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Using Multibiomarker Approach as a Tool to Improve the Management Plan for a Private Reserve of Natural Heritage (RPPN)

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Abstract This study aimed to monitor an aquatic ecosystem during two different periods (dry and rainy season) on a protected area located inside a sugarcane farm, using the fish *Astyanax* sp. as bioindicator. An integrated approach was adopted by combining the responses of well-known biomarkers: acetylcholinesterase, lipid peroxidation (LPO), catalase (CAT), glutathione S-transferase (GST), micronucleus test, and liver histopathology. The activity of enzymes CAT and GST was increased after the rainy season. This can be explained mainly by the intensification of rain density, which drags substances into the streams, especially pesticides applied on agriculture. LPO and micronucleus test also suggested some effects of contamination in the surrounding area during this season. The results have supported a discussion about the effectiveness of protected areas in agricultural regions, emphasizing the biomonitoring as a tool for improving management plans in protected areas.

Keywords Biomonitoring · Protected areas · Freshwater fish · Biochemical biomarkers · Histopathology

Private Reserve of Natural Heritage (RPPN) is an important Brazilian category of protected area. They are private areas of relevant ecological interest, which are voluntarily demarcated by the owner, aiming to protect biodiversity; it is allowed to use indirectly its natural resources. Brazil has 1,073 RPPN, protecting over 699,000 ha around the country; they are used as an additional tool to strengthen the system of protected areas and to extend ecological corridors. From all RPPN presented in Brazil, 734 of them protect the Atlantic Forest Biome, where this study was focused.

Agricultural activities are carried out in the surroundings of the RPPN, especially in the South and Southeast of Brazil. The aquatic environment in agricultural complexes can be directly affected by pesticides and some of them recommended for sugarcane production are periodically employed, especially herbicides such as glyphosate and diuron (Armas et al. 2005). The glyphosate (*N*-(fosfonometyl) glicin) and diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea) were the pesticides applied at the study region as informed by Farm Barbacena managers.

Barbacena Farm Reserve is a RPPN with 555 ha area, located in southern Brazil. It is a seasonal semi deciduous forest (Atlantic Forest Biome) and some of its vegetal and animal species are considered rare or even endangered. It is placed on a predominantly agricultural region with a very fertile soil; therefore, this RPPN can be affected by the activities developed on its buffer zone. In this way,

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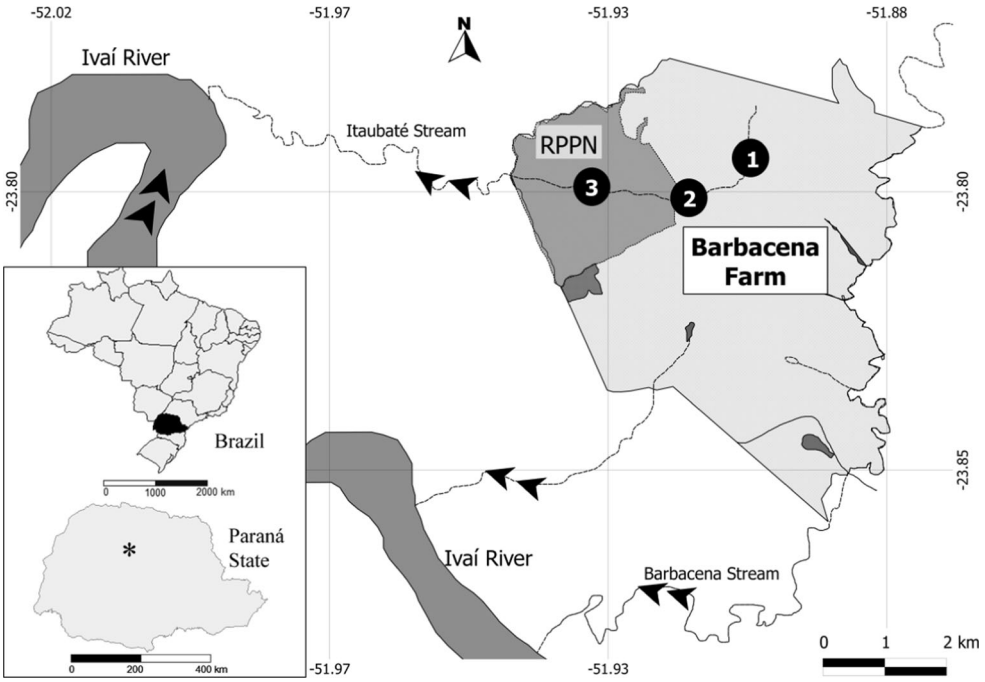

Fig. 1 RPPN Barbacena Farm (*asterisk*) in Paraná State, Brazil. Sampled sites: (*1*) Sugarcane; (*2*) Surrounding area; (*3*) Inner RPPN site. *Black arrow* head indicate the river flow

biomonitoring studies can be useful as a tool for improving management plans for RPPN.

The aim of this study was to monitor an aquatic ecosystem, during two different periods (dry and rainy season), on a RPPN placed inside a sugarcane farm, using the fish *Astyanax* sp. as bioindicator and also to discuss biomonitoring as a tool for integrating the management of RPPN with their surroundings areas.

Materials and Methods

The study was carried out on the RPPN Barbacena Farm, inside Barbacena Farm, in São Pedro do Ivaí city, central northern of Paraná State, Brazil (Fig. 1). This RPPN was created in 2004 by Ordinance 207/2004. This region is covered by seasonal semideciduous forests (Atlantic Forest biome). RPPN Barbacena Farm is surrounded by sugarcane plantations by Usina do Vale do Ivaí Company, also responsible for managing it.

For the biomonitoring the Neotropical native fish, *Astyanax* sp. (Teleostei, Characidae), were collected during the dry (September 2006) and rainy (March 2007) seasons. Thirty fishes were collected at each sampling point and season (Fig. 1): (1) tank formed at the sugarcane plantation, (2) tank formed at the producing area surrounding the RPPN, and (3) in the river inside RPPN. The RPPN is located downstream from the agricultural area and the three collecting points were about 2,000 m away from each other. Fishes were anesthetized with benzocain and blood samples were collected from the caudal vein and smeared on clean microscope slides for the micronucleus test. The liver was collected for histopathology and biochemical biomarkers (GST, CAT, and LPO), and the muscle was collected for acetylcholinesterase determination (AChE).

AChE activity was measured as described by Silva de Assis (1998) at 412 nm and expressed as nmol min^{-1} mg $protein^{-1}$. GST activity was analyzed according to Habig and Jakoby (1981) method at 340 nm and expressed as nmol of glutathione 1-chloro-2,4-dinitrobenzene (GSH-CDNB) conjugate min^{-1} mg $protein^{-1}$. CAT activity was measured following the method recommended by Aebi (1984), with a spectrophotometer (Ultrospec 2000, UV/Visible) at 240 nm. The activity was expressed as μmol min^{-1} mg $protein^{-1}$. LPO was measured according by Hermes-Lima et al. (1995), using the ferrous oxidation-xylenol assay at 570 nm and expressed as nmol of hydroperoxides concentration min^{-1} - mg $protein^{-1}$. Protein quantification on the samples was carried out according to the Bradford (1976) method, using bovine serum albumin as standard. The Sunrise TECAN microplate spectrophotometer was used for the measurements.

The liver samples were submitted to histopathology protocols and stained with hematoxylin and eosin. Lesions were recorded and the histopathologic index was calculated by Bernet et al. (1999). For the Piscine Micronucleus Test, a drop of fish blood was drawing over the slide to form a thin smear which was air-dried for 24 h, fixed with absolute methanol for 1 h and stained for 20 min with 5 % (w/v) Giemsa in pH 6.8 phosphate buffer. For each fish, 2000 erythrocytes were examined under 1,000× magnification and scored for the presence of both typical micro-nuclei and nuclear alterations.

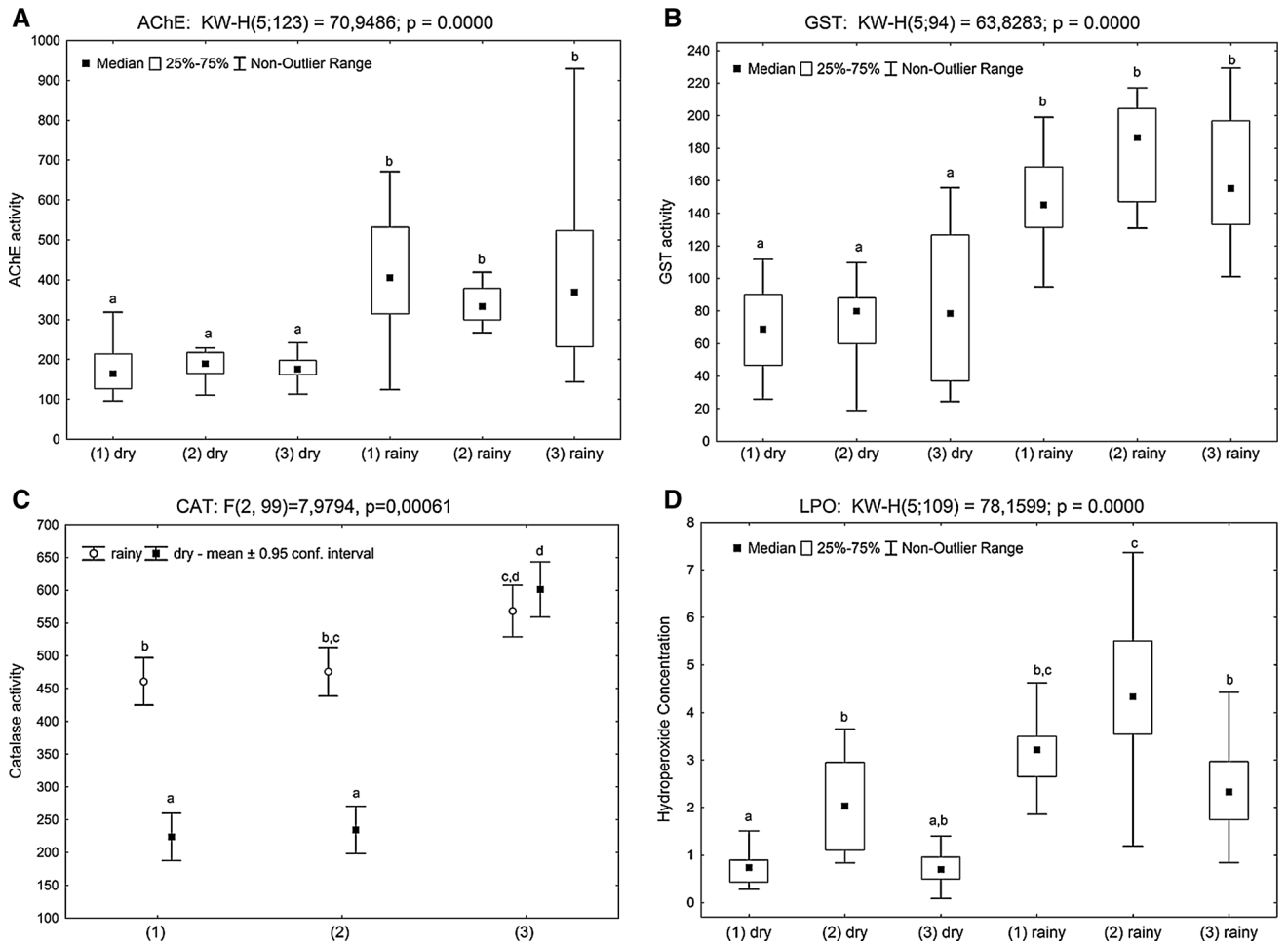

Fig. 2 Biochemical biomarkers in *Astyanax* sp. sampled in (*1*) Sugarcane, (*2*) Surrounding area, and (*3*) RPPN. **a** Acetylcholinesterase activity in muscle (nmol min^{-1} mg $protein^{-1}$). **b** Glutathione S-transferase (nmol min^{-1} mg $protein^{-1}$) in liver. **c** Catalase activity (μmol min^{-1} mg $protein^{-1}$). **d** Hydroperoxide concentration in liver (nmol mg $protein^{-1}$). *Different letters* indicated statistical differences ($p < 0.05$). KW-H: Kruskal–Wallis test results, F: two-way ANOVA result

All data were first tested for normality and homocedasticity (Kolmogorov–Smirnov and Levene's test respectively). Enzyme activities were analyzed by one-way ANOVA, two-way ANOVA, or Kruskal–Wallis test, depending on the results of the assumptions test. Group comparisons were carried out by Fisher's Least Significant Difference (LSD). Index of histopathological lesions and genetic alterations were tested by one-way ANOVA, followed by the LSD test. The level of significance adopted was 0.05.

Results and Discussion

The AChE and GST activities showed no statistically significant difference among the three sites during the same sampled period. Between periods, the activity of both enzymes was significantly higher in the rainy season (Fig. 2a, b). In dry season, the CAT activity increased only in RPPN, while between sugarcane and surrounding points no difference was found (Fig. 2c). The samples of the rainy season presented higher CAT activity values compared those of the dry season (except RPPN samples that presented similar values). The hydroperoxide concentration (LPO) in a same point generally was higher in the rainy season. The highest LPO value was in the surrounding area in the rainy season (Fig. 2d). The results suggest that rain density intensification can interfere in the activity of biochemical biomarkers because the xenobiotics are dragged into the streams, especially pesticides applied on agriculture. This fact was already demonstrated by Tejeda-Vera et al. (2007). Moreover, different seasons can create different species patterns: species have specific breeding seasons and particular periods in ontogeny that are more sensitive to pesticides (Relyea and Hoverman 2006).

In the present study the muscle AChE activity was not inhibited in *Astyanax* sp. as already observed in a lab study with glyphosate and diuron in fishes of the same genus (Rossi et al. 2011). On other hand, another herbicide clomazone inhibited muscle AChE in the fish *Leporinus obtusidens* (Miron et al. 2008). This variation may be produced by the differences in herbicide formulations and exposure time, fish size and sensitivity to the effects on AChE.

The increased GST activity during the rainy period might be indicative of an attempt of metabolic adequacy eliminated through glutathionization in cases of continued exposure to contaminants. The increase of GST activity has also been reported in areas with different contamination profiles in the freshwater fish *Leuciscus albumoides* (Lopes et al. 2001).

In the aquatic environment, the increase on enzyme activities related to antioxidant defense, such as CAT, has been already demonstrated by authors through different studies using fishes (Vasylkiv et al. 2011). Fishes exposed to the herbicide mixture (including diuron and glyphosate) have showed alterations in oxidative stress enzymes, as CAT (Gehin et al. 2006). These pesticides are widely sold and used around Barbacena Farm region (Agricultural and Supply Paraná State Secretary/SEAB *-pers. com.)* and it could be part of the causes of increasing CAT activity during the rainy season.

About LPO variations, a study showed an increase in the LPO levels in *Ameca splendens* and *Good atripinnis*, which was related to pesticides used on sugarcane production, especially diuron (Tejeda-Vera et al. 2007). This study corroborates our results.

The results for micronucleus and morphological alterations are presented in Fig. 3a. Consistent variations from the normally smooth and elliptical shape of the erythrocyte nucleus were apparent. The maximum nuclear alterations rate was found in the surrounding area in rainy season. All other points presented smaller values not statistically different. We observed a similar behavior between lipoperoxidation and genetics alterations: the highest values were found in surrounding area in rainy season. Lipid hydroperoxides can interact with other fatty acid initiating an autocatalytic chain of lipid peroxidation that can lead to a structural change of biological membranes (Abuja and Albertini 2001), prompting irreversible damages, such as nuclear morphological alterations. There is almost none previous research showing the effects of effluents from sugarcane production on the genetic damage rate. However, there are countless studies showing the nuclear damage and micronucleus formation in fish species exposed to different classes of pesticides (e.g. Ghisi and Cestari 2013).

Statistical differences were found in the histopathological indexes of sugarcane plantation and RPPN during the dry season (Fig. 3b). In the rainy season, fewer alterations were seen and no difference in the three points. The normal aspect of the liver tissue in *Astyanax* sp. is shown in Fig. 4a. In all samples lesions were identified as nuclear alterations (Fig. 4b), cytoplasmic vacuolization (Fig. 4d), leukocyte infiltration (Fig. 5a), tissue differentiation, and for the presence of free melanomacrophages (Fig. 5d). Parasites were also identified (Fig. 5b, c).

Necrosis was the most evident histopathological lesion found on all sampling points (Fig. 4c).

This type of alteration has been reported in fishes from areas impacted by multiple contaminants (Abdel-Moneim et al. 2012). Necrosis cause functional and structural damages in the liver of fishes, which may cause its collapse

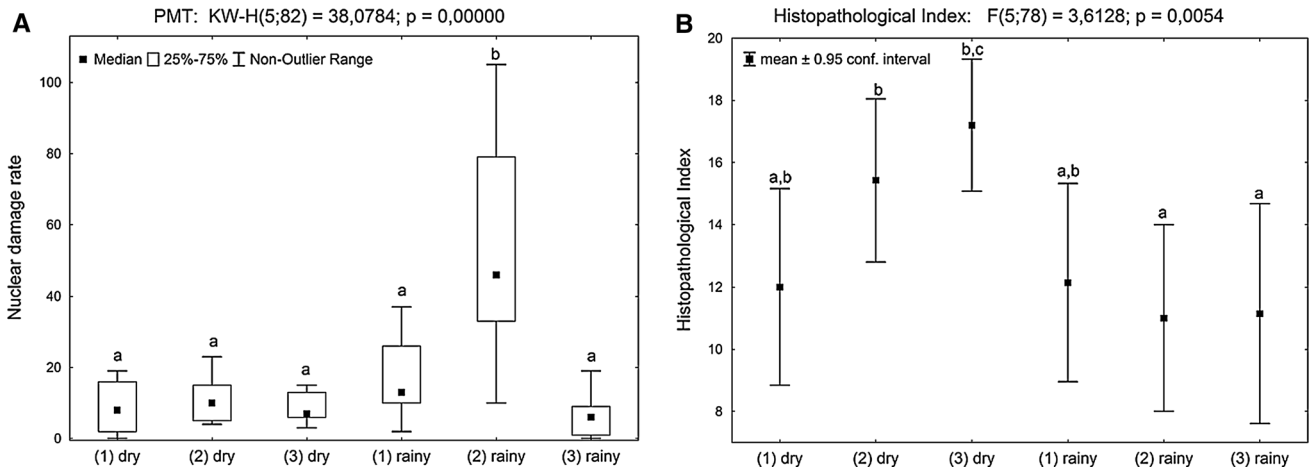

Fig. 3 Alterations rate in *Astyanax* sp. sampled in (*1*) Sugarcane, (*2*) Surrounding area, and (*3*) RPPN. **a** Nuclear alterations and micronucleus (PMT). **b** Histopathological lesions index in the liver according to Bernet et al. (1999). *Different letters* indicate a significant difference ($p < 0.05$). KW-H: Kruskal–Wallis test statistical result, F: ANOVA result

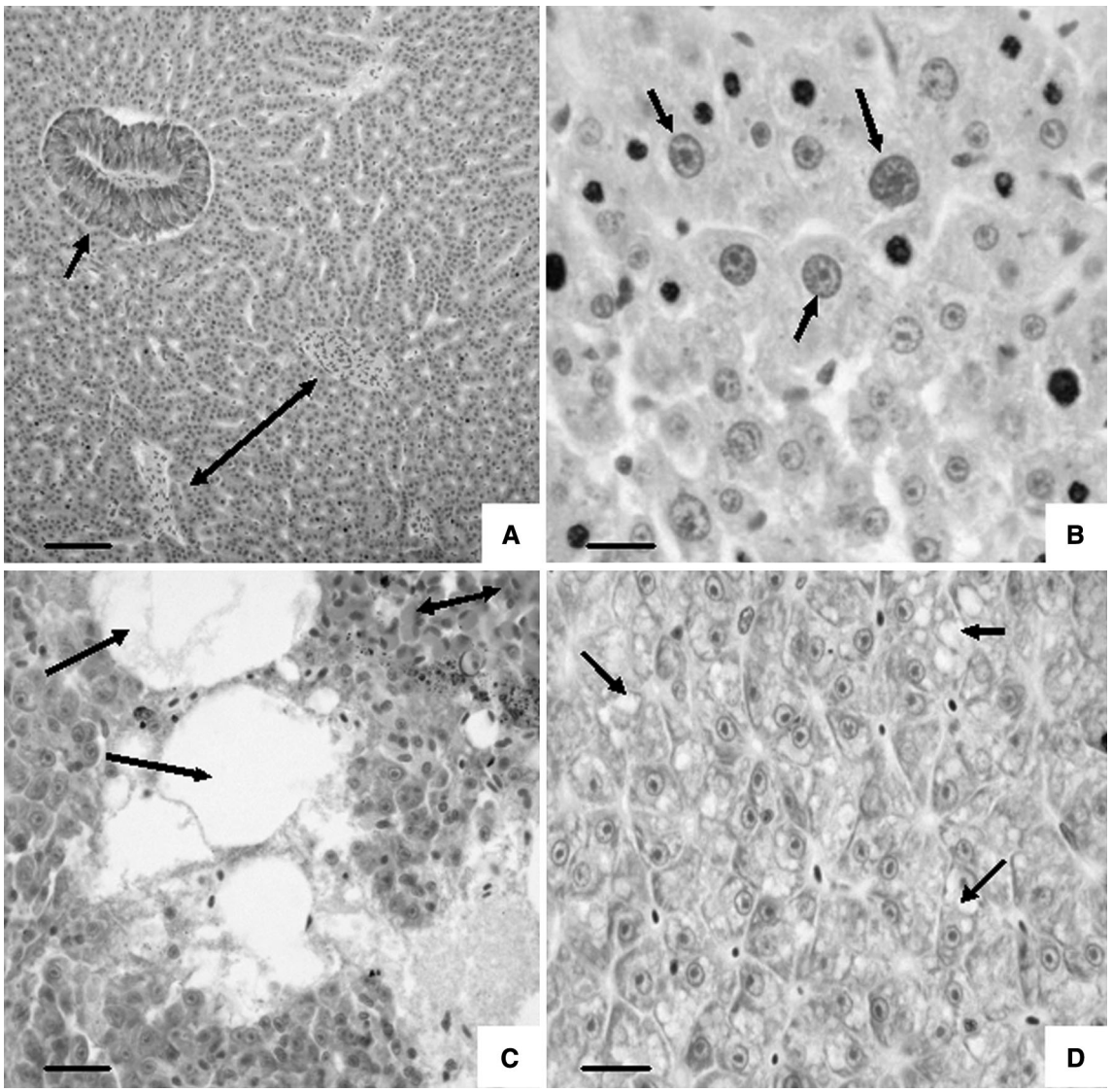

Fig. 4 Histopathological findings in the liver of *Astyanax* sp. from the Barbacena Farm, São Pedro do Ivaí, PR, during dry and rainy seasons and stained with hematoxylin and eosin. **a** Liver normal organization: (*right arrow*) indicates pancreatic tissue and (*left right arrow*) indicates sanguineous vases (*scale bar* 50 μm). **b** Nuclear alterations (*right arrow*) (*scale bar* = 10 μm). **c** Area of focal necrosis (*right arrow*) (*scale bar* 20 μm). **d** Cytoplasmic vacuolization (*right arrow*) (*scale bar* 20 μm)

(Stentiford et al. 2003). The presence of necrosis in individuals inside RPPN and also on areas near the sugarcane plantation indicates that the water resources in the area are compromised.

Liver lesions can be related to the continued exposure to pollutants due to absorption and metabolization processes (Bussolaro et al. 2010). Melanomacrophages are pigmented cells or set of cells in organs like the liver, kidney, and intestine. In the present study, a high incidence of free melanomacrophages was observed on organisms that also presented necrosis, might indicate that melanomacrophages are involved on removing necrotic cells. Moreover, the eosinophilic center on fishes from Barbacena Farm can be considered at preneoplasia stage, it can be also used as a potential biomarker of exposure, because the infiltration of defensive cells is response to the presence of contaminants (Bernet et al. 1999).

The cytoplasmic vacuolization observed in the liver of *Astyanax* sp. from our study can represent an immobilization mechanism of compounds more lipophilic, as described by Oliveira Ribeiro et al. (2005). This vacuolization might indicate that animals accumulate xenobiotics as an attempt to reduce its concentration on the circulation and other tissues.

Finally, we can observe that the simple creation of protected areas in agricultural regions does not automatically lead to the improvement of life quality of organisms inhabiting streams in this area. The legal status of the protected

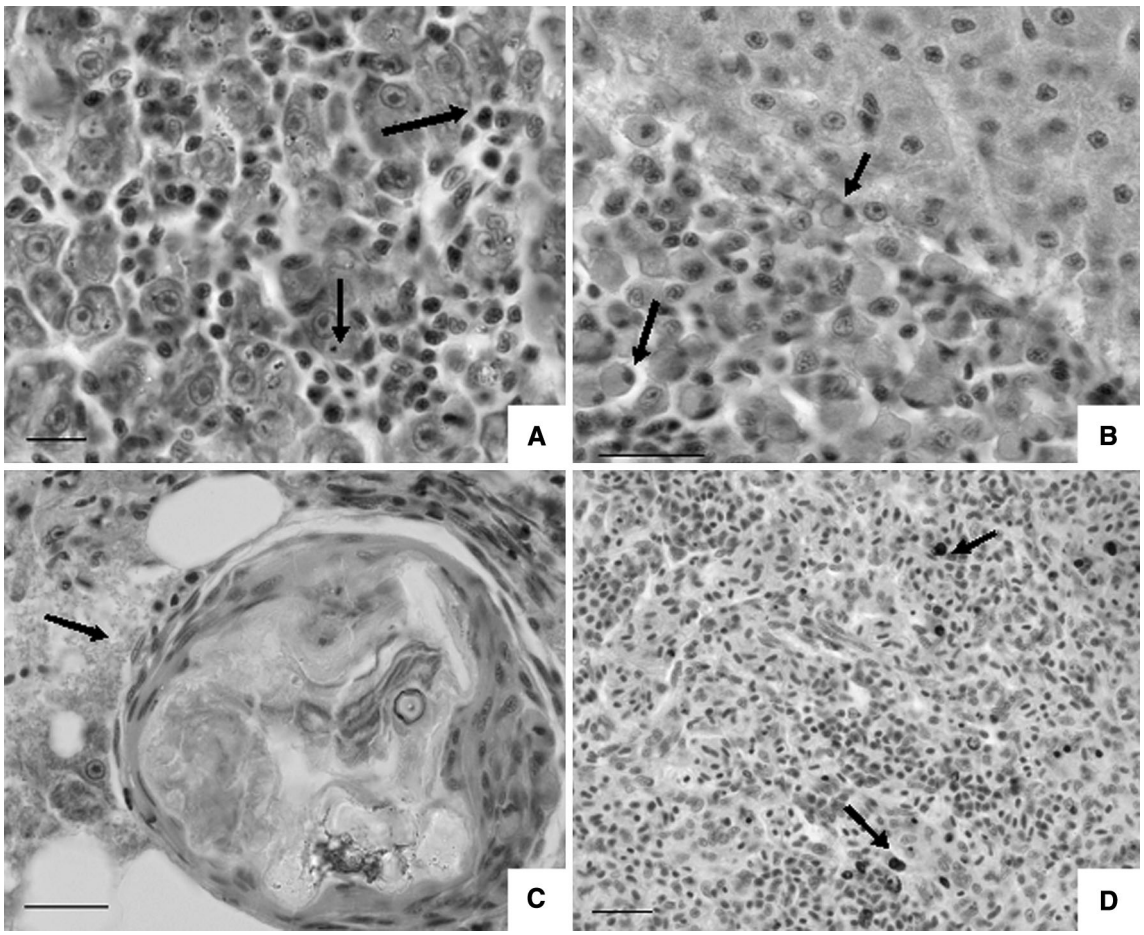

Fig. 5 Histopathological findings in the liver of *Astyanax* sp. collected from Barbacena Farm, São Pedro do Ivaí, PR, during dry and rainy seasons and stained with hematoxylin and eosin. **a** Leukocytes Infiltration (*right arrow*) (*scale bar* 10 μm). **b** Parasites in liver (*right arrow*) (*scale bar* = 10 μm). **c** Parasites in liver (*right arrow*) (*scale bar* = 20 μm). **d** Free melanomacrophages in liver with tissue differentiation and leukocytes infiltration (*right arrow*) (*scale bar* 20 μm)

area is not sufficient to preserve the ecological integrity in most anthropogenic altered areas. It is necessary to have a more effective investment in order to care and manage the protected area and its surrounding zones. Moreover, it should be considered that, since the water runs without respecting borders, it has a connecting function and plays a fundamental role as a pollution carrier. River conservation should be considered as a component of integrated catchment management (Nel et al. 2007).

In addition, protecting a drainage basin or even part of it might increase the functionality of the whole catchment. For example, sustainable management of buffer areas, such as the riparian zones, could improve the system functionality, preventing pollutants from running-off and draining into to water bodies (Lowrance et al. 1997). The strong dependence of running waters on the surrounding terrestrial environment is widely recognized by stream ecologists (Harding et al. 1998)—in general, more anthropogenic altered landscape leads to higher deterioration of freshwater biological quality.

Brazil contains 74 % of all protected areas around the world (Jenkins and Joppa 2009), and still lacks studies to assess and monitor the biological quality of its reserves. In this sense, our study highlights the importance of inserting monitoring programs in management plans of RPPN. Furthermore, studies are needed to discuss the conservation effectiveness of private land inserted in productive surroundings.

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