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Multi-biomarker responses in fish (*Jenynsia multidentata*) to assess the impact of pollution in rivers with mixtures of environmental contaminants

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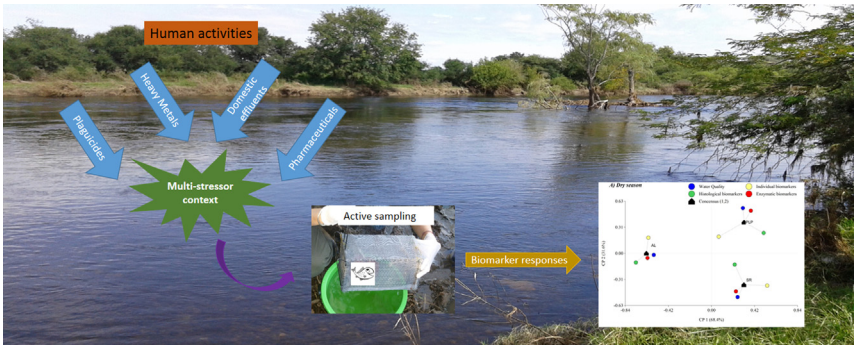
HIGHLIGHTS

- Fish caging studies allowed us to determine stations with moderate pollution.
- Changes at histological level were marked during wet season.
- Biochemical makers responded better during dry season.
- Multiple biomarker responses were useful to evaluate a multistressor context.

GRAPHICAL ABSTRACT



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ABSTRACT

Aquatic biotas frequently inhabit a multi-stressor environment that affects their structure and function. The hypothesis of this work is that differential responses at different levels of organization will be found in *J. multidentata* exposed to the multi-stressor context of the Ctalamochita River (Córdoba, Argentina), and that those responses will be more pronounced in sites impacted by anthropic activity (cultivated and industrial areas, city settlements). The study was carried out at four sites along the Ctalamochita River, during the wet and the dry seasons. A seven-day active sampling used caged fish. After exposure, biomarker levels were measured at individual (somatic indexes, behavioral parameters), histological (semi-quantitative indexes in gills and liver) and biochemical (oxidative stress, Acetyl- and butyrylcholinesterase enzymes) levels. The biomarkers had an individually differential response depending on the hydrological seasons, the histological biomarkers (increased histopathological gill and total indexes) being more sensitive during the wet season and the biochemical and behavioral (increased catalase in liver, increased AChE and BChE in muscle, time spent in the lateral section of the tank) during the dry season. Multivariate analysis demonstrated our hypothesis that contamination of the Ctalamochita River occurs in sites impacted by anthropic activity.

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1. Introduction

Aquatic biotas are usually exposed to diverse stress situations, including regulated and emerging pollutants, including pharmaceuticals, personal care products, pesticides, etc., reaching the environment from many different sources (Gorga et al., 2015; von der Ohe et al., 2011). Bioindicators can determine the overall effects of the pollutants interacting within the complexity of natural systems. In biomonitoring studies including environmental risk assessment it is important to use sentinel species (Friberg et al., 2011). Fish have proved to be sensitive organisms for assessing the condition and functioning of aquatic ecosystems (Abdel-Moneim et al., 2012). However, their normal mobility (to seek food or as a result of river floods that re-locate them in another sites) generates uncertainty about how much of the water quality is actually reflected in individuals captured at one site (Kerambrun et al., 2011). A caging strategy in field experiments may provide more realistic results about the effects of xenobiotics, because the exact location and duration of exposure are known (Kerambrun et al., 2011; Oikari, 2006) and thus the results from different sites are validly comparable.

In the multi-stressor context, contaminants can affect the structure and function of biological systems, causing responses (biomarkers) at molecular, biochemical, histological, and behavioral levels before the community level is affected. The Ctalamochita River basin (Córdoba, Argentina) is not only a traditional area of agriculture and livestock activities, but also of numerous industries (dairy, chemical products, slaughterhouses, even a tannery, among others) established around the heavily populated cities of Río Tercero and Villa María (DiPAS., UNC, 2007; Gualdoni et al., 1994). Previous studies have registered toxic substances related to chemical industries such as nitric acid, hydrogen peroxide, petrochemicals and heavy metals (DiPAS., UNC, 2007; O'Mill, 2012). There is thus a multi-stressor situation in the Ctalamochita River basin that could be causing differential responses at different levels of organization in fish.

The live-bearer fish *Jenynsia multidentata* (Jenyns, 1942) (Anablepidae, Cyprinodontiformes) is an abundant native neotropical fish of the Ctalamochita River (Haro et al., 1996), with a wide distribution in South America (Malabarba et al., 1998). This species usually feeds on mosquito larvae and is therefore important in the vector control of mosquito-transmitted diseases (Bonifacio et al., 2014; Haro and Bistoni, 2007). Because of its ecological and morphological characteristics, the species has been successfully used as a regional model to assess water quality and the effects of different chemicals on biological processes (Guyón et al., 2012; Hued and Bistoni, 2007; Monferrán et al., 2011).

The hypothesis underlying this work is that differential responses at different levels of organization will be found in *J. multidentata* exposed to the multi-stressor Ctalamochita River environment, and that those responses will be more pronounced at sites located below the Piedras Moras Dam, where anthropic activities such as industry, agriculture and large cities occur. This study aimed to evaluate biomarker responses in the native fish *J. multidentata* in a multi-stressor context, at points with different degrees of contamination along the Ctalamochita River through a field caging experiment.

2. Material and methods

2.1. Study area and water quality

The Ctalamochita River is the second largest basin in Córdoba Province and an exorheic course that ends in the Parana River (Fig. 1). Its source is in the Piedras Moras Dam and it flows from west to east for 300 km across the province with a mean flow of 27.6 m^3/s (DiPAS., UNC, 2007). The hydrological regime is pluvial, with a maximum period of rainfall from October to March, and a minimum from April to September. The mean annual rainfall is about 630 mm (DiPAS., UNC, 2007). To assess the effect of industrial and domestic sewage as well as of the nearby cultivated fields, four sampling sites were selected with different contamination conditions (Fig. 1):

1) A reference site with little human activity located upstream in the basin, before the city of Santa Rosa (SR, 32°9′30.67″S; 64°30′42.88″W) - population ≅ 12,000 (INDEC, Instituto Nacional de Estadisticas y Censos, 2010).
2) Almafuerte station (AL, 32°10′2.97″S; 64°14′28.45″W) located 1 km downstream from Piedras Moras dam, surrounded by cropfields

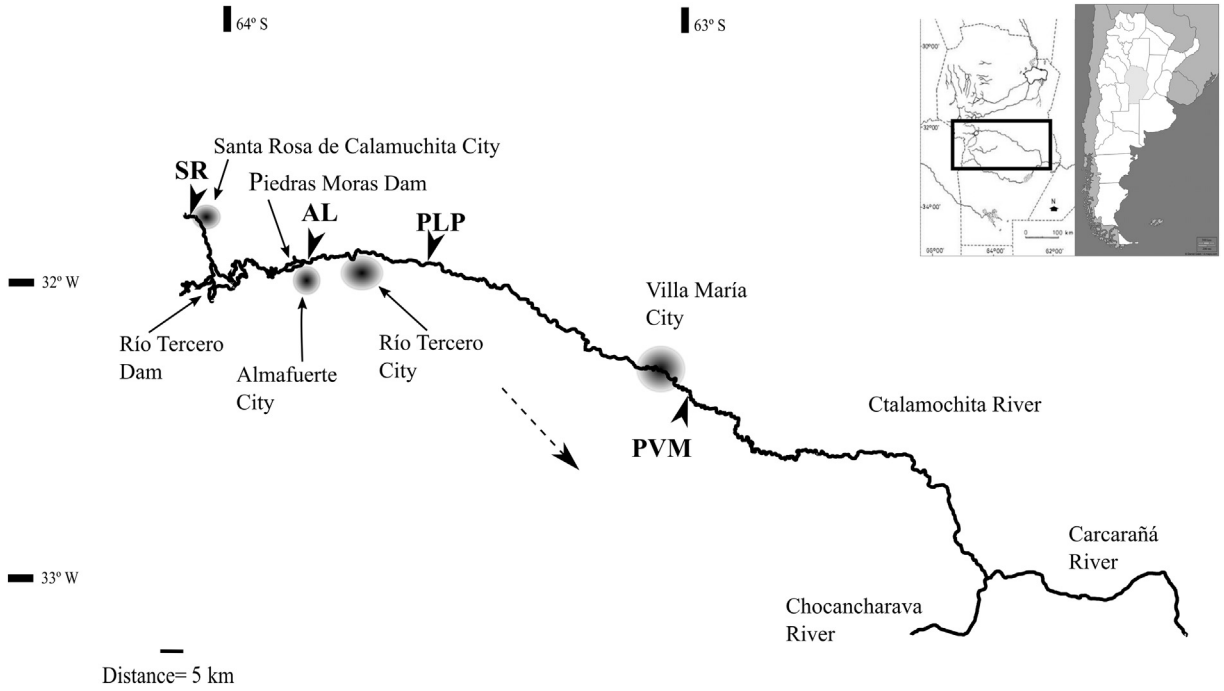

Fig. 1. Study area and location of sampling sites in Ctalamochita River. References: SR = Santa Rosa; AL = Almafuerte; PLP = Puente los Potreros; PVM = Post-Villa María. Arrow with dotted line represents the direction of the water flow.

(51 km. from SR) - Almafuerte population ≅ 12,000 (INDEC, Instituto Nacional de Estadisticas y Censos, 2010).

3) Puente Los Potreros station (PLP, 32°9′2.92″S; 64°1′39.08″W), 10 km downstream from several industries in the city of Río Tercero and surrounded by cropfields (25 km from AL) - Río Tercero population ≅ 48,000 (INDEC, Instituto Nacional de Estadisticas y Censos, 2010).
4) The last sampling station was 5 km after the sewage treatment plant of the city of Villa María (PVM, 32°27′50.01″S; 63°10′52.73″W), the most populated city in the basin (115 km from PLP) - Villa María population ≅ 100,000 (INDEC, Instituto Nacional de Estadisticas y Censos, 2010).

The experiments were carried out during the two hydrological seasons, dry (July 2013) and wet (March 2014). At each sampling station, water temperature, pH and conductivity were measured in situ using multiparametric equipment (WTW Multiline F/Set 3). Four replicates of river water were collected in amber glass bottles (without headspace, ca. 30 cm below the river surface). Samples were ice-refrigerated and transported to the laboratory for chemical and biological analyses of nitrite, nitrate, phosphate, ammonia, chlorides, sulphate and total coliform (APHA, American Public Health Association, 1995). These parameters were measured in duplicate. To characterize the physicochemical conditions of the sampling sites, the water quality index (WQI) proposed by Pesce and Wunderlin (2000) was calculated. This index gives a percentage of water quality: a value of 100% represents the highest water quality, and values of 50% are considered to have a negative impact on the biota.

2.2. Test organism

Adult male individuals of live bearer (*J. multidentata*, standard length = 26.8 ± 0.6 mm and weight = 0.36 ± 0.01 g) were used as a bioindicator because of their higher sensitivity to pollutants compared with females (Ballesteros et al., 2007). The use of experimental animals was carried out considering the guidelines of the Committee on Animal Bioethics and Welfare of the Consejo Nacional de Investigaciones Científicas y Técnicas (Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, 2005, Res 1047), Argentina.

2.3. Experimental design

Individuals of *J. multidentata* were collected with a trawl net from the Yuspe River (31°14′17.6″S 64°31′14.7″ W), considered a quasi-pristine site (Hued and Bistoni, 2005). Fish were transported to the laboratory in 20 L tanks and kept in 15 L glass aquaria with dechlorinated tap water for 15 days (room temperature and natural light) prior to the experiment to evaluate the health status of the fish batch. They were fed twice a day *ad-libitum* with commercial fish pellets (TetraMin®, USA). After acclimation, fish were transported in 20 L tanks to the sampling sites on the Ctalamochita River. Two cages (24 × 12 × 12 cm length × height × wide, volume = 3.46 L, lined with wire mesh to allow water circulation through the cage) were placed in the river at each site at a depth of 10 cm. Each cage contained eight fish, randomly assigned. Individuals were exposed for 7 days, following Oikari (2006). The cage was tightly anchored to the surrounding vegetation to prevent any losses. To avoid overcrowding, the density of individuals did not exceed 1 g of fish per L of water in each cage. When the exposure in the field was finished, the surviving fish were recorded to calculate the percentage of survival and they were transferred back to the laboratory in 20 L tanks containing river water.

2.4. Laboratory measurement of biomarkers

2.4.1. Swimming activity

Once back in the laboratory, each fish was recorded individually for 10 min in a glass tank (30 × 25 × 8.5 cm, length × height × width, water column kept at 15 cm height) located in an isolated room to avoid disturbance. Each recording was made with a digital camera (Panasonic ®, Model DMC-FH20) placed in front of the fish tank. Filming was carried out in the same time slot (from 9:00 to 12:00 h) to avoid fluctuations due to natural circadian rhythm. Fish were placed in the aquarium 5 min before recordings began as a period of acclimation to the new tank. The videos were analyzed using ANY-maze software (ANY-maze®, Stoelting CO, USA).

The following variables were analyzed:

- Swimming speed, SS (m/s): calculated as the total distance traveled for the specific time period (10 min).
- Time in the top section, TS (s): refers to the time that fish remains in the upper portion of the tank (5 cm below the water level).
- Time in the bottom section, TB (s): refers to the time that fish remains in the bottom portion of the tank (5 cm from the floor of the aquarium).
- Time in the lateral section, TL (s): refers to the time that fish remains in one of the lateral portions of the tank (5 cm from the wall of the aquarium).
- Immobile time, IT (s): time interval in which the fish is considered inactive, with movement only of their fins and operculum.
- Immobile episodes, IE: Number of immobile episodes for the specific time period.

2.4.2. General health status of fish

Each individual was weighed and standard length recorded. They were killed by transecting the spinal column behind the opercula. Then they were dissected and the brain, gills, liver and muscle stored according to the biomarkers analyzed. This experimental procedure was carried out following Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (2005).

To assess the general health status of the fish, the Fulton Condition Factor (CF) was calculated. The Hepatosomatic Index (HSI) was also calculated (Van der Oost et al., 2003).

2.4.3. Histological and morphometrical analysis

For histological purposes, liver and gills were stored in buffered formalin at 10%, processed routinely and stained with hematoxylin and eosin (H&E). Each slide was examined, blinded, with a light microscope (Olympus X-785) and photographed with a digital camera (Moticam Camera 2300, 3 Megapixels). In gills, five primary filaments were observed (10 secondary lamellae per filament). The number of secondary lamellae with a particular alteration *was* registered to calculate their frequency. In liver, five random areas were observed and the percentage of area affected with a particular alteration was registered. For each sample site an average of frequency of each alteration in gills and liver was calculated.

Histopathological indices of gill (I_{gill}) and liver (I_{liv}) were estimated using a semi-quantitative protocol following Bernet et al. (1999), modified by Rautenberg et al. (2014). Briefly, alterations were classified into four major reaction patterns: RP_1, circulatory disturbances (gills: hemorrhage, aneurysm, edema; liver: dilatation of sinusoids, vascular congestion, hemorrhage); RP_2, regressive changes (gills: epithelial lifting, lamellar disorganization and shortening; liver: vacuolar degeneration, nuclear alteration, fibrosis, necrosis); RP_3, progressive changes (gills and liver: cell hypertrophy and hyperplasia); and RP_4, gill and liver inflammation (leukocyte infiltration). Then, I_{gills} and I_{liv} were calculated based on two factors: the pathological importance of the lesions (importance factor, W) (range 1–3) and the extension of pathological change (score value, a) from 0 (unchanged) to 8 (extreme occurrence). Finally, a total histopathological index (I_{tot}) was calculated by adding gills and liver indices of each individual fish. A greater value of the I_{tot} reflects a more severely affected individual.

2.4.4. Enzyme extraction and measurement

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Corporation ® (USA). To assess enzyme activity, liver, gills,

brain and muscle were frozen immediately after being dissected using liquid nitrogen, and kept at −80 °C until analysis. Enzyme extracts from each organ were prepared from individual fish following Cazenave et al. (2006) with modifications (Bonifacio et al., 2016). Briefly, organs were homogenized in 0.1 M potassium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol (DTE) using a glass homogenizer (Potter Elvehjem), affording a tissue weight of ca 10% per volume. The samples were centrifuged at 10,000 × *g* for 10 min to separate cell debris. The resultant supernatant was used to assess all the enzyme activities. Enzyme activities were determined in triplicate using a Biotek Synergy HT® microplate reader. The activity of Glutathione-S-Transferase (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate at 340 nm, following Habig et al. (1974). Catalase activity (CAT) was determined using H_2O_2 as a substrate at 240 nm following Beutler (1982). Acetyl- and butyrylcholinesterases (AChE and BChE) were measured at 412 nm following Ellman et al. (1961) using acetylthiocholine iodide and butyrylthiocholine as substrates, respectively. Enzyme activity was calculated in terms of the protein content of the sample (Bradford, 1976) and is reported in nanokatals per milligram of protein (nkat (mg prot)$^{-1}$), where 1 kat is the conversion of 1 mol of substrate per second.

2.5. Statistical analysis

Statistical analyses were carried out using the R Software Package (R Core Team, 2015). First, differences in survival rates between pairs of sampling sites were calculated using the Test of Equal or Given Proportions. Second, all data were assessed for Normality and Homogeneity of Variance using the Shapiro Wilks and Levene tests, respectively, with the R "car" package (Fox and Weisberg, 2011). Differences between sampling sites (for each hydrological season separately) were assessed by a One-way Analysis of Variance (ANOVA), followed by a Tukey test using the same R package. When parametric assumptions were not fulfilled, Kruskal-Wallis was used followed by multi-comparison Dunn's tests. Significance was set at $\alpha = 0.05$. Figures were constructed using the "R "sciplot" package (Morales, 2012).

Finally, to analyze the different multi-stressor contexts globally at each sampling station, groups of biomarkers (groups named "individual" – including somatic indices and behavioral responses; "histological" – including histological indexes; and "enzyme" – enzyme activities) and a water quality (physicochemical parameters) group, a Generalized Procrustes analysis (GPA) was applied. Specifically, GPA builds a consensus configuration of a group of datasets by applying mathematical transformations (rotation, translation and scaling). More specifically, each item of data has a multivariate spatial configuration of the chosen group of variables and the GPA analysis attempts to superimpose them. Therefore, GPA theory and algorithms can be applied to different groups of biomarkers, determining if the variables used were suitable for characterizing the studied sampling sites. Sampling seasons were analyzed separately because of a lack of information of the biomarkers at the PVM sites during the dry season. As a criterion for prior selection of the variables and to avoid redundant information in the analysis (as the GPA requires), the variables selected were those which were statistically significant in the univariate analysis. This analysis was carried out using Infostat software (Di Rienzo et al., 2016).

3. Results

3.1. Water quality

The PVM site showed lower concentrations of dissolved oxygen (DO) in both hydrological seasons than the reference station (SR) (Table 1), and also the highest conductivity value. During the dry season, there was an increasing concentration of the parameters listed in Table 1 along the basin, with higher values at the last two sampling sites (PLP and PVM). The increase in the concentration of Total Coliforms at PVM was remarkable, with two orders of magnitude greater than the other sampling sites. In the wet season, there was no difference of the parameters between sites. The WQI decreased downstream in the basin during the dry season, with the highest and lowest values being those of SR and PVM respectively (Table 1). These trends were not evident during the wet season and the highest value of WQI was recorded at AL.

3.2. Survival rates

During the dry season, the survival rate at AL was significantly higher than at SR (Table 2). However, all individuals at PVM died during the experiment. During the wet season, survival at SR was significantly higher than at the last two sampling sites. When the hydrological seasons are compared at the same sampling station, there are statistical differences between the dry and wet seasons at SR and PVM, with the highest survival rates in the wet season. At PLP station, the highest survival rate occurred during the dry season while at AL there were no statistical differences between hydrological seasons.

3.3. Biomarkers

Because of the mortality of every individual at the PVM station during the dry season, we could not perform a two-way ANOVA with interactions. Therefore, the statistical analyses of the biomarkers measured in the caged fish were carried out for each hydrological season separately.

3.3.1. Swimming activity

Behavioral variables are displayed in Table 3. In the dry season, immobile episodes (IE) were significantly lower at AL and PLP than at SR. The time in the lateral zone (TL) was significantly higher at these sites than at the reference site. Moreover, a tendency for IT and TS to decrease

Table 1
Physicochemical parameters measured in the Ctalamochita River.

Sampling station	Hydrological season	pH	DO (mg/L)	EC (μS/cm)	T (°C)	Nitrite mg/L NO_2^-	Nitrate mg/L NO_3^-	Ammonia mg/L NH_4^+	Phosphate mg/L PO_4^{3-}	Sulphate mg/L SO_4^{2-}	Chloride mg/L Cl^-	Total coliforms MPN/100 mL	WQI
SR	Dry	7.9	9.5	268	6.6	0.011 ± 0.001	0.68 ± 0.20	0.016 ± 0.002	<LOD	0.160 ± 0.149	14.0 ± 6.6	2200	84
AL		9.0	12.0	163	10.6	0.012 ± 0.001	0.52 ± 0.17	0.017 ± 0.001	0.019 ± 0.005	4.697 ± 0.564	28.0 ± 0.0	8800	76
PLP		8.6	8.2	253	10.0	0.072 ± 0.002	1.94 ± 0.10	0.041 ± 0.002	0.035 ± 0.012	5.034 ± 0.089	135.4 ± 6.6	2200	75
PVM		8.2	8.8	300	12.5	0.127 ± 0.009	1.12 ± 0.06	0.069 ± 0.001	0.145 ± 0.005	6.273 ± 0.297	144.8 ± 6.6	8×10^5	67
SR	Wet	8.0	9.2	230	18.0	0.010 ± 0.000	3.57 ± 0.19	0.008 ± 0.002	0.279 ± 0.030	0.013 ± 0.000	14.0 ± 4,7	NA	81
AL		8.0	11.5	176	22.3	0.011 ± 0.001	0.01 ± 0.10	0.011 ± 0.001	0.010 ± 0.001	0.645 ± 0.100	168.1 ± 9.3	280	90
PLP		8.1	9.7	197	22.2	0.012 ± 0.001	2.07 ± 0.26	0.009 ± 0.001	0.140 ± 0.020	1.672 ± 0.110	88.7 ± 4. 7	12,900	75
PVM		8.2	8.4	249	19.0	0.013 ± 0.000	3.05 ± 0.16	0.011 ± 0.001	0.337 ± 0.010	2.618 ± 0.040	102.7 ± 4.7	36×10^5	71

References: EC = Electric Conductivity, DO: Dissolved Oxygen, T: Temperature, MPN: Most Probable Number/100 mL, WQI: Water Quality Index, SR = Santa Rosa; AL = Almafuerte; PLP = Puente los Potreros; PVM = Post-Villa María. NA = Not Analyzed, <LOD, below detection limit (LOD for Phosphate: 0.005 mg/L).

Table 2
Survival rate, Condition Factor (CF) and Hepatosomatic Index (HIS) of caged individuals of *Jenynsia multidentata* in Ctalamochita River.

Sampling station	Survival (%)		CF		HSI	
	Dry	Wet	Dry	Wet	Dry	Wet
SR	47a*	86a	1.5 ± 0.2a	2.2 ± 0.1a	1.1 ± 0.2a	1.3 ± 0.1b
AL	80b	67ab	1.4 ± 0.1a	2.2 ± 0.1a	1.4 ± 0.1b	1.2 ± 0.1b
PLP	60ab*	33c	1.5 ± 0.2a	2.1 ± 0.2a	1.1 ± 0.1a	0.8 ± 0.1ab
PVM	0c*	53bc	NA	2.0 ± 0.1a	NA	0.6 ± 0.1a

References: SR: Santa Rosa, AL: Almafuerte, PLP: Puente los Potreros, PVM: Post-Villa María. Different letters indicate statistical differences among sampling sites ($p < 0.05$).
* Indicate statistical differences between hydrological stations for each station ($p < 0.05$).

and for SS and TB to increase was observed at the AL and PLP sites. In the wet season, there were no significant differences in behavioral variables among sampling sites, but only a tendency to increased IT in PLP and decreased IT in PVM was observed with respect to reference sites.

3.3.2. General health status of fish

The Condition Factor (CF) showed no statistical differences between sampling sites at both seasons ($p > 0.05$), although a trend to be higher in the wet season can be observed (Table 2). The HSI, however, registered a significant increase at AL during the dry season and a significant decrease at PVM during the wet season.

3.3.3. Gill and liver histomorphology and histological indexes

Gill and liver alterations were observed in both hydrological seasons and at all sampling sites (Table 4 and Fig. 2a). In gills, there was a significantly higher occurrence of lamellae fusion at PLP station compared with AL during the dry season. This alteration was also significantly higher during the wet season at all the sampling sites compared with SR. Finally, there was a significant increase in shortening of secondary lamellae during the wet season at all sampling sites compared with the reference site.

In liver, the occurrence of lipid degeneration was significantly higher at AL than SR during the dry season (Table 4 and Fig. 2b). The opposite occurred in the wet season where this alteration was significantly lower than at the other sampling sites. Also, the occurrence of fibrosis was significantly higher at AL and at PLP than at SR during the dry season, whereas in the wet season, the increase was significant only at AL. Finally, during the wet season, hydropic degeneration was higher at AL than at the other sites.

The index of histopathological damage in gills (I_{gill}) showed no significant differences among sampling sites during the dry season (Table 4). However, during the wet season, AL and PLP showed significantly higher values of I_{gill} than SR (Fig. 2b). Significant differences were found among sampling sites in the index for liver histopathology (I_{liv}) during the dry season only (Table 4), with the highest value at AL. Finally, the Total Index (I_{tot}) was significantly higher in the wet season (Table 4), and tended to be higher in the dry season, at all sampling sites than at the reference station SR.

3.3.4. Enzyme activity

During the dry season, GST activity in gills was significantly greater than at the reference site SR only at AL and PLP (Table 5). CAT activity was significantly inhibited in liver only at AL with respect to SR. There were significant differences in brain AChE activity among the three sampling sites, being highest at PLP and lowest at AL. In muscle tissue, this enzyme activity registered a significant increase only at PLP compared with the reference site. BchE activity in muscle was significantly higher at PLP than at the reference site SR, but showed no significant differences in liver. During the wet season, GST activity in brain was significantly inhibited at PLP and PVM with respect to the reference station SR but in the other organs this enzyme showed no significant changes. CAT activity showed significant inhibition only in brain at all sites with respect to the reference site SR. AChE activity in brain and muscle showed no significant differences among sampling sites. However, BChE activity in liver was significantly higher at PLP than at the reference site SR.

3.4. Multivariate analysis

The ANOVA test results grouped single variables by level of organization: enzyme (GST activity in brain and gills, CAT activity in liver, AChE activity in brain and muscle, BChE activity in liver and muscle), histological (I_{gill} and I_{liv}), and individual (somatic indexes: HSI; behavioral parameters: TL). All the physicochemical variables were included in the analysis. The analysis produces a configuration of the different study sites in each sampling season separately that reflects the consensus among the four groups (Fig. 3a and b). During the dry season, the consensus configuration of four groups of variables, drawn with its first and second principal axis, explained 68.4% and the 31.6% of the variability among sampling sites (Fig. 3a). The group closest to the consensus form was the enzyme group followed by the water quality group, and these thus contributed most to separating the sampling sites. On the other hand, during the wet season, the first and second principal axis explained 57.9% and the 30.9% of the variability among sampling sites

Table 3
Swimming behavior variables measured in caged individuals of *Jenynsia multidentata* in Ctalamochita River.

Hydrological season	Swimming variables	Sampling station			
		SR	AL	PLP	PVM
Dry	SS	0.012 ± 0.002a	0.017 ± 0.001a	0.016 ± 0.004a	NA
	TS	352.5 ± 64.7a	163.3 ± 33.1a	219.5 ± 69.7a	NA
	TB	155.0 ± 65.0a	351.4 ± 36.3a	301.6 ± 68.3a	NA
	TL	197.7 ± 47.1a	441.7 ± 34.6b	413.4 ± 69.1b	NA
	TI	61.4 ± 17.3a	47.6 ± 11.3a	47.8 ± 16.9a	NA
	IE	12.3 ± 3.7b	6.4 ± 1.2a	7.6 ± 2.5a	NA
Wet	SS	0.033 ± 0.003a	0.027 ± 0.003a	0.030 ± 0.005a	0.031 ± 0.004a
	TS	365.4 ± 27.5a	341.6 ± 55.4a	477.6 ± 47.7a	377.7 ± 42.6a
	TB	191.7 ± 24.4a	229.1 ± 52.2a	125.5 ± 45.2a	151.7 ± 37.8a
	TL	283.7 ± 26.2ab	178.6 ± 28.9a	294.0 ± 42.9ab	396.5 ± 52.5b
	TI	18.2 ± 4.7a	19.9 ± 11.0a	30.0 ± 10.1a	8.5 ± 2.3a
	IE	3.6 ± 0.9a	4.0 ± 2.0a	4.5 ± 1.5a	1.5 ± 0.3a

Data expressed as mean ± standard error (SE). References: SR: Santa Rosa, AL: Almafuerte, PLP: Puente los Potreros, PVM: Post-Villa María. Different letters indicate statistical differences among sampling sites ($p < 0.05$). SS: swimming speed (m/s), TS: time in the surface (s), TB: time in the bottom (s), TL: time in the lateral zone (s), TI: time immobile (s), IE: immobile episodes NA: not analyzed.

Table 4
Frequencies of histological alterations and Histopathological Indices in caged individuals of *Jenynsia multidentata* in Ctalamochita River.

Organ	Reaction pattern	Histological alteration	Hydrological season							
			Dry				Wet			
			SR	AL	PLP	PVM	SR	AL	PLP	PVM
Gills	Circulatory disturbances (RP1)	Aneurysms	NR	3.1 ± 1.7a (0–12)	0.5 ± 0.5a (0–2)	NA	1.0 ± 0.5a (0–4)	1.6 ± 0.8a (0–4)	1.0 ± 0.8a (0–2)	1 ± 1a
		Blood congestion	4.6 ± 4.6a (0–14)	NR	6.5 ± 5.9a (0–24)	NA	NR	NR	NR	NR
	Regressive changes (RP2)	Epithelial lifting	12.7 ± 3.7a (8–20)	14.6 ± 3.6a (0–30)	19.0 ± 5.2a (10–34)	NA	21.3 ± 3.9a (2–30)	16.4 ± 1.9a (12–22)	12.0 ± 6.0a (6–18)	22.5 ± 5.9a (14–40)
		Shortening of secondary lamellae	36.0 ± 17.5a (12–70)	14.6 ± 4.9a (0–28)	35.5 ± 10.4a (12–54)	NA	16.5 ± 4.3a (0–32)	44.4 ± 5.8b (32–64)	49.0 ± 1.0b (48–50)	24.5 ± 7.3ab (4–38)
		Secondary lamellae fusion	8.7 ± 5.9ab (0–20)	4.3 ± 1.5a (0–12)	18.5 ± 5.7b (4–32)	NA	2.0 ± 0.9a (0–6)	19.2 ± 2.33b (12–24)	23.0 ± 7.0b (16–30)	21 ± 6.5b (12–40)
		Chloride cells hyperplasia	13.3 ± 13.3a (0–40)	18.6.9 ± 13.9a (0–100)	10.0 ± 9a (0–40)	NA	17.5 ± 7.9a (0–60)	44.0 ± 13.3a (0–80)	40.0 ± 0.1a (40–41)	29.0 ± 12.8a (6–60)
		Epithelial cells hypertrophy	24.0 ± 18.3 (0–60)	NR	NR	NA	NR	NR	NR	NR
		Epithelial cells hyperplasia	10.6 ± 4.8a (4–20)	NR	19.5 ± 4.2a (8–28)	NA	14.8 ± 7.6a (80–66)	17.6 ± 2.6a (8–22)	20.0 ± 10.0a (10–30)	21.0 ± 5.5a (6–30)
	I_{gill}		30.0 ± 1.2a	22.6 ± 4.7a	37.0 ± 4.1a	NA	22.0 ± 3.4a	42.4 ± 2.2b	47.0 ± 5.0b	18.0 ± 6.1a
Liver	Circulatory disturbances (RP1)	Vascular congestion	9.1 ± 2.8a (4–14)	2.3 ± 1.3a (0–9)	9.0 ± 4.2a (3–17)	NA	3.1 ± 0.8a (0–6)	5.6 ± 2.3a (0–12)	2.9 ± 0.9a (2–4)	4.4 ± 1.9a (2–10)
		Hemorrhage	0.8 ± 0.8a (0–2)	NR	1.0 ± 0.6a (0–2)	NA	0.4 ± 0.4a (0–3)	1.4 ± 1.4a (0–7)	0.5 ± 0.5a (0–1)	3.2 ± 3.2a (0–13)
		Sinusoid dilatation	NR	NR	NR	NA	0.4 ± 0.3a (0–2)	NR	NR	0.9 ± 0.5a (0–2)
	Regressive changes (RP2)	Hydropic degeneration	17.7 ± 10.4a (0–36)	23.1 ± 3.2a (12–38)	41.0 ± 10.7a (23–60)	NA	16.7 ± 4.6a (2–42)	50.3 ± 7.7b (25–38)	1.0 ± 1.0a (0–2)	14.33 ± 12.8a (0–53)
		Lipid degeneration	1.5 ± 1.0a (0–3)	32.1 ± 8.5b (4–59)	NR	NA	22.6 ± 8.8b (2–77)	1.4 ± 1.4a (0–7)	44.7 ± 11.7b (33–56)	20.0 ± 11.9b (0–51)
		Fibrosis	1.9 ± 0.3a (0–2)	9.1 ± 1.5c (5–14)	7.7 ± 1.2bc (1–10)	NA	1.5 ± 0.8a (0–9)	5.1 ± 1.2b (0–1)	0.4 ± 0.3ab (0–1)	0.3 ± 0.2a (0–1)
		Necrosis	5.7 ± 3.8a (0–13)	12.0 ± 2.1a (3–20)	2.3 ± 1.9a (0–6)	NA	4.0 ± 1.1a (0–1)	7.2 ± 1.4a (5–11)	11.3 ± 3.7a (0–2)	10.9 ± 2.5a (0–1)
	Inflammatory changes (RP4)	Leukocyte infiltration	NR	NR	NR	NA	0.1 ± 0.1a (0–1)	0.2 ± 0.1a (0–1)	1.05 ± 0.95a (0–2)	NR
	I_{liv}		13.3 ± 2.9a	34.3 ± 3.0b	12.5 ± 4.3a	NA	16.5 ± 2.2a	22.4 ± 1.9a	23.0 ± 1.0a	26 ± 4.2a
I_{tot}			43.3 ± 4.1a	56.9 ± 7.0a	49.5 ± 7.4a	NA	38.5 ± 5.2a	64.8 ± 4.1b	70.0 ± 4.0b	46.3 ± 10.1b

Data expressed as mean percentage of occurrence (%) ± standard error (SE). Between brackets: Range of occurrence. References: SR: Santa Rosa, AL: Almafuerte, PLP: Puente los Potreros, PVM: Post-Villa María. I_{gill}: Gill Index; I_{liv}: Liver Index; I_{tot}: Total Index. Different letters indicate statistical differences among sampling sites ($p < 0.05$). NA: not analyzed.NR: Not registered.

(Fig. 3b). The configuration of groups in this season showed the reference site SR and PVM well-separated in the multivariate space. In this case, the water quality and enzyme groups contributed most to this configuration. Finally, sites AL and PLP did not separate properly.

4. Discussion

4.1. Water quality

Water quality parameter values in this study increased gradually from the reference station up to those located below the Piedras Moras Dam, in both hydrological seasons. The increased concentration of ammonia, nitrate, nitrite, phosphate, chloride, and total coliform bacteria at PLP and PVM is indicative of contamination by domestic effluents mainly from the large cities (Rio Tercero and Villa Maria, with populations of over 48,000 and 100,000 inhabitants respectively, INDEC (Instituto Nacional de Estadisticas y Censos) (2010)), and by artificial fertilizers from nearby cultivated areas. The concentrations of most of these parameters did not exceed the maximum allowed levels of the aquatic biota protection and drinking water guidelines (Boletín Oficial, 1993). Wastewater discharge also tends to increase conductivity due to a higher ion concentration (USEPA, 1997) and thus the lower basin sites yielded higher values for this parameter.

Most physicochemical parameters registered during the wet season were lower than those of the dry season. During this period, prior to our sampling, exceptionally strong rainfalls were recorded, increasing the water flow. Chapman (1996) pointed out that a high flow of water dilutes the particles and soluble ions present in the body of water. However, the nitrate and phosphate values were seen to increase in this season, probably because it is the pesticide and fertilizer application period in the nearby crops. These contaminants reach the river by leaching, run-off or aerial spraying.

At PLP and PVM, the increase in coliform bacteria in the wet season could be due to higher temperatures than in the dry season (winter) and the input from cattle feces due to runoff. It is remarkable that the total coliform levels at PVM in the present study were as high as those previously registered by O'Mill (2012), when Villa María did not have a sewage treatment plant. Total coliform count includes bacteria that normally inhabit river waters and fecal bacteria from animal and domestic sewage (WHO, World Health Organization, 2006), and therefore their count could be only an indirect indicator of fecal contamination. Typically, raw municipal sewage contains from 10 to 10 million for coliform bacteria (Chapman, 1996). Therefore, our findings may indirectly indicate high fecal contamination but within the normal range.

The WQI registered a gradual decrease from the upper to the lower sampling sites during the dry season, with the lowest values at PVM. In all sampling seasons and sites, WQI values were higher than those reported for a highly contaminated river in the same area, the Suquía River (with WQI values around 40 to 50 at the highly-contaminated sites) (Monferrán et al., 2011). The Ctalamochita river sites downstream from the large cities of Río Tercero and Villa María thus had moderate chemical alteration.

4.2. Survival rates

According to Oikari (2006), an acceptable mortality rate is around 10–15% in quasi-pristine sites, which is the rate effectively registered during the wet season at the reference site (SR). However, during the dry season (winter), mortality here was around 40%. This can be explained by the low water temperatures that individuals in the cages had to face. Even when fish were acclimated during 20 min at the sampling site before release in the cages, this time was not enough. Besides, the cages at the site SR were placed near nightfall and thus the fish faced adverse temperature conditions overnight. Low temperatures could also be the cause of the high mortality registered at PVM, where the cages were placed early in the morning. Added to this effect, the contamination of the station itself, located right below the sewage treatment plant, produced 100% mortality. The low prevailing temperatures during the dry season (coinciding with the coldest month of the year) proved to be the major constraint for the caged fish method throughout this study.

During the wet season, survival rates decreased in the downstream sampling sites (PLP and PVM), evidencing the deterioration of the river after passing through the cities of Río Tercero and Villa María, matching the decrease of WQI mentioned before.

4.3. Biomarkers

The quantification of physicochemical and biological parameters and of the presence of contaminants as the only source of information for the study of water quality in the environment may not reflect the multi-stressor context that the biota must deal with. However, under field conditions, the response of most biomarkers is not related to exposure to a specific contaminant but rather to the complex mixture of substances present in the environment.

4.3.1. Swimming activity

J. multidentata has been described as a species inhabiting shallow vegetated coasts of the rivers in the first centimeters of the water column. These ecological traits, plus its morphological characteristics (the mouth in an upper position), enable feeding on mosquito larvae (Ringuelet, 1975). More recently, its normal swimming behavior was quantified considering the variables of swimming speed and movement

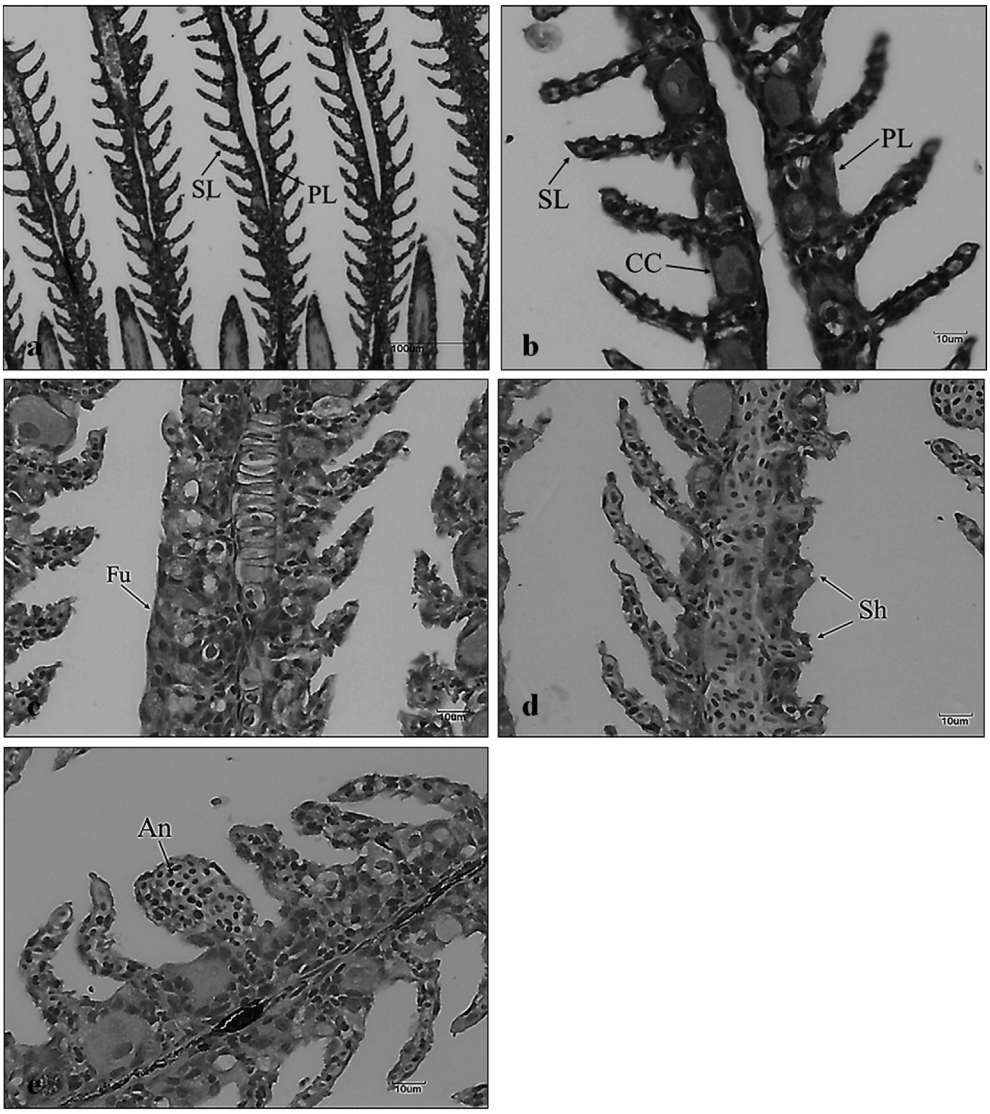

Fig. 2. a) Photomicrographs of gills of caged individuals of *Jenynsia multidentata*. a–b: Normal gill structure. c–e: Gill histopathological alterations. Abbreviations: PL = Primary Lamellae. SL = Secondary Lamellae. CC = Chloride Cells. Lif = Epithelial lifting. Fu = Secondary lamellae fusion. Sh = Secondary lamellae shortening. An = Aneurism. H&E stain (10× and 40×). b) Photomicrographs of liver of caged individuals of *Jenynsia multidentata*. a: Normal histology. b–e: Liver histopathological alterations registered in caged fish. Abbreviations: Hd = Hydropic degeneration. Nec = Necrosis. Ld = Lipidic degeneration. Bs = Congestion of blood sinusoids. Fib = Fibrosis. H&E stain (40×).

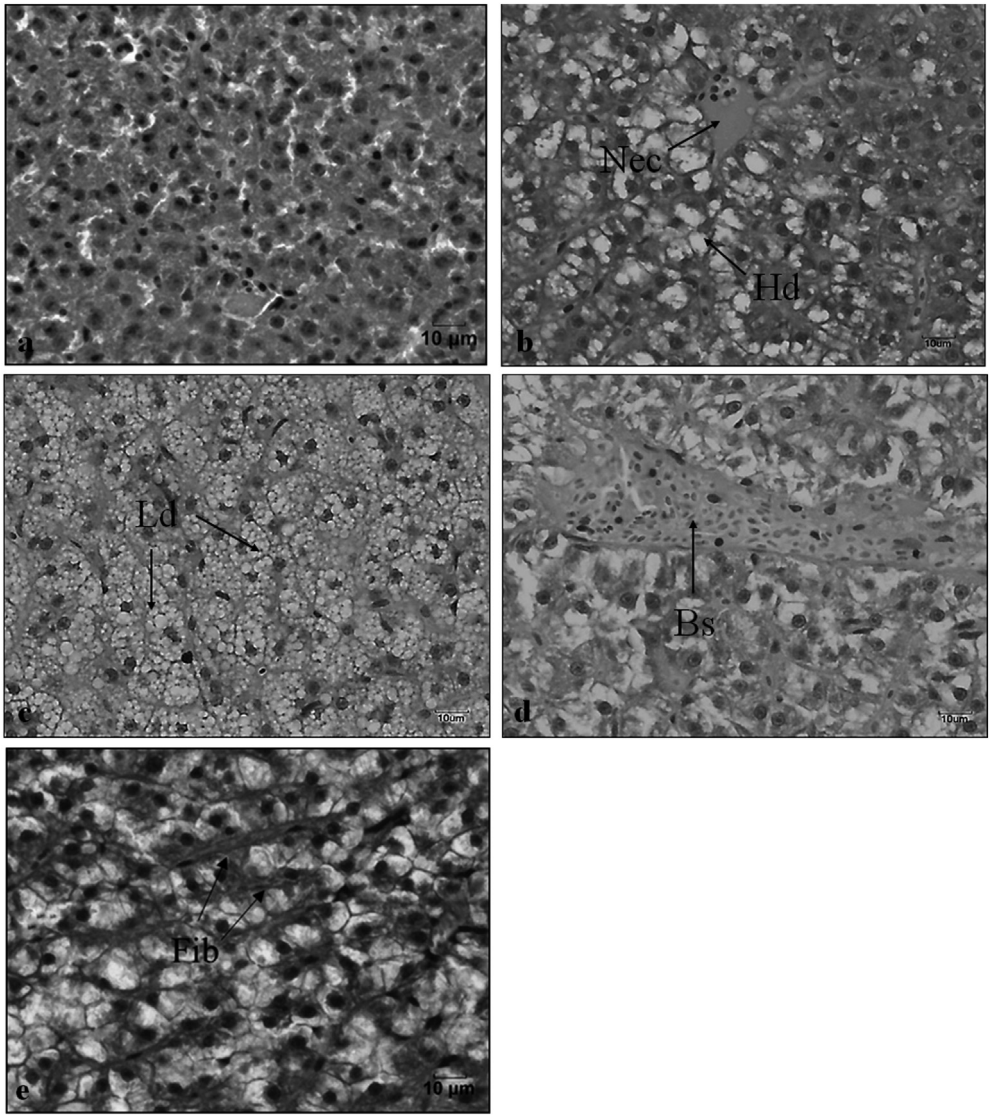

Fig. 2 (*continued*).

percentage (Cazenave et al., 2008), characterizing the species as diurnal with higher swimming speed. In the present study, the dry season showed the most altered swimming behavior with decreased IE and increased TL at AL and PLP, accompanied by a tendency to increase swimming speed. These altered behaviors can disrupt several activities such as feeding and reproduction, as well as make fish more susceptible to predation (Little and Finger, 1990). An increase in mean speed has been interpreted by other authors as an indirect index of erratic movements, since this behavior occurs with sharp increases in speed (Champagne et al., 2010). The erratic movement is considered an accurate indicator of acute stress response (Kalueff et al., 2013). Moreover, during this season, fish exposed at the AL and PLP sites remained longer in the lateral section of the tank (an effect known as thigmotaxis) than fish from the reference site SR. The literature describes this as a defensive response, seeking refuge and protection at the edges of the tank (Maximino et al., 2010). However, these same altered behaviors have been described in laboratory conditions of fish exposed to herbicides (atrazine) and pharmaceutical drugs (nicotine) (Schmidel et al., 2014; Stewart et al., 2015). Our results suggest that the water quality in the sites downstream of Piedras Moras dam in the dry season alters the normal exploratory strategy of the fish.

Behavioral responses are often more sensitive than other indicators of toxic exposure (Little and Finger, 1990). However, against our expectations, in the wet season, the behavioral variables showed no significant differences between sites.

4.3.2. General health status of fish

The CF index showed no differences among sampling sites. Other similar studies using the caged fish method in wastewater-contaminated rivers also showed no differences in this biomarker between contaminated and reference sites during seven days exposure (Cazenave et al., 2014). Since the experimental design guaranteed access to food, one explanation could be the time of exposure; seven days was not enough to cause a significant decrease in weight.

HSI responses differed among sampling sites in the dry and the wet season. In the dry season, caged fish in AL had a higher HSI value than SR, and in the wet season PVM had the lowest. This differential response matches Almeida et al. (2005) who observed both increases and decreases in the index values in *Prochilodus lineatus* exposed to sediment collected at different sites on a river contaminated with urban pollution. These authors interpreted that a decrease in HSI could be due to a depletion of glycogen stores in the liver related to the increase in energy demand from the stress of pollution. They also pointed out that the index

Table 5
Enzyme activity (nkat/min) measured in caged individuals of *Jenynsia multidentata* in Ctalamochita River.

Hydrological season								
Dry					Wet			
	SR	AL	PLP	PVM	SR	AL	PLP	PVM
GST								
Liver	2.8 ± 0.5a	2.9 ± 0.1a	3.5 ± 1.3a	NA	3.6 ± 0.5a	2.8 ± 0.2a	2.8 ± 0.43a	2.6 ± 0.7a
Gills	0.7 ± 0.2a	1.9 ± 0.2c	1.3 ± 0.1b	NA	0.9 ± 0.2a	0.7 ± 0.1a	0.9 ± 0.1a	1.2 ± 0.6a
Brain	1.9 ± 0.6a	1.6 ± 0.5a	2.5 ± 0.4a	NA	2.4 ± 0.1b	2.9 ± 0.1b	1.31 ± 0.1a	1.6 ± 0.2a
Muscle	0.4 ± 0.1a	0.2 ± 0.03a	0.5 ± 0.03a	NA	0.3 ± 0.1a	0.3 ± 0.02a	0.25 ± 0.04a	0.2 ± 0.1a
CAT								
Liver	1469.1 ± 257.9b	622.5 ± 113.6a	1438.9 ± 116.4b	NA	751.5 ± 78.3a	865.0 ± 116.9a	839.8 ± 68.0a	870.3 ± 244.5a
Gills	34.9 ± 12.0a	43.1 ± 4.0a	37.0 ± 9.1a	NA	17.8 ± 3.2a	23.0 ± 3.7a	18.0 ± 2.1a	19.8 ± 11.5a
Brain	16.0 ± 2.9a	24.4 ± 4.1a	35.2 ± 12.5a	NA	18.6 ± 1.7b	11.6 ± 0.5a	11.9 ± 2.0a	7.1 ± 0.7a
Muscle	6.1 ± 1.3a	5.2 ± 0.8a	3.9 ± 0.1a	NA	3.5 ± 0.7a	3.1 ± 0.2a	5.8 ± 1.8a	4.8 ± 1.7a
AChE								
Brain	22.0 ± 1.7b	11.4 ± 2.4a	46.7 ± 4.3c	NA	23.2 ± 2.4a	19.2 ± 3.8a	17.7 ± 3.6a	15.7 ± 1.8a
Muscle	4.1 ± 0.6a	4.4 ± 0.6a	7.58 ± 0.7b	NA	3.9 ± 0.8a	3.3 ± 0.2a	4.4 ± 0.8a	3.6 ± 0.9a
BChE								
Liver	11.7 ± 2.1ab	5.7 ± 1.9a	18.5 ± 2.62b	NA	4.3 ± 1.2a	8.0 ± 1.0ab	9.1 ± 1.6b	3.7 ± 1.3a
Muscle	1.4 ± 0.4a	1.5 ± 0.149a	2.9 ± 0.15b	NA	1.6 ± 0.4a	0.9 ± 0.3a	2.0 ± 0.7a	1.5 ± 0.1a

Data expressed as mean ± standard error (SE). References: SR: Santa Rosa, AL: Almafuerte, PLP: Puente los Potreros, PVM: Post-Villa María. Different letters indicate statistical differences among sampling sites ($p < 0.05$). GST: Glutathione-S-Transferase; CAT: Catalase; AChE: Acetyl-cholinesterase: BChE: Butyryl-cholinesterase; NA: not analyzed.
Graphical abstract

may increase because of hypertrophy and hyperplasia of hepatic cells related with detoxification processes. Similarly, the review study by Van der Oost et al. (2003) showed that in other field and laboratory studies, where the fish were exposed to PAHs (polycyclic aromatic hydrocarbons), PCBs (polychlorinated biphenyls) and OCPs (organochlorine pesticides), the HSI also decreased or increased. We were not able to associate the HIS changes either with histological damage or with enzyme activities analyzed in this study. The responses of the liver (reflected in that index) can thus be highly variable and can be explained only with parallel studies that include other variables that contribute to clarifying the observed pattern.

4.3.3. Gill and liver histomorphology and histological indexes

Numerous studies describe a wide variety of toxic substances in the aquatic environment that affect the structure and function of the gills and liver (Sensini et al., 2008; Wood, 2001). With this unspecificity, the same histological alterations were found, at all the sampling sites and in both hydrological seasons. In gills, most of the damage was of the regressive and progressive type (RP1 and RP2), such as lifting and hyperplasia of epithelial cells, shortening and fusion of secondary lamellae and chloride cell hyperplasia. These were more frequently observed in the middle basin sampling sites during both seasons. The most frequent liver alterations were hydropic and lipid degeneration, followed by necrosis and fibrosis, included in the RP1 and RP2 patterns. Such damage has been reported as a response to exposure to various toxicants, regardless of their chemical nature. Fibrosis and necrosis are the most serious damage registered since they are irreversible and their presence indicates the total loss of tissue function in the affected area. Necrosis usually is accompanied by leukocyte infiltration to remove dead cells, as was recorded in this study. Although these regressive and progressive changes in gills and liver cannot be associated with the presence of a specific contaminant, they have been observed in fish exposed to anthropogenic pollutants such as agrochemicals (Ballesteros et al., 2007), heavy metals (Arellano et al., 1999) and wastewater (Bernet et al., 2004), as well as natural toxins (Prieto et al., 2008).

It should be noted that both I_{gill} and I_{liv} reflected alterations in different hydrological seasons. The overall index for histopathological gills (I_{gill}) showed significant differences in the wet season only, with a higher score at AL and PLP than at SR. On the other hand, liver was the most damaged organ during the dry season. This was significantly higher at AL only during the dry season. During the wet season, no changes were reflected in the index. In both organs, the main alterations corresponded to circulatory and regressive patterns.

The total histopathological index (I_{tot}) showed that, during the wet season, all sampling stations below Piedras Moras Dam exceeded a score of 70 (the higher score the more affected the organ or individual), which was significantly different from the reference site, SR. In contrast, during the dry season, there was only a tendency to increase in the index, which was only close to 60.

4.3.4. Enzyme activity

A differential response was observed by fish organs and sampling sites in the enzyme involved in the biotransformation of xenobiotics, GST, as well as in the oxidative stress enzyme, CAT. Greater GST activity was recorded in gills at AL and PLP than at the reference site during the dry season, while its inhibition in the brain was seen at sites PLP and PVM during the wet season. It is widely known that the activity of biotransformation and oxidative stress enzymes can be increased or inhibited under chemical stress, depending on the nature of the compound, the intensity and duration of the stress, as well as the susceptibility of the species (Cheung et al., 2001). Our results agree with those of Maggioni et al. (2012) and Monferrán et al. (2011) in gills and brain of females of *J. multidentata* collected in contaminated sites on the Suquía River.

Inhibition of CAT activity was recorded in livers exposed to the AL site during the dry season and in brain at all sampling sites in the wet season. Inhibition of this enzyme can result in an excess of hydrogen peroxide (H_2O_2) within the cell, generating an oxidizing atmosphere therein. This compound can cross biological membranes, causing damage in DNA and proteins, as well as increasing the occurrence of lipid peroxidation (Ansari et al., 2014). These effects were also observed in laboratory experiments with specific toxic organic as well as inorganic compounds. For example, Pandey et al. (2001) reported a significant increase in GST activity in gills and CAT inhibition in the liver of the catfish *Channa punctatus* exposed to endosulfan. CAT inhibition has also been found in livers of *Rhamdia quelen* exposed to 1.21 $mg \cdot L^{-1}$ of glyphosate (Ferreira et al., 2010) and in *Cyprinus carpio* exposed to different concentrations of atrazine and chlorpyrifos (Xing et al., 2012). As was mentioned previously, sites located below the Piedras Moras Dam (AL, PL and PVM) are close to cultivated areas whose final destination may be the river. Future studies remain to be made on the presence of these pesticides in the basin.

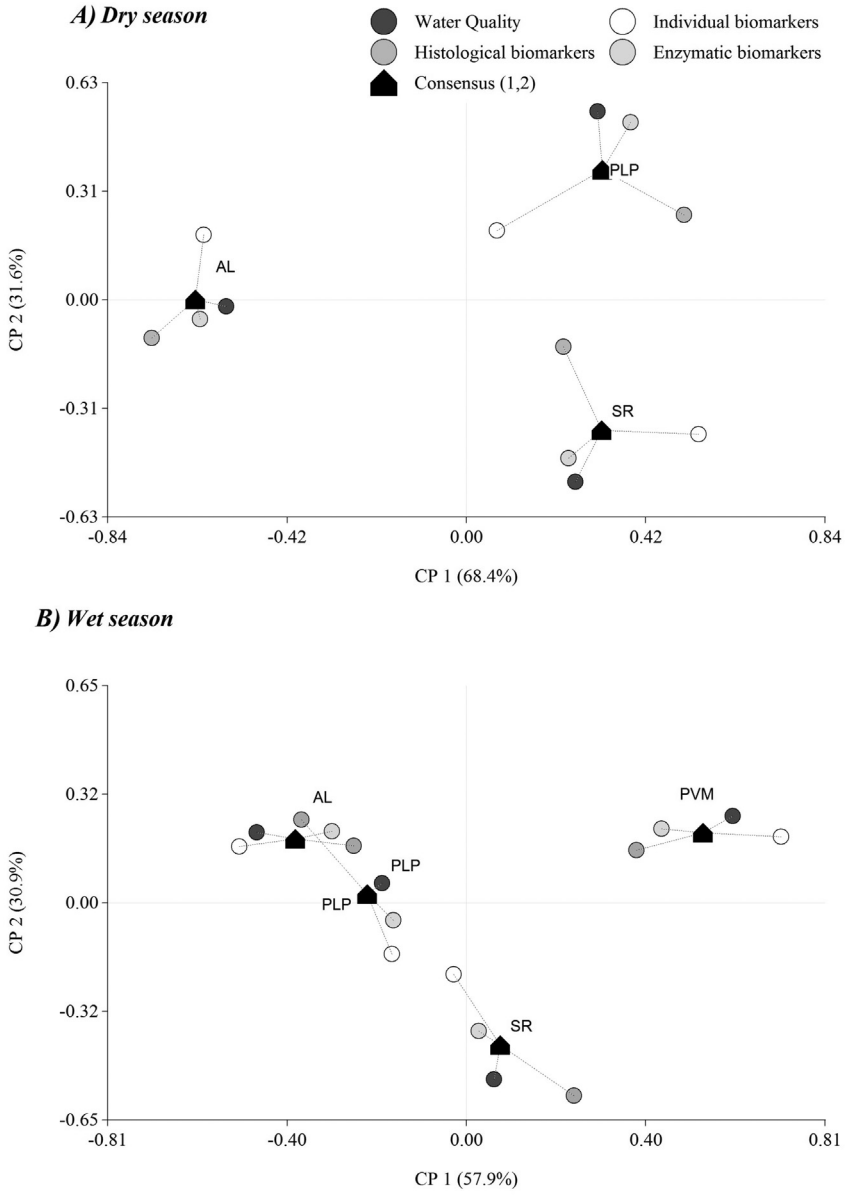

Fig. 3. Consensus space from Generalized Procrustes Analysis of water quality variables and biomarkers in caged individuals of *Jenynsia multidentata*. References: SR = Santa Rosa; AL = Almafuerte; PLP = Puente los Potreros; PVM = Post- Villa María.

Inhibition of AChE in the brain was observed only during the dry season in caged fish exposed at AL, while an increase was registered in this organ and in muscle at PLP. Organophosphorus compounds are known to produce inhibition of AChE and BChE activity (Aker et al., 2008; Barbieri and Ferreira, 2011). Only a few studies report increases in AChE activity in fish in the presence of toxic compounds. An increase of AChE was registered in the brain of fish exposed to the detergent sodium dodecylbenzenesulfonate and heavy metals such as copper, cadmium and aluminum (Jifa et al., 2006, Maheswari et al., 2014). In this study, high total metal levels were recorded in water and sediments with the presence of heavy metals such as copper, cadmium and aluminum at PLP and AL (O'Mill, 2012). Salles et al. (2006) pointed out that organisms with high amounts of BChE in liver or serum may be resistant to poisoning by organophosphate pesticides. In this study, a significant increase of BChE activity in liver was found at PLP in the wet season and in muscle in the dry season. BChE may thus be playing a detoxification function in the liver.

4.4. Multivariate analysis

The results obtained through the Generalized Procrustes Analysis (GPA) gives us an idea about the relationship among groups of variables at a particular sampling station. During the dry season, where contaminants are concentrated in water due to the lower water flow and lack of rains, all sampling sites were separated in the multivariate space by the combination of groups of biomarkers. On the other hand, during the wet season, the GPA could only separate SR and PVM individually, leaving AL and PLP together. The distance from AL to PLP is only 25 km, much less than between the other sites. Because these intermediate sites are surrounded by crops, there is a continuous input of pesticides that may homogenize the situation and clearly separate the control station from PVM as the worst environmental condition. At both sampling seasons, the groups of biomarkers that best explain the contamination situations where the enzyme group first, followed by the physicochemical group. The level of enzyme organization is considered an early response, compared with the other groups of biomarkers

(histological and individual groups) (Van der Oost et al., 2003). Therefore, the results of this analysis showed the relationship between the biological and physicochemical parameters that determine the particular multistressor context in each sampling season.

5. Conclusions

- This study is the first integral study of Ctalamochita River analyzing physicochemical and bacteriological parameters together with biomarkers at different levels of organization.
- Mortality, the increase or decrease of enzyme activities, histological alterations in gills and liver, and behavioral changes were observed in individuals of *J. multidentata* exposed in cages at different sites of the river.
- Through the field caging study, we demonstrated our hypothesis that contamination of Ctalamochita River occurs at sites impacted with different anthropic activity. This hypothesis was validated because the biomarkers proposed responded according to the sampling site and hydrological season. Considering that the study area has different sources of contamination (agriculture, industries, sewage discharges), the individual responses registered in the biomarkers cannot be associated with a specific contaminant, but it is evident that a complex mixture of substances produces effects on the biota.
- The GPA showed that the combined use of groups of biomarkers and the water quality parameters enabled us to differentiate sampling sites. The differences are related with the hydrological season and highlight the importance of evaluating biomarkers at different levels of organization in the multi-stressor context of a river. Consequently, the use of a battery of complementary biomarkers is recommended for the study of exposure to water that receives different sources of contamination.

Acknowledgments

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