Neotropical Ichthyology, 13(3): 569-578, 2015

DOI: 10.1590/1982-0224-20140146

Spatial and temporal biomarkers responses of *Astyanax jacuhiensis* (Cope, 1894) (Characiformes: Characidae) from the middle rio Uruguai, Brazil

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Due to intense agricultural activity in the rio Uruguai (South Brazil), there is the potential for aquatic contamination by agrochemicals. In this region, there are many reservoirs to meet the water demand for rice fields, forming lentic environments. In line with this information, the aim of this study was to show a comparative analysis of some biomarkers, such as lipid peroxidation (TBARS), gluthatione S-transferase (GST), non-protein thiols (NPSH), amino acids (AA) and piscine micronucleus tests (MNE) in *Astyanax jacuhiensis* from lentic and lotic environments in the middle rio Uruguai region, comparing warm and cold seasons. Eight pesticides were found in water samples, with propoxur having the highest concentration found in both environments and seasons. Fish from the warm season showed higher levels of biochemical biomarkers, and fish from the cold season showed higher levels of MNE and AA. TBARS and AA presented higher levels in fish from the river, while GST, NPSH, MNE and AA presented higher levels in fish from dams. These environments have different characteristics in terms of redox potential, aeration, sedimentation, trophic structure, agrochemicals input and others, which may affect the physiological and biochemical responses of fish in against adverse situations.

Devido à intensa atividade agrícola no rio Uruguai (Sul do Brasil), há potencial para contaminação aquática por agrotóxicos. Há muitos reservatórios para atender a demanda de água de campos de arroz, formando ambientes lênticos. De acordo com estas informações, o objetivo do presente estudo foi mostrar uma análise comparativa de alguns biomarcadores como a peroxidação lipídica (TBARS), glutationa S-transferase (GST), tióis não-protéicos (NPSH), aminoácidos (AA) e teste písceo de micronúcleos (MNE) em *Astyanax jacuhiensis* amostrados em ambientes lóticos e lênticos da região do médio rio Uruguai, comparando estações quentes e frias. Oito pesticidas foram encontrados em amostras de água, sendo propoxur a maior concentração encontrada em ambos os ambientes e estações. Peixes da estação quente apresentaram maiores níveis de biomarcadores bioquímicos e peixes da estação fria apresentaram maiores níveis de MNE e AA. TBARS e AA apresentaram maiores níveis nos peixes de rio, enquanto GST, NPSH, MNE e AA apresentaram níveis mais elevados em peixes da represa. Estes ambientes têm características diferentes, com potencial redox, aeração, sedimentação, estrutura trófica, a entrada de agroquímicos e outros que podem afetar as respostas fisiológicas e bioquímicas de peixe contra situação adversa.

Keywords: Biomonitoring, Lotic and lentic environments, Pampa, Pesticides.

Introduction

Brazil is recognized as a country of rich hydrography, with more than 8,500,000 km^2 of hydrographic regions. In Southern Brazil, is the Uruguai is the main river that establishes the border between Brazil and Argentina. This river is distinguished by agribusiness activities and by electric potential. Uruguai watershed has 177,494 km^2 in Brazilian territory and shows as major environmental problems, such as the direct discharge of sewage (only about 10% of the wastewater is treated), erosion and siltation, and contamination by pesticides and other pollutants, such as metals. Despite the recognized degradation of this basin, official reports claim a shortage of data about the environmental problems, especially pesticides contamination (Agência Nacional de Águas (ANA), 2005).

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The middle rio Uruguai region shows good conditions for rice production, mainly by intense solar exposition and a predominance of flat areas. However, the rainfall pattern is not enough to meet the water demand of rice crops, requiring artificial irrigation, held mainly by water drainage of natural streams or artificial reservoirs, like weirs and dams. There is an estimate of 165,000 ha of dammed water used in rice crops in the middle rio Uruguai region (Instituto Rio Grandense do Arroz (IRGA), 2006). These physical barriers create lentic environments where wildlife is in constant contact with pesticides used during rice cultivation (Marchesan *et al*., 2007). Furthermore, these pesticides and metabolites eventually are carried to tributaries before emptying into the main river, also reaching rio Uruguai organisms, such as fish.

Astyanax has almost 150 species (Eschmeyer, 2015), which includes the fishes popularly known as lambari and piaba (Géry, 1977). Some eco-toxicological studies have pointed to the *Astyanax* species as an adequate bioindicator for evaluating contaminated environments (Schulz & Martins-Junior , 2001; Ribeiro *et al*., 2002; Alberto *et al*., 2005; Carrasco-Letelier *et al*., 2006; Silva *et al*., 2009; Prado *et al*., 2011; 2014; Trujillo-Jiménez *et al*., 2011; Santos *et al*., 2012). Among these, *Astyanax jacuhiensis* (Cope, 1894) is a non-migratory species, distributed by throughout Argentina, Brazil and Uruguay and is characterized by a fusiform body with a dark oval spot, arranged horizontally, just behind the head and yellow caudal fin (Reis *et al*., 2003). Some studies about the use of *A. jacuhiensis* were performed, using histological (Flores-Lopes & Malabarba, 2007) and mutagenic (Lemos *et al*., 2008; Goldini & Silva, 2012) biomarkers.

Pollutants may be metabolized by fish, generating reactive metabolites and reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals, leading to impairment of normal oxidative metabolism and finally to oxidative damage (Lushchak, 2011). At the biochemical level, lipid peroxidation is a parameter that can be measured through the quantification of compounds, such as malondialdehyde (MDA), which is formed by the degradation of initial products of the free radical attack, being the reaction with the thiobarbituric acid (Liu *et al*., 1997; Oost *et al*., 2003). At the genetic level, DNA damage can be assessed using a micronucleus test cytogenetic technique through the visual examination of the supernumerary nucleus formed in any type of dividing cell when whole or fragmented chromosomes lag behind the other chromosomes during the anaphase because of an aneuploidic or clastogenic event (Carrasco *et al*., 1990).

The organisms have antioxidant defenses against oxidative damage. An important parameter is the activity of glutathione-S-transferase (GST), catalyzing the conjugation of several xenobiotics with glutathione (GSH), protecting lipids from peroxidation during the detoxification process (Cairrão *et al*., 2004). The non-protein thiols (NPSH) also show antioxidant capacity acting against the formation of free radicals in the maintenance of the cell redox balance and in defense against electrophilic agents (Reischl *et al*., 2007). Moreover, metabolic parameters, like the amount of total amino acids, can reveal a stress response in which the organism is exposed.

Accordingly, the present study aims to show a comparative analysis of some biochemical responses of fish from lentic and lotic environments in the middle rio Uruguai were analyzed, *A. jacuhiensis* as a bioindicator, verifying the seasonal effects.

Material and Methods

Sampling sites and fish collection. This work was undertaken in the region of middle rio Uruguai, at Uruguaiana city, Rio Grande do Sul State, Brazil. Fish were sampled from a lotic environment, rio Uruguai (between 29º45'05"S and 57º05'64"W to 29º30'32"S and 56º50'67"W) and from a lentic environment, a dam at the Universidade Federal do Pampa (29º50'12.9"S and 57º05'09"W) (Fig. 1). The lotic sampling was conducted between two points in the rio Uruguai in order to verify any difference along of this river section. Given that no difference was found, all data from rio Uruguai is analyzed together.

In total, 40 specimens of *A. jacuhiensis* were used as bioindicator in this study (7.92 ± 3.3 cm and 12.8 ± 4.7 g). Fish were collected in the cold season (April, May and June 2012, and April 2013) and in the warm season (November and December 2012 and February and March 2013), being 10 specimens for each environment (lotic and lentic) and season (cold and warm). The fish were collected by local fishermen with fishing nets and transferred into a live box containing aerated river water and transferred to the Laboratório de Biologia at the Universidade Federal do Pampa, in Uruguaiana city. The fish were killed by section of spinal cord behind the opercula and the liver, muscle and gills were quickly removed for biomarkers analyses. These tissues were kept frozen until processing of samples.

The climate of this region is characterized as Cfa (humid tropical) by the Köppen classification. Average precipitation during the study period was 214.1±118.03 mm in the warm season and 84.8±53.7 mm in the cold season. The rainfall data were obtained by Instituto Nacional de Meteorologia from Brazil, INMET (www.inmet.gov.br). Average temperature in the warm season was 23.4±2.0 ºC and in the cold season was 16.4±3.1 ºC.

The investigation was approved by the Ethics Committee on Animal Use of Universidade Federal do Pampa, protocol 001/2012, and the fish sampling was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), number 32304-1.

Water analysis. Simultaneously with fish collection data from water quality at each study site were recorded in situ: pH, dissolved oxygen, temperature, salinity and conductivity, through Hanna® HI 9828 multiparameter (approximately 20 cm deep). Additionally, samples of water

were taken (one liter per sampling site packed in amber bottle) for determining the concentrations of pesticides, by high performance chromatography according to Sabin *et al*. (2009) and Martins *et al*. (2013).

Biomarkers analyses. The TBARS, GST, NPSH and AA in liver, muscle and gills were analyzed. TBARS assay (thiobarbituric acid-reactive substances) was estimated by malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured according to Buege & Aust (1978). Gluthatione S-transferase (GST) activity was assayed according to Habig *et al*. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. NPSH were determined by the method of Ellman (1959). AA quantification was assayed according to Spies (1957). The protein was determined according to Bradford (1976) using bovine serum albumin as standard, being the absorbance of samples measured at 595 nm.

For piscine micronucleus test, slides were prepared according described by Vicari *et al*. (2012) and 2.000 cells were examined under light microscope. The frequency of nuclear alterations and micronuclei were observed according Carrasco *et al*. (1990).

Statistical analyses. Statistical analyses were performed using statistical software GraphPad Prism®, version 5.0 for Windows (San Diego, USA.). Normality was determined by Shapiro-Wilk and Kolmogorov-Smirnov test (alpha = 0.05). For biochemical, physicochemical and pesticide concentration the values are presented as means ± standard error (SD) and for piscine micronucleus test are presented in medians and quartiles (q1 - q3). The data with normality distribution were analyzed through unpaired t test with Welch's corrections. The analysis of non-parametric data were performed through Mann-Whitney test. The minimum significance level was set at $p < 0.05$.

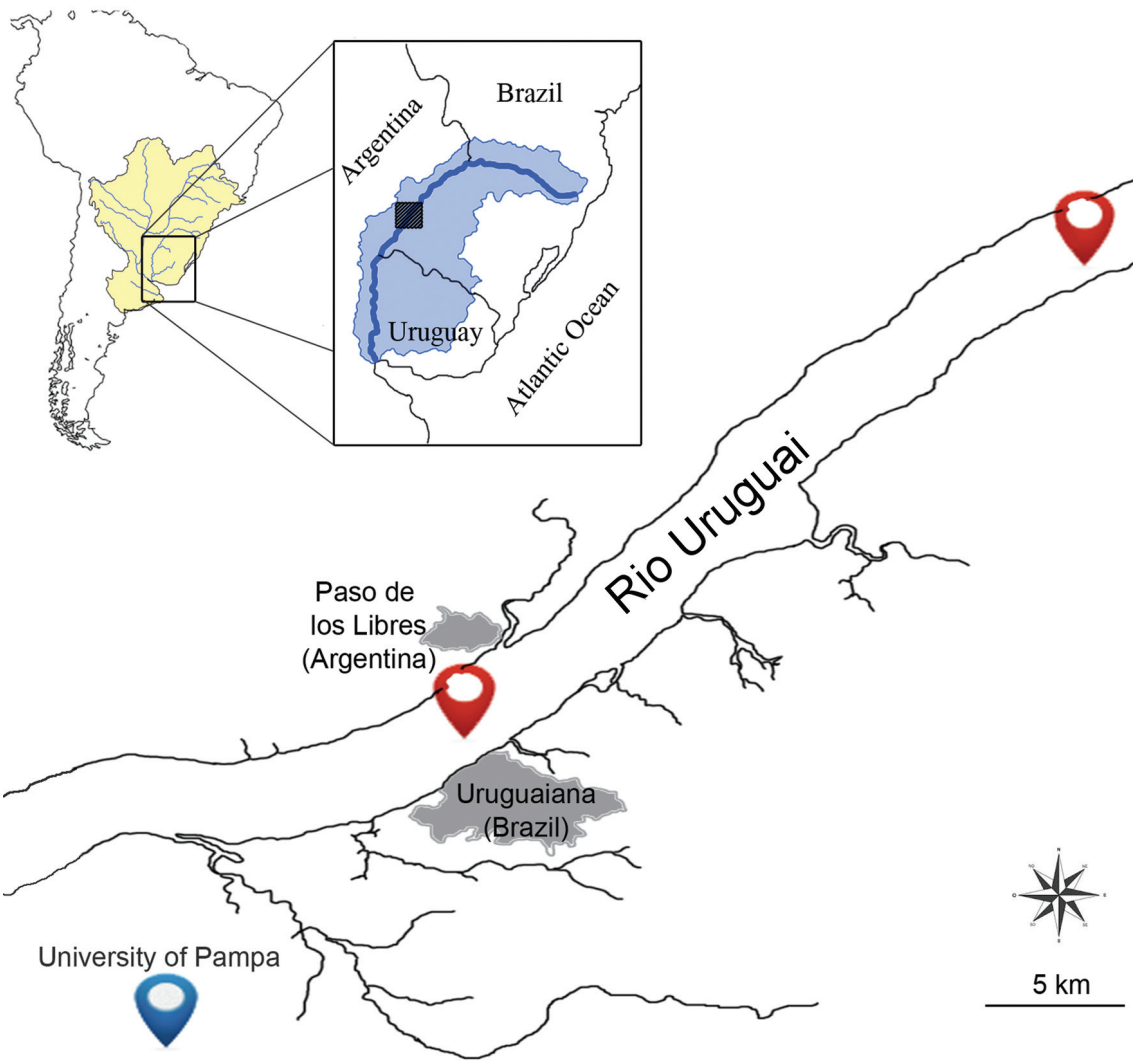

Fig. 1. Map of sampling sites. Red points indicate the sites of sampling in lotic environment, and blue point indicates the dam (lentic environment). The La Plata basin in the South America is highlighted in yellow and the Uruguai basin in blue. Urban areas of Paso de los Libres and Uruguaiana cities are indicated by a gray area.

Results

During the warm season, only conductivity and salinity showed significant differences between the river and the dam, with the river higher for both parameters. In the cold season, pH from the dam was lower when compared to the river. All physicochemical parameters in the river did not show difference between the seasons. In the dam, temperature and pH were higher in the warm season (Table 1).

Propoxur insecticide showed high concentration in the lentic environment, with an even higher occurrence of different pesticides during the cold season. Carbofuran, thiuran, paraoxon-methyl, clomazone and propyzamide were found exclusively during the cold season. Carbofuran and thiuran were observed only in the river, while paraoxon-methyl, clomazone and propyzamide were found only in the dam. Propoxur, pirimiphos-methyl and atrazine occurred in both sites and seasons (Table 2).

For all biomarkers analyzed, the higher levels were observed in the warm season (Fig. 2, Table 3). Lipid peroxidation levels were higher in fish collected from the river in all of the tissues analyzed, whereas the highest levels were observed in the gills of fish from the river during the warm season and the lowest values were observed in the muscle of fish from the dam during the cold season. The fish collected during the warm season in the river also showed a higher amount of AA in the three tissues when compared with the fish from the dam during the same season. In the cold season, fish from the river also showed higher levels of AA, not being significant from muscle.

On the other hand, the fish from the dam showed more GST enzymatic activity in hepatic and gill tissue when compared with fish from the river, although there was not a significant difference in the muscle. Fish from both the river and the dam showed higher levels of GST activity in the warm season for all tissues, except for the gills in the fish from the dam. The gills of fish from the river showed lower means of GST activity, and the higher means were observed in liver of fish from the dam. Similar results were obtained with NPSH levels. Fish from the dam showed higher levels of NPSH for all tissues analyzed, with the levels higher in the warm season.

Table 1. Means of physicochemical parameters of water found in the sites and seasons of studied period. Different letters in a row represent significant differences between seasons of an environment (river or dam). Asterisks represent significant differences between environments in a same season. By one-way ANOVA unpaired t test with Welch's corrections.

	River		Dam	
	Warm	**Cold**	**Warm**	**Cold**
Temp. (ºC)	25.2 ± 3.3^{a}	19.1 ± 4.1^{a}	24.9 ± 1.6^{a}	18.4 ± 1.1^{b}
pH	7.6 ± 0.8^{a}	$7.5 \pm 0.1^{a*}$	7.5 ± 0.1^{a}	$6.2 \pm 0.2^{b*}$
DO (mg L^{-1})	5.1 ± 0.7^{a}	6.3 ± 0.2^{a}	5.9 ± 0.6^{a}	5.1 ± 0.8^{a}
Cond. (mS cm^{-1})	$0.066 \pm 0.02^{a*}$	0.053 ± 0.002^{a}	$0.043 \pm 0.006^{a*}$	0.043 ± 0.005^{a}
Sal. (ppt)	$0.029 \pm 0.009^{a*}$	0.023 ± 0.005^{a}	$0.016 \pm 0.005^{a*}$	0.020 ± 0.001^{a}

Table 2. Means of multiresidue of pesticides (µg L^{-1}) found in the sites and seasons during studied period. Limit of detection: 0.037µgL^{-1}. Limit of quantification: 0.12µgL^{-1}. (n.d: not detected).

Pesticides	River		Dam	
(µgL^{-1})	**Warm**	**Cold**	**Warm**	**Cold**
Atrazine	4.15	2.6	5.4	3.9
Carbofuran	n.d	0.1	n.d	n.d
Clomazone	n.d	n.d	n.d	5.0
Paraoxon-methyl	n.d	n.d	n.d	7.5
Pirimiphos-methyl	4.7	4.5	4.9	3.6
Propoxur	5.3	18.1	5.0	29.1
Propyzamide	n.d	n.d	n.d	7.5
Thiuran	n.d	2.1	n.d	n.d

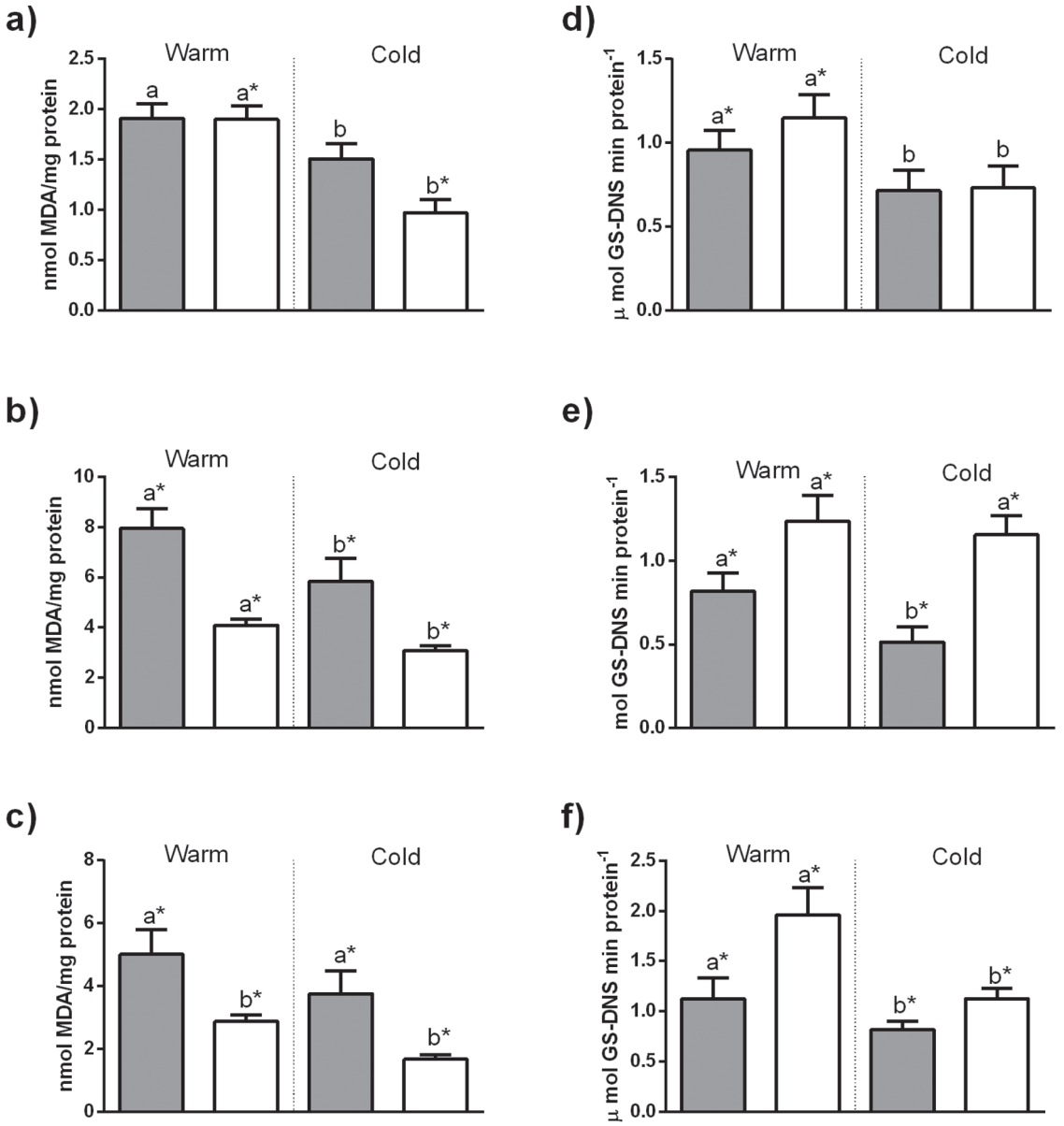

Fig. 2. Levels of the biomarkers TBARS (a - c) and GST (d - f) in muscle (a, d), gill (b, e) and liver (c, f) of *Astyanax jacuhiensis* from river (gray bars) and dam (white bars) sampled in warm and cold seasons from Uruguai basin. N = 10. Different letters represent significant differences between seasons of the same environment (river or dam). Asterisks represent significant differences between environments in the same season. By unpaired t test, with Welch's correction.

Table 3. Variations of biomarkers in *Astyanax jacuhiensis* samples obtained from two environments from rio Uruguai basin. NPSH expressed in μmol SH g tissue^{-1}, and AA expressed in μmol g tissue^{-1} (n = 10). Different letters in a column represent significant differences between seasons of an environment (river or dam). Asterisks represent significant differences between environments in a same season. By one-way ANOVA test fallowed by unpaired t test, with Welch's correction.

		NPSH			AA		
		Muscle	**Gill**	**Liver**	**Muscle**	**Gill**	**Liver**
River	**Warm**	0.209 ± 0.015 ^{a*}	0.478 ± 0.018 ^{a*}	0.394 ± 0.023 ^{a*}	22.98 ± 1.739 ^{a*}	41.28 ± 1.217 ^{a*}	43.38 ± 0.701 ^{a*}
	Cold	0.173 ± 0.007 ^{a}	0.278 ± 0.019 ^{b*}	0.261 ± 0.016 ^{b*}	17.49 ± 0.974 ^{b}	29.17 ± 2.306 ^{b*}	22.90^{b} ± 1.188*
Dam	**Warm**	0.350 ± 0.018 ^{a*}	0.687 ± 0.035 ^{a*}	0.907 ± 0.030 ^{a*}	17.00 ± 0.829 ^{a*}	33.22 ± 0.935 ^{a*}	31.94 ± 1.197 ^{a*}
	Cold	0.195 ± 0.015 ^{b}	0.382 ± 0.018 ^{b*}	0.647 ± 0.047 ^{b*}	17.69 ± 0.890 ^{a}	21.82 ± 1.289 ^{b*}	18.28 ± 0.935 ^{b*}

A significant difference of micronuclei and nuclear abnormalities analysis between fish from dam of warm and cold seasons was observed (p = 0.0050) and in fish of from river and dam environments during the cold season (p = 0.0043) (Fig. 3, Table 4).

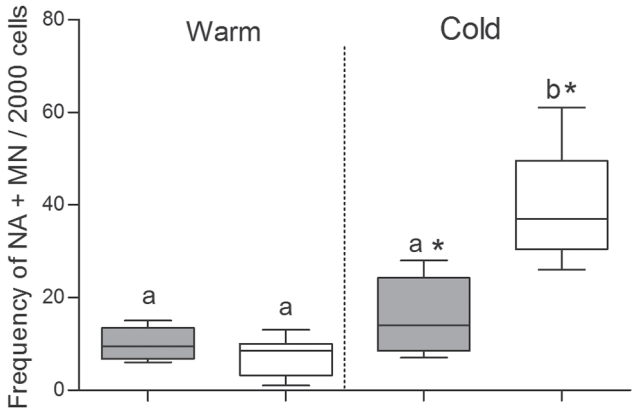

Fig. 3. Piscine micronucleus test, (2000 peripheral red blood cells per fish) in *Astyanax jacuhiensis* from river (gray boxes) and dam (white boxes) sampled in warm and cold season from Uruguai basin. N = 6. Different letters represent significant differences between seasons of the same environment (river or dam). Asterisks represent significant differences between environments in the same season.

Table 4. Frequency of micronucleus and nuclear alterations in erythrocytes of *Astyanax jachuiensis* samples from two environments of the rio Uruguay basin, in warm and cold season.

		N	NA	MN
			Medians (q1/q3)	*Medians (q1/q3)*
River	**Warm**	6	7 (4 / 9.5)	0 (0 / 0.5)
	Cold	6	12 (9 / 19.5)	1.5 (0.25 / 2)
Dam	**Warm**	6	8.5 (5 / 9)	0 (0 / 0)
	Cold	6	34 (32 / 51)	6 (2 / 6)

Discussion

Comparative studies assessing pollutant toxicity between lotic and lentic ecosystems are scare in the literature. Biomonitoring studies are important for better understanding how the ecological, hydrological and biochemical characteristics of each type of ecosystem can modulate pollutant behavior in different environments. The environments considered here have different characteristics, such as higher redox potential in lotic aquatic systems, due to constant aeration by the water flow. In addition, lentic environments have higher pesticide sedimentation rates and there are differences in the chemical, hydrological and ecological parameters. The variation between different environments can also affect sedimentation rates and trophic structure (Simmons & Wallschlager, 2005).

We found some differences in the physicochemical parameters between the river and dam. Lentic environments showed significant low levels of pH (cold season), conductivity and salinity (warm season). Furthermore, this environment showed higher pesticide concentration. Differences could be found in the amount of pesticides between the seasons. In the longer period of rain (warm), the amount of pesticide was lower, with only three kinds detected, while in the cold season, with the lowest average rainfall, we found eight different pesticides. These differences are possibly due also the plantation management, with differing applications of pesticides throughout the year. Considering the chemical groups of pesticides, we found the herbicides atrazine, clomazone, thiuran, propyzamide and the insecticides pirimiphos-methyl, paraoxon, propoxur and carbofuran in the water of the river and dam (Table 2). N-methylcarbamate propoxur was the pesticide that showed higher levels in both season and environments. It interferes in nervous transmission across the synaptic gap through the inhibition of acetylcholinesterase and is classified as moderately hazardous class II according to the World Health Organization (WHO, 2002) and United States Environmental Protection Agency (USEPA, 1997). However, it draws attention the concentration of herbicide atrazine. The Brazilian legislation establishes the maximum concentration of 2 µg/L (Conselho Nacional do Meio Ambiente (CONAMA), 2005) and during our study period, the mean concentration in all samples was higher. Atrazine is moderately toxic for aquatic animals according to present legislation. However, biochemical, histopathological and genetic effects were demonstrated in several fish species (Ventura *et al*., 2008; Paulino *et al*., 2012; Santos & Martinez, 2012). Biomarkers responses in *A. jacuhiensis* from the rio Uruguai basin showed different responses in both environments and seasons. Fish from rivers showed higher amount of AA, possibly due to increased energy demand needed in the lotic environment, since the energy demand of fish is strongly related with environmental characteristics. Moreover, for almost four biomarkers used in three tissues (12 biomarkers responses) significant difference between sites and seasons were observed. Similar results were highlighted in many studies from around the world (Ozmen *et al*., 2008; Cazenave *et al*., 2009; Güngördü & Ozmen, 2011; Carvalho *et al*., 2012; Güngördü *et al*., 2012; Kantati *et al*., 2013; Silva *et al*., 2014), showing the importance of spatiotemporal analyses for assessing different effects of pollutants in the environment.

GST conjugates xenobiotics or their metabolites with glutathione, making them less toxic and more easily able to be excreted (Oost *et al*., 2003). Many works have been show changes in GST activity in the organs of fish exposed to different pesticides used in rice culture (Cattaneo *et al*., 2011; Moraes *et al*., 2011; Clasen *et al*., 2012; Toni *et al*., 2013). The lower GST activity in fish from river may be due to exposure to a different complex mixture and the hydrodynamics of contaminants from the river. A variety of

chemicals and chemicals mixtures are known to inhibit GST activity in fish due to a general impairment of the chemical metabolism, interfering with mechanisms involved in GST induction. Furthermore, it is known that in aquatic systems, seasonal changes, such as dissolved oxygen, temperature and pH, are environmental variables that generally influence the oxidative process of aquatic organisms (Güngördü *et al*., 2012).

NPSH also shows the antioxidant capacity acting against the formation of free radicals, in the maintenance of the cell redox balance, as well as in the defense against electrophilic agents (Reischl *et al*., 2007). Our results demonstrate consistency between antioxidant analyses because both NPSH and GST showed higher levels in gill and liver of fish from dam. Some researches have highlighted the potential antioxidant from NPSH in fish exposed to contaminants in bioassays studies (Menezes *et al*., 2011, 2012, 2013). However, nothing was reported in the scientific literature about NPSH as a biomarker for contaminated environments. Besides responses observed for GST and NPSH, the higher levels of lipid peroxidation (LPO) were observed in fish from the river. Cazenave *et al*. (2009), studying *Prochilodus lineatus* from Río Salado basin (Argentina) highlighted that induced ROS could not be totally scavenged by antioxidant enzyme due to elevated levels of LPO observed in different fish organs. It is possible that fish from the dam are constantly exposed to pollutants due to lower water flux, and the induction of antioxidants defenses might have also contributed to oxidative damage. Similar responses were observed in *Wallago attu* (Siluridae) from the Yamuna River in India (Pandey *et al*., 2003).

The fish from the dam also presented more genotoxical damage when compared with fish from river, primarily during the cold season. These results corroborate with biochemical analysis explained above, showing the dam as an environment with a higher toxic potential for *A. jacuhiensis*. Lemos *et al*. (2008) analyzed the petrochemical complex impact through the piscine micronucleus test in *A. jacuhiensis,* finding no seasonal difference in the frequency of the micronucleus. These authors highlighted the importance of establishing baseline values for the biomarker in sentinel organisms in environmental studies performed using native populations. The researchers also proposed the mean of 2.0 ± 3.3 x 10^{-4} for micronucleus erythrocytes. This frequency baseline is similar to those presented in several fish species studied fish species (Al-Sabti & Metcalfe, 1995; Grisolia & Starling, 2001; Lemos *et al*., 2001; 2007; Bolognesi *et al*., 2006). Considering this baseline value, *A. jacuhiensis* from rio Uruguai basin present in both environments and seasons analyzed values higher than the one pointed as baseline for Lemos *et al*. (2008). Therefore, considering the high concentration and variety of pesticides found, the results of the piscine micronucleus test, together with biochemical parameters shows us the genotoxic damage and toxic impact on fish fauna from rio Uruguai basin. Cleary, more analysis on factors such as like heavy metals and other biomarkers should be performed for better understand the environmental impacts caused by anthropogenic action in this basin. Nevertheless, these results call attention for the potential pollution in rio Uruguai, in its middle portion, highlighting the need for more studies in this environment.

Astyanax jacuhiensis from rio Uruguai basin show different responses in lotic and lentic environments, as well as in warm and cold seasons, for the biomarkers GST, NPSH, and TBARS, in addition to the amounts of amino acids. The biomarkers showed distinct responses in the different tissues, showing that fish survive changes, inducing on physiological and biochemical strategies against environment stressors.

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Submitted September 26, 2014
Accepted May 01, 2015 by Bernardo Baldisserotto
Published September 25, 2015