújo, Pinto, & Teixeira, 2009). This species has been reported as an excellent sentinel species in biomonitoring programs, because it is widely distributed through all river basin, and due to its benthic behavior, it reflects better the pollutants that accumulate into the substratum.

This study aimed to characterize the histological structures of the liver of the long-whiskered catfish *P. maculatus* an abundant and widely distributed fish species in rivers and reservoirs of the Neotropical Region.

We also try to find changes in these tissues that could serve as a baseline contribution, which enables comparisons with similar fish species in other tropical aquatic systems in further studies.

Material and methods

Study area

The Funil Reservoir (22°30′S, 44°45′W, altitude 440 m) is located in the middle reaches of the Paraíba do Sul River basin, in southeastern Brazil. The reservoir has a surface of 40 km^2, mean depth of 22 m, maximum depth of 70 m, and total volume of 890 × 106 m^3. It was built in 1969 and it is used for domestic and public drinking water source, irrigation, industrial self-supply systems, aquaculture and hydroelectric power generation. Hydraulic residence time varies between 10 and 50 days, dictated by seasonal variation in precipitation. There is little vegetation cover around the reservoir because of previous agricultural use for coffee plantation and pasture. Reforestation programs have been implemented by the power generation company that uses the reservoir waters and by other industries in the adjacent areas.

Fish handling procedure

Twenty-one specimens of *P. maculatus* were collected using gill nets in the 2012 winter. Their average size and body weight ranged from 11.2 to 29.5 cm of total length (TL) and 40.3 to 345.9 g of body weight (BW), respectively. Immediately after collection, all fish were anaesthetized in benzocaine hydrochloride (50 mg L^{-1}), killed by immersion in water at 4°C, and dissected. The euthanasia procedure is in accordance with the ethical principles of animal handling, established by the Brazilian College of Animal Experimentation, considering the ethics, well-being and regulations regarding animal use in research. The livers were removed and a portion of each lobe was fixed in Bouin's solution during 8 hours, being subsequently transferred to 70% ethanol. Afterwards, they were dehydrated and embedded in paraffin wax. Cross-sections, of 4–6 μm thick, were made in a rotary microtome (Leica RM 2135, Wetzlar, Germany), stained with hematoxylin and eosin, and mounted on glass slides for light microscopy scrutiny. The microphotography was taken with a Nikon Coolpix 4300 digital camera coupled to an Olympus (Tokyo, Japan) B941 microscope.

Histopathological procedures

The histological changes in the liver tissues were classified according to the system proposed by Haschey and Rousseaux (1996). From each fish, 10 sections of each tissue were examined by light microscope, and 20 fields per section of each tissue were observed. The presence of histological alterations for each organ was assessed semi quantitatively by the degree of tissue change (DTC), which is based on the severity of the lesions. The alterations were classified in progressive stages of damage to the tissue: stage I alterations, which do not alter the normal functioning of the tissue; stage II, more severe alteration that impairs the normal functioning of the tissue; and stage III, alterations that are very severe and cause irreparable damage. A value of DTC was calculated for each individual following the formula: $DTC = (1 \times SI) + (10 \times SII) + (100 \times SIII)$, where I, II, and III correspond to the number of alterations of stages I, II, and III, respectively, and S represents the sum of alterations in a given stage. The DTC value obtained was used to calculate the average index. DTC values ranging between 0 and 10 indicate the normal functioning of the organ; values between 11 and 20 indicate slight alterations, values between 21 and 50 indicate moderate changes in the organ, whereas values between 51 and 100 indicate severe lesions, and values above 100 indicate irreversible damage to the organ (Poleksic & Mitrovic-Tutundzic, 1994).

Results

Twenty-one individuals of *P. maculatus* were used, and among them, 13 presented alterations in the liver. The classification of the severity of histopathological changes observed were: Stage I: cytoplasmic vacuolation, inflammatory infiltrate, infiltration of adipocytes; Stage II: congestion and steatosis; Stage III: granuloma.

Six types of liver alterations were found: 1) cytoplasmic vacuolization; 2) congestion; 3) inflammatory infiltrated; 4) adipocytes infiltration; 5) steatosis; 6) cytoplasmic granules. Normal hepatic parenchyma of fish is shown in Figure 1 (a,c,e). Figure 2a exhibits well-defined hepatocytes, with polyhedral shape. The main alterations found in the liver were cytoplasmic vacuolization (Figure 1b), congestion (Figure 1d and Figure 2b), inflammatory infiltrated (Figure 1f, g, and Figure 2b), infiltration of adipocytes in the pancreas (Figure 2d, f), steatosis (Figure 1d, g), and granuloma (Figure 1b). The majority of the alterations found in the liver were stages I and II, i.e., the tissue varied from slightly to moderately damaged.

Overall, the histopathological changes found in the liver included irregular arrangements of hepatocytes. The hepatic steatosis was characterized by the presence of clear, rounded cytoplasmic vacuoles, with hepatocytes of increased volume and dislocated nuclei. Several MMC of different sizes containing pigments such as melanin and hemosiderin were observed associated to perivascular inflammatory infiltrate. Diffuse hepatodistrophy characterized mainly by congestion, cytoplasmic vacuolization and granulomatous process (Figure 2d) were observed. We also observed intrahepatic segments surrounded by adipocytes infiltration, and pancreocytes with vacuoles in their interior.

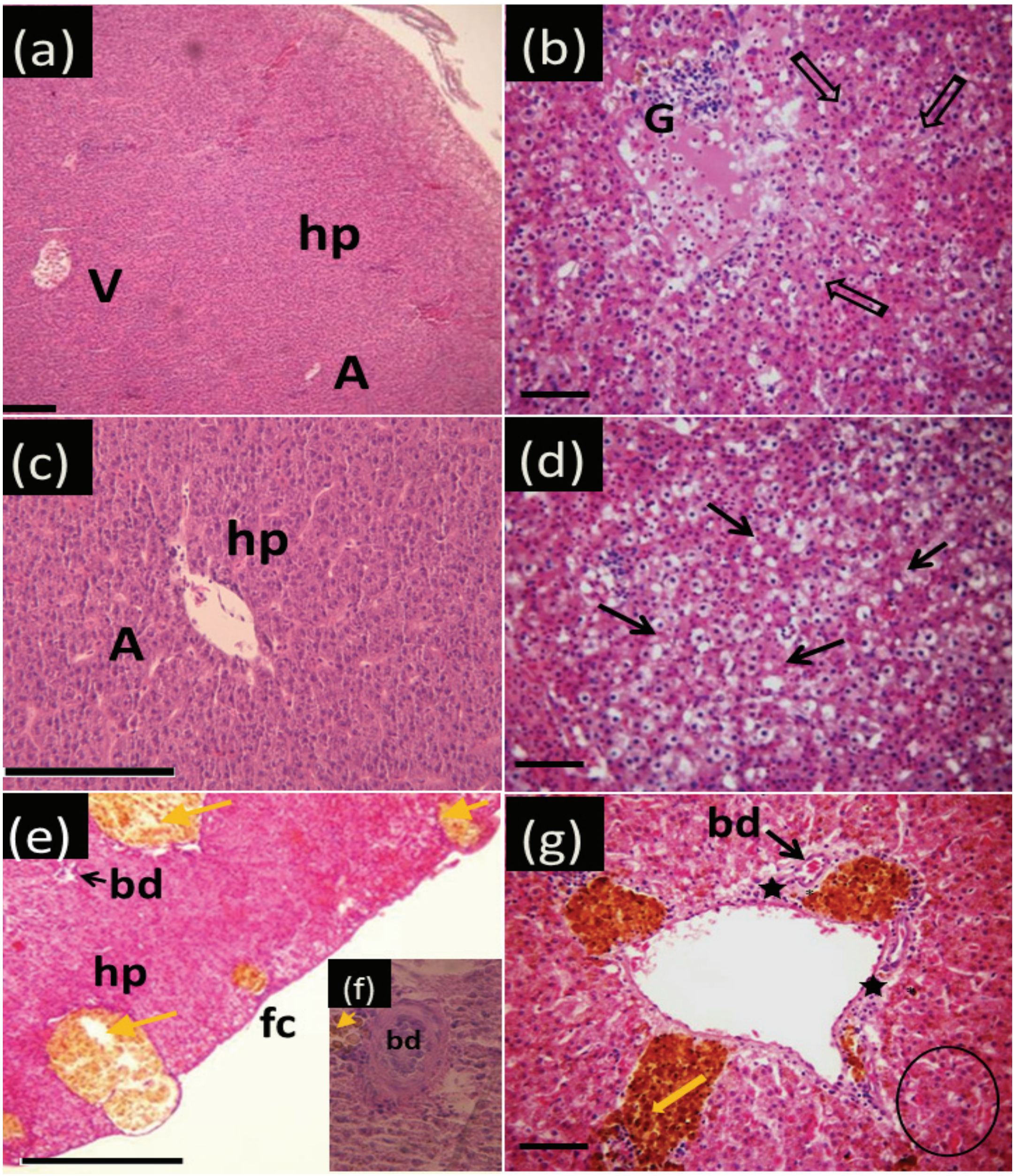

Figure 1. Photomicrographs of the liver tissue in normal (a, c, and e) and altered (b, d, f and g) conditions of *Pimelodus maculatus*. (a) Portal portion in the hepatic parenchyma (hp), V= hepatic vein, A= hepatic artery. (b) Granulomatous process, hepatocytes filled with cytoplasm of granulation (G), Cytoplasmic vacuolation (empty arrow). (c) Hepatic artery (A) between the anastomosing cords of hepatocytes (hp = hepatic parenchyma). (d) Steatosis (arrow). (e) Portal portion and periphery of the hepatic parenchyma (hp) with melano-macrophages centers (yellow arrows), bd = bile duct, fc = fibro connective. (f) Melano-macrophages centers (yellow arrow) in the region of the bile duct (bd). (g) Melano-macrophages centers (yellow arrow) associated to periportal iinflammatory infiltrate (star), dilation and congestion of sinusoid (circle). Hematoxylin and eosin (H&E) stain, bar = 50 µm.

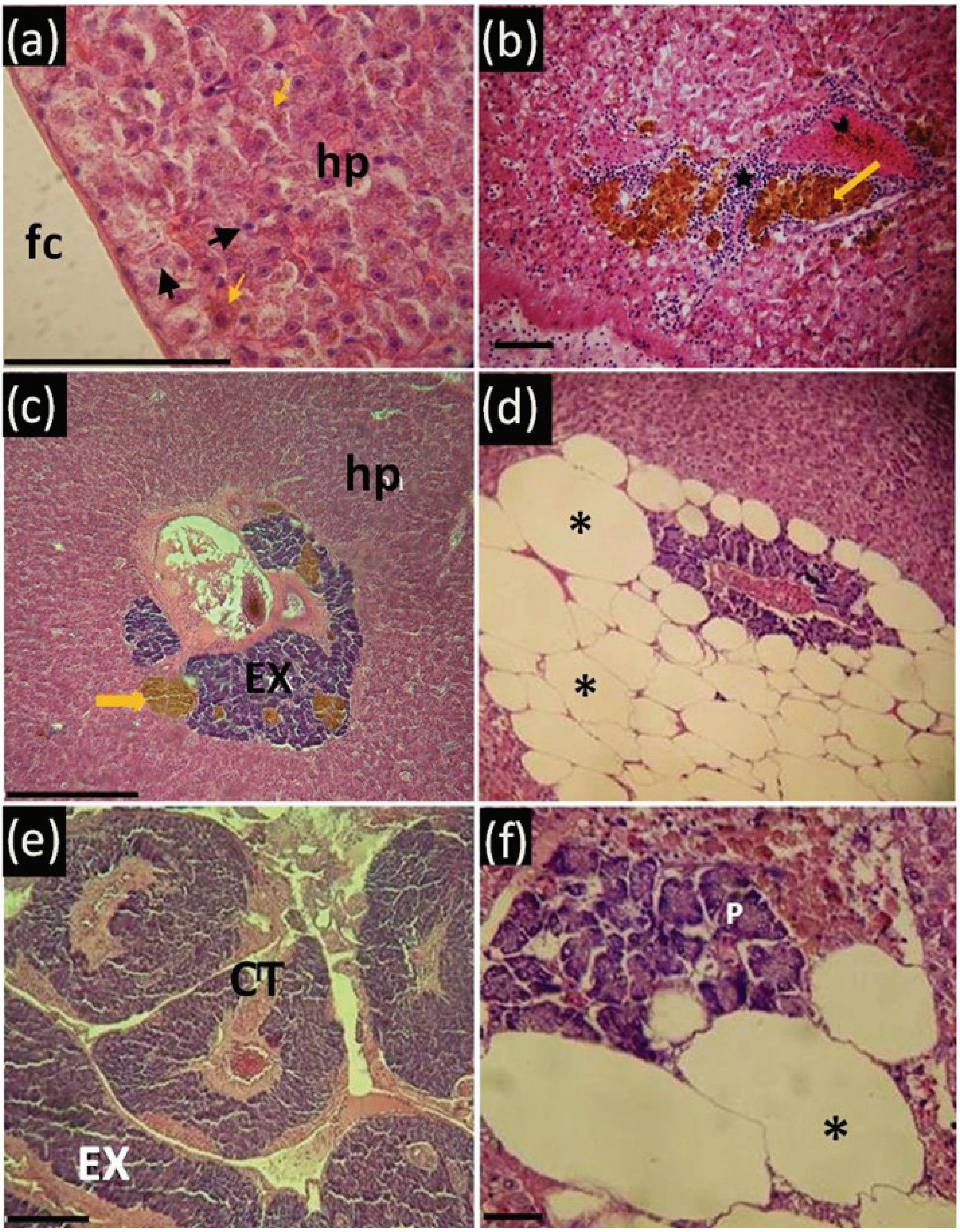

Figure 2. Photomicrographs of the liver tissue and pancreas tissue in normal (a, c, and e) and altered (b, d and f) conditions of *Pimelodus maculatus*. (a), (b) Liver tissue: (a) Edge of the hepatic parenchyma (hp), fibro connective (fc) delimiting the cords of hepatocytes; melano-macrophages centers (yellow arrow), (b) Congestion (arrowhead) and melano-macrophages centers (yellow arrow) associated to perivascular inflammatory infiltrate (star). (c, d, e, f) Pancreas tissue: (c) Intra-hepatic (hp= hepatic parenchyma, EX= exocrine portion, yellow arrow = melano-macrophages centers), (d) Intrahepatic segment surrounded by infiltration adipocytes (asterisk), (e) Exocrine portion (EX), CT= conjunctive tissue), (f) Intrahepatic segment with infiltration adipocytes (asterisk) and pancreocytes (P) with vacuoles in their interior. Hematoxylin and eosin (H&E) stain, bar = 50 μm.

Discussion

Histological analysis of *P. maculatus* liver parenchyma revealed that this species has the general pattern of teleosts, with a thin connective tissue capsule and a simple columnar epithelium. It was not always possible to distinguish the hexagonal subdivisions, which are characteristics of the hepatic lobules. However, we observed a radial arrangement of hepatocyte cords, a classic organization of liver lobules.

The liver of *P. maculatus* have a continuous compact field of weakly eosinophilic, rounded hepatocytes with scattered islands of connective tissue that enclose the bile ducts and blood vessels. These findings differed from those of Bodammer and Murchelano (1990) for *Pseudopleuronectes americanus* (Walbaum,

1792) and of Pack et al. (1996) for *Brachydanio rerio* (Hamilton, 1822), that reported a continuous compact field of the eosinophilic polygonal hepatocytes.

In our investigation, the hepatopancreas was found for *P. maculatus*, being distinguished from the hepatic parenchyma by a thin layer of connective tissue. However, pancreatic tissue inside the liver is not present in all kinds of fish (Petcoff, Diaz, Escalante, & Goldenberg, 2006; El-Bakary & El-Gammal, 2010). The exocrine pancreatic tissue is formed around the portal vein, and during ontogenesis it penetrates deeply into the liver parenchyma (Vicentini et al., 2005), while its proportion depends on the species of fish (Gonzalez, Crespo, & Brusle, 1993). Both diffuse and compact pancreas have been reported elsewhere for different fish species, as the cases of *Chalcalburnus tarichi* (Güldenstädt, 1814) (Ünal, Çetinkaya, Kankaya, & Elp, 2001) and *Ctenopharyngodon idella* (Valenciennes, 1844) (Mokhtar, 2015). Moreover, Eastman and Devries (1997) stated that the notothenioid fish lack intrahepatic pancreas.

Melano-macrophage centers are distinctive groupings of pigment-containing cells normally located in the stroma of the haemopoietic tissue of the kidney (Agius & Roberts, 2003; Steinel & Bolnick, 2017). In some fish, they are also found in the liver (Passantino et al., 2014; Sayed & Younes, 2017), as the case of *P. maculatus*. They may also develop in association with chronic inflammatory lesions elsewhere in the body and during ovarian atresia. In higher teleosts, they often exist as complex discrete centers, containing lymphocytes and macrophages, and may be primitive analogues of the germinal centers of lymph nodes. The melano-macrophage centers (MMCs) or macrophage aggregates enclosed the absorbing region (artery/vein) and excretion (bile region) of the liver, and it is associated with metabolism of toxic compounds (Diaz, Garcia, & Goldemberg, 2005; Passantino et al., 2014; Steinel & Bolnick, 2017). In the present study, we found MMCs in both absorbing and excretion regions of the liver. According to Agius and Roberts (2003), MMCs play an important role in the sequestration of the products of cellular degradation and in potentially toxic tissue materials, such as free radicals and catabolic breakdown products. High occurrence of MMCs in the liver of *P. maculatus* was related to the toxicity at the more contaminated sites in a tropical reservoir (Paulino et al., 2014). Furthermore, large liver areas exhibiting disorganization of the tubular cord-like structure, large melanomacrophage aggregates close to the blood vessels, and sinusoid dilatation are considered as indicators of severe alteration in the environment (Brito et al., 2012; Sayed & Younes, 2017).

Liver is the main organ of metabolism that comes into contact with xenobiotics absorbed from the aquatic environment, also, liver lesions are often associated with exposure to aquatic pollutants (Fernandes, Fontainhas-Fernandes, Rocha, & Salgado 2008; Sayed & Younes, 2017). Morphological modifications, such as the vacuolization of the hepatocytes, glycogen depletion, inflammations, changes in the shape of the sinusoid vessels, and neoplasms may be interpreted as a response to environmental stress or a pathological process caused by infections or parasites (Köhler, Deisemann, & Lauritzen, 1992; Teh, Adams, & Hinton, 1997). Cytoplasmic vacuolization was the most frequent hepatocyte change and it can be a signal of degenerative processes involving metabolic damage (Pacheco & Santos, 2002). Inflammatory responses have been associated to leukocyte infiltration activation and clustering (Brito et al., 2012). Histopathological analysis of liver fragments examined showed some types of changes. *Pimelodus maculatus* showed steatosis and vacuolization, indicating a mobilization of glycogen and fat. This type of lipid deposition is a pathological process that can be used as an indicator of hepatic disturbances in the fat metabolism (Ghittino, 1978; Ferramosca & Zara, 2014). Spisni, Tugnoli, Ponticelli, Mordenti, & Tomasi (1998) described steatosis as a liver alteration due to an excessive dietary intake of lipid, which saturates the physiological capability of the liver. Similar hepatic lipid deposition due to the excessive caloric intake has been described for other vertebrates such as poultry, which indicates a pathological situation (Stake, Fredrickson, & Bourdeau, 1980).

Steatosis and congestion found in this species may be related to poisoning processes that are occurring in the Funil Reservoir. According to Cotran et al. (2005), the extent and severity of damage depends on the type, time, severity of aggression and physiological state of cell involved. Congestive hepatopathy is a liver dysfunction due to venous congestion, usually as a result of dysfunction of the heart, which is also known as congestive heart failure. The fish liver is especially liable to chemical products thanks to the slow blood flow in relation to the cardiac output. Campos, Moraes, and Moraes (2008) observed steatosis in fish maintained in contaminated environments, although Santos, Ranzani-Paiva, Felizardo, and Rodrigues (2004), and Camargo & Martinez (2007) found no lesions whatsoever, even in polluted environments. Therefore, it is very difficult to establish relations among this condition and toxic substances in water.

The hepatocytes have only small lipids droplets that tend to increase in diseased specimens due to adipocytes infiltration, and this change was common in *P. maculatus*, that had large and numerous lipids droplets. These changes are common in the carnivorous species such as *Dissostichus mawsoni* Norman, 1937 (Eastman & DeVries, 1981), *Salmo truta* Linnaeus, 1758 (Rocha, Monteiro, & Pereira, 1994), and *Anguilla anguilla* (Linnaeus, 1758) (Rodríguez, Gisbert, Rodríguez, & Castelló-Orvay, 2005). Conversely, a few and small lipid droplets are found in herbivorous species such as *Labeo niloticus* Linnaeus, 1758 (Weis, 1972), and *B. rerio* and *Gambusia affinis* (Baird & Girard, 1853) (Giari, Simoni, Manera, & Dezfuli, 2008).

These histopathological hepatic observations suggest that *P. maculatus* are suffering from exposure to sub lethal concentrations of many pollutants discharged into Funil Reservoir. This reservoir has poor water quality and it receives large amounts of phosphorus during the inflow increases of the Paraiba do Sul River. Chlorophyll (mean = 9.23 $\mu g\ L^{-1} \pm 18.6$ sd), pH (7.1 ± 0.8) and total phosphorus (0.07 $mg\ L^{-1} \pm 0.06$), in several cases, were recorded above the recommended Brazilian guidelines in this reservoir (Branco et al., 2002). Liver alterations, such as those observed in this study, could result in severe physiological problems and provide reliable information on stress to a broad range of environmental pollutants. However, we cannot associate such changes with the eutrophic conditions of the reservoir, because we have no available reference areas to compare. These findings are baseline contributions that enable comparisons with similar species in other tropical aquatic systems in further studies.

Conclusion

The liver has a parenchyma, which is divided into irregular lobules that contain the exocrine pancreas (hepatopancreas), with pancreatic cells arranged around a branch of the portal vein. The hepatocytes are diffuse and arranged in cords. The organization of exocrine pancreatic tissues is similar to the acinar morphology of many teleosts. Six types of liver alterations were found: 1) cytoplasmic vacuolization; 2) congestion; 3) inflammatory infiltrated; 4) infiltration of adipocytes; 5) steatosis; 6) cytoplasmic granules. The majority of the alterations found in the liver were stages I and II, i.e., the tissue varied between slightly to moderately damaged. The incidence of melano-macrophage centers was also observed, suggesting an immune response to contaminants. However, we cannot associate directly such changes with the eutrophic conditions of the reservoir, because we have no available reference area to compare. These findings are a baseline contribution that enables comparisons with similar fish species in other tropical aquatic systems in further studies.

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