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Inhibition of the brain cytochrome P450 aromatase isoform expression in *Jenynsia multidentata* reflects changes in water quality

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Abstract

Cytochrome P450 aromatase is the steroidogenic enzyme that catalyzes the conversion of androgens to estrogens. Teleosts express two structurally and functionally different P450 aromatase isoforms, termed Cytochrome 19a1a and Cytochrome 19a1b. The first is preferentially expressed in ovary while the second is constitutively expressed in brain. The aim of the present study was to evaluate changes in *cyp19a1b* expression in brain of *Jenynsia multidentata* collected in Suquía river (Córdoba, Argentina) in order to assess if this biological response reflects changes in water quality. For this purpose we first identified *cyp19a1b* parcial cDNA sequence and adjusted the method to quantify mRNA expression by qRT-PCR (quantitative RT-PCR). Then, water and fish samples were collected in five monitoring stations located along a pollution gradient in Suquía river. The qRT-PCR analysis of female brains reveals that *cyp19a1b* mRNA levels change as water pollution does, showing the highest levels at both unpolluted and recovered areas, with suppression as pollution increases. Even when *cyp19a1b* expression in *J. multidentata* will need further studies, the results here presented indicate that this biological response appear as a promising biomarker of water pollution that may also point out the probable presence of endocrine disruptors.

Keywords: Aromatase; Fish; Real time RT-PCR; Water pollution.

La inhibición de la expresión de Citocromo P450 aromatasa en cerebro de *Jenynsia multidentata* refleja cambios en la calidad de agua

Resumen

La enzima Citocromo P450 aromatasa cataliza la conversión de andrógenos en estrógenos. En teleósteos se expresan dos isoformas (Citocromo 19a1a y Citocromo 19a1b) que difieren en su estructura y funcionalidad. La cyp19a1a se expresa preferentemente en ovario mientras que cyp19a1b se encuentra en cerebro. El objetivo del presente trabajo fue evaluar cambios en la expresión de *cyp19a1b* en cerebro de *Jenynsia multidentata* recolectadas en el río Suquía (Córdoba, Argentina) para evaluar si esta respuesta biológica refleja cambios en la calidad de agua. Con este fin se identificó la secuencia parcial del cADN de *cyp19a1b* y se ajustó un método para cuantificar la expresión de mARN por qRT-PCR (RT-PCR cuantitativa). Posteriormente se recolectaron muestras de agua y peces en cinco estaciones de monitoreo con distinto grado de contaminación en el río Suquía. El análisis en la expresión de *cyp19a1b* mARN en cerebros de hembras mostró los mayores niveles de expresión en áreas de baja contaminación e inhibición en los sitios con mayor contaminación. Si bien se necesitan aún estudios complementarios, los resultados encontrados son promisorios en el uso de expresión de *cyp19a1b* en *J. multidentata* como biomarcador de contaminación acuática que podría indicar la presencia de disruptores endócrinos.

Keywords: Aromatasa; *Jenynsia multidentata*; RT-PCR en tiempo real; Contaminación acuática.

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INTRODUCTION

Cytochrome P450 aromatase is the steroidogenic enzyme that catalyzes the conversion of androgens (C19) to estrogens (C18). The enzymatic complex consists of the Cytochrome P450 aromatase, product of the *cyp19* gene, and the flavoprotein NADPH-cytochrome P450 reductase (Simpson *et al.*, 1994).

Euteleosts express two structurally and functionally different P450 aromatase isoforms, termed Cyp19a1a and Cyp19a1b. The first is preferentially expressed in ovary, playing important roles in sex-differentiation and oocyte growth, while the second is constitutively expressed in brain and is probably involved in the development of central nervous system, survival, morphology, synaptogenesis, neurogenesis, neuroplasticity, neuroprotection, sex differentiation and sexual and aggressive behavior (Kishida *et al.,* 2001; Kwon *et al.,* 2001; Trant *et al.,* 2001; Gracía-Segura *et al.,* 2003; Blázquez *et al.,* 2008; Clotfelter & Rodriguez, 2010; Diotel *et al.,* 2010).

Since aromatase genes are potential targets for endocrine disruption (reviewed in Cheshenko *et al.*, 2008) considerable research effort has been done to detect changes in *cyp19* expression or Cyp19 activity, which could indicate the presence of endocrine disrupting compounds (EDCs) in the environment (Noaksson *et al.,* 2001, 2003a, 2003b; Orlando *et al.,* 2002; Greytak *et al.,* 2005).

Endocrine disruption in fish is strongly linked to natural or man-made chemicals that enter rivers and streams. Sewage treatment works, for example, release a complex mixture of natural and synthetic chemicals (domestic, industrial and/or agricultural waste) into the aquatic environment (Jobling *et al.,* 2004). As urban areas tend to increase, the concentrations of these compounds in rivers is also likely to increase.

Research on endocrine disruption in wild freshwater fishes has been limited to a small number of northern hemisphere fish species of commercial importance. Among neotropical fishes, *cyp19* genes were described for only two species *Odontesthes bonariensis* **(**Strobl-Mazzulla *et al.,* 2005), and eastern mosquitofish *Gambusia holbrooki* (Orlando *et al.,* 2002).

Jenynsia multidentata (Anablepidae, Cyprinodontiformes) is a native fish inhabiting the neotropical region, from the Río Negro Province (Argentina) to the city of Río de Janeiro (Brazil) (Malabarba *et al.,*1998). It is frequently used for experimental studies (Cazenave *et al.,* 2008; Pesce *et al.*, 2008; Amé *et al.,* 2009; Ballesteros *et al.,* 2009a, 2009b). However, the biomarkers investigated in *J. multidentata* are considered as non-specific ones, since several pollutants can modify directly or indirectly their responses (Monserrat *et al.*, 2007).

The aim of the present study was to assess changes in *cyp19a1b* expression in the brain of *J. multidentata* in an urban polluted river. For this purpose we identified *cyp19a1b* parcial cDNA sequence and adjusted the method to quantify mRNA expression. We decided to evaluate the *cyp19a1b* expression considering previous reports showing that aromatase activity in brain is several times higher than in ovary and that *cyp19a1b* could be used as sensitive exposure biomarker (González & Piferrer, 2002).

MATERIALS AND METHODS

Isolation of cyp19a1b partial cDNA sequence and RT-qPCR

Adult females of *J. multidentata* were collected from an unpolluted site (San Antonio River, Córdoba, Argentina) (Wunderlin *et al.,* 2001; Hued & Bistoni, 2005) using a backpack electrofisher. Fish were immediately transported to the laboratory in water tanks (20 L), acclimatized for two weeks at 21±1ºC, with a photoperiod of 12-h light: 12-h dark. They were kept in a 15 L aerated glass aquarium, containing aquarium water (Best *et al.,* 2002).

After dissection, brain was quickly removed, snap-frozen in liquid nitrogen and stored in RNA*later* (QIAGEN) at -80ºC until analysis. Total RNA from a ten fish pool was extracted by the guanidine thiocyanate-phenol chloroform extraction method according to Chomczynski and Sacchi (1987). Non-specific reverse transcription was performed on 5 µg RNA using a first strand complementary DNA synthesis method (Amé *et al.,* 2009).

The resulting cDNA was used as template to amplify *cyp19a1b* using a polymerase chain reaction (PCR) strategy. Degenerate primers were designed based on conserved nucleotide sequences with high interspecies homology of fish *cyp19a1b* cDNAs available in GenBank (*Cyp19a1b* Forward 5'AAYTAYTTYGARACNTGGCA3', Reverse 5'NCCRCANCCRAANGGYTGRAA3'). PCR was performed and the expected 660bp amplicon was cloned into pCR 2.1 plasmidic vectors (Invitrogen) and transformed in *E. coli* competent cells. The plasmidic cDNA was sequenced in Macrogen Inc. (Korea), and submitted to Blast for comparison with previously reported teleostean *cyp19a1b* cDNA sequences accessible in GenBank Database (http://www.ncbi.nlm.nih.gov). From the partial cDNA sequence obtained, new specific and non-degenerate primers for real-time RT-PCR were designed (*Cyp19a1b* Forward 5'GAAACATCATTAACAAAACTGAGAAACTG3', Reverse 5' GAGAGCTCCCCATGGTTCTG3') with Primer Express Software (Applied Biosystems).

β-actin was used as housekeeping gene. Primers for real-time PCR (*β-actin* Forward 5'-CCA AAG CCA ACA GGG AGA AGA TGA-3'; Reverse 5'-TCG GCT GTG GTG GTG AAG GAG T-3') were designed based on *J. multidentata β-actin* sequence previously described (Genbank accession numbers EF362747, Amé *et al.*, 2009).Quantitative PCR was used to amplify and measure the transcript abundance of *cyp19a1b* in brain. Real-time PCR was performed on a Bio-Rad iQ cycler with 1 µL of a 1/10 dilution of cDNA (25 ng reverse-transcribed total RNA), the SYBR Green PCR Master Mix spiked with fluorescein 10 nM (Applied Biosystems) and 7.5 pmol primers in a final volume of 20 µL. Cycling conditions were as follows: 1 x 95ºC, 15 min; 45

x [94ºC, 15 sec, 61.2ºC, 30 sec and 72ºC, 30 sec]. Real-time PCR reactions were run in triplicate for each cDNA sample and melting curves were carried out to monitor the quality of amplicons and reactions. Amplifications were also performed on 25 ng of non-retrotranscribed RNA as negative control to check absence of genomic contaminants. To estimate efficiencies, a standard curve was generated for each primer pair based on known quantities of cDNA (10-fold serial dilutions corresponding to cDNA transcribed from 100 to 0.01 ng of total RNA). The relative expression levels (fold change) of the gene, calculated using the relative expression software tool (REST©), were based on mean threshold cycle differences between each site and the sampling station located before Córdoba city (Pfaffl *et al.,* 2002).

Cyp19a1b measurement in wild fish

In order to assess the changes in *cyp19a1b* expression associated to urban pollution, we used adult female brains of *J. multidentata* collected in July 2006 (non-reproductive season) at different sampling stations of Suquía River basin (Fig. 1). Sampling stations were selected according to previous studies on water quality and fish assemblages, reporting high anthropogenic impact in locations downstream from the sewage discharge of Córdoba City (Argentina) (Pesce & Wunderlin, 2000; Wunderlin *et al.,* 2001; Hued & Bistoni, 2005). Thus, five sites with different pollution levels ranging from quasi pristine to heavily polluted along Suquía River were sampled. La Calera (LC) is located 30 km upstream from Córdoba City (considered as quasi pristine site); Villa Corazón de María (CM) is 15 km downstream from the Córdoba Waste Water Treatment Plant (WWTP) discharge. Further stations: Capilla de los Remedios (CR), Río Primero (RI) and Santa Rosa de Río Primero (SR) are situated 35, 70 and 110 km downstream from the WWTP, respectively. Water quality parameters were determined at each sampling site to describe the environmental conditions of the study area. Dissolved oxygen, conductivity, pH and temperature were monitored in the field, while water samples were analyzed in the laboratory for 5-day biological oxygen demand (BOD-5), chemical oxygen demand (COD), ammonia-nitrogen, nitrite-nitrogen, nitrate-nitrogen, orthophosphate-phosphorous, chloride, sulphates, total solids, and total coliforms (all determinations were performed according to AOAC, 1995 and APHA, 2005). These physicochemical parameters were integrated into a Water Quality Index (WQI, Pesce & Wunderlin, 2000). The construction of WQI requires normalization for each parameter on a 0-100 scale to avoid interferences arising from different magnitudes of the parameters measured, with 100 being the optimal and 0 the worst water quality. Further to normalization, WQI requires the application of a weighting factor to each measured parameter, reflecting its importance on the water quality (Pesce & Wunderlin, 2000). WQI gives then a non-dimensional number that can be associated with a quality percentage.

Seven fish were collected at each site as described in 2.1 and transported to the laboratory in fresh water tanks. Fish were kept within their respective river water with aeration, and dissected within 12 h of capture. The means of the standard length (SL) and mass were 42.0 ± 4.1 mm and 1.45 ± 0.11 g, and were not statistically different between sampling stations. Brain was quickly removed and treated as previously described for RNA extraction.

Statistical analysis

All values are expressed as mean ± standard deviation. Statistical analyses were carried out using Infostat Software Package (Di Rienzo *et al.,* 2011). Normal distribution of data was analyzed by Shapiro Wilks test, and Levene test was used to test the homogeneity of variance. One-way ANOVA followed by DGC's (Di Rienzo *et al.,* 2002) test was performed to compare the sampling sites. Spearman correlation test was used to determine association between WQI and *cyp19a1b* expression. Significance was set at $p < 0.05$.

The link between water quality parameters and *cyp19a1b* expression in *J. multidentata* was assessed by means of a Principal Component Analysis (PCA) performed on the standardized variables in order to avoid misclassifications

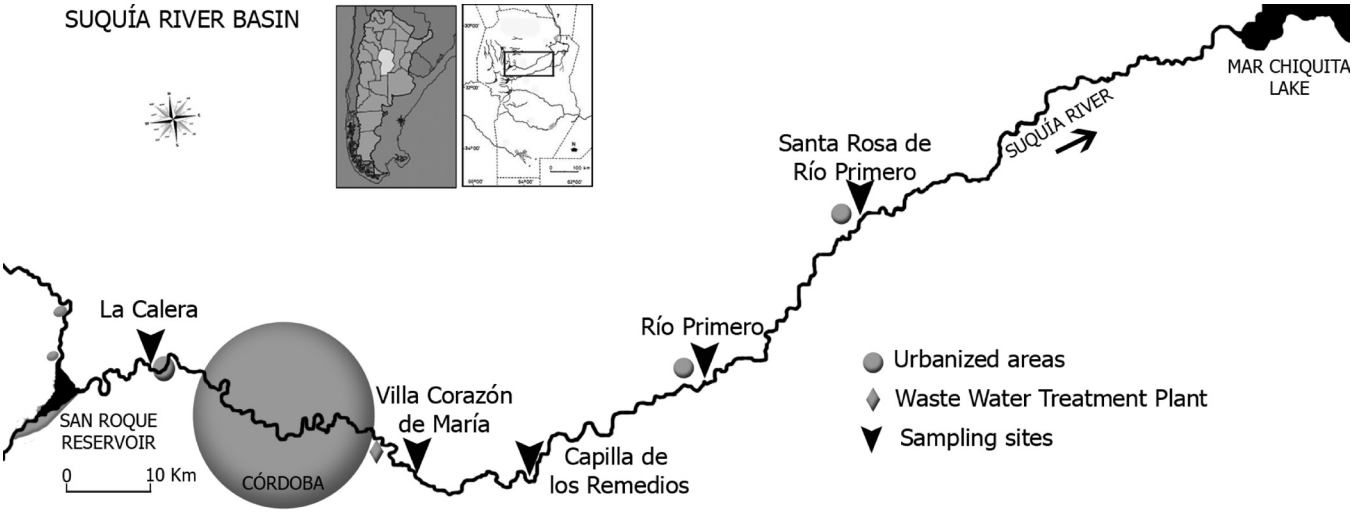

Figure 1 - Sampling stations selected along the Suquía River Basin.

arising from the different orders of magnitude of both numerical value and variance of the parameters analyzed (Wunderlin *et al.,* 2001; Pereira *et al.,* 2009).

RESULTS

Cyp19a1b in Jenynsia multidentata

GenBank accession number of the partial cDNA sequence for *cyp19a1b* of *J. multidentata* is EU851873. The characterized partial fragment of *Cyp19a1b* was 660bp in length, encoding a 184 amino acid polypeptide with a predicted molecular weight of 21.3 kDa. A BLAST search revealed the highest homology of *cyp19a1b* with *Poecilia reticulata* (90%) and *Fundulus heteroclitus* (89%).

The amplification efficiencies of real-time RT-PCR obtained for *cyp19a1b* and *β-actin* were 1.88 and 1.87, respectively. The slopes used to calculate the amplification efficiency were -3.65 and -3.69 for *cyp19a1b* and *β-actin*. The use of *β-actin* as housekeeping gene was tested in our experiments. Non significant differences were observed between sampling stations.

Field study

Water quality parameters

As it can be observed from Table 1, water quality was greatly altered by Córdoba city as showed by significant differences for all the measured parameters between LC and CM. This deterioration could be mainly attributed to urban run-off and to the city sewage discharge. Evidences of these facts are the drastic drop in dissolved oxygen and the increase in BOD, COD, total inorganic nitrogen (TIN=additive amounts of ammonia, nitrates and nitrites nitrogen), phosphorous, sulfates and chloride concentrations as well as in the proliferation of coliform bacteria at CM (Table 1). However, different patterns were observed downstream this monitoring station. Ammonia and nitrites-nitrogen, BOD and DO displayed a slow recovery from CM to SR. In contrast, chloride, phosphorous, sulfates, total solids, conductivity and temperature remained with similar levels throughout CR, RI and SR. Nitrates- nitrogen increased approximately two times from CM to CR and continue with this level toward SR. It is remarkable that the rise in TIN at stations directly affected by the city sewage discharge was due to ammonia nitrogen content of water (CM and SR) while in other monitoring stations the main contributor to TIN was nitrates- nitrogen. TIN pattern observed in our study is in good agreement with previous reports (Pesce & Wunderlin, 2000; Wunderlin *et al.,* 2001). COD displayed a significant maximum at CM and lower values with punctual variations at the other monitoring stations.

The applied WQI (Table 1) showed a significant drop after Córdoba city. The higher impact of urban pollution was observed at CM, CR and RI (WQI= 36.6, 42.6 and 51.5 respectively) contrasting with the 73.7 observed in the sampling station located upstream from Córdoba (LC). Further downstream, at SR the river tend to restore its water quality, with WQI reaching means of 60.1.

Cyp19a1b expression in wild fish

As shown in Fig. 2, when expressed levels of *cyp19a1b* were analyzed by one way ANOVA, significant differences were obtained. In naturally exposed fish, the highest mRNA levels of *cyp19a1b* were found at the extremes of the gradient studied, in LC (reference site) and SR (Fig. 1). CM, CR and RI had a significant inhibition of mRNA expression in comparison with the reference site.

Association between cyp19a1b expression and water quality

Principal component analysis (PCA) is an unbiased method which indicates associations between samples and/or variables. These associations, based on similar magnitudes and variations in physiological responses, chemical and physical constituents, may indicate the presence of seasonal or human influences (Wunderlin *et al.,* 2001).

Three principal components were defined as explaining the major amount of total variance (97%). The first principal component (PC1) accounts for 64% of the variance and shows the association between *cyp19a1b* expression in brain of *J. multidentata* with the WQI, dissolved oxygen and pH levels in the water samples of Suquía River (Table 2 and Fig. 3). These parameters showed lower levels at the most impacted sites. PC1 has positive scores at the highly polluted sites (CM, CR and RI) and negative scores for the less contaminated stations (LC and SR, Table 2 and Fig. 3). The PC2 and PC3 account for the 25 % and 9% of the total variance respectively. PC2 separates those parameters with different variation pattern through the river basin as has been previously described (3.2.1). PC2 has positive scores for the parameters with higher levels at the low basin (chloride, phosphorous, sulfates, total solids, conductivity, pH and temperature) and negative scores for the variables that show recovery after CM (Total coliforms, BOD, COD, nitrites and ammonia-nitrogen) (Fig. 3).

DISCUSSION

It is widely accepted that human activities affect not only the physical, chemical and biological process in a stream, but also the resident biota and the associated human population. Suquía River is not an exception. As most water courses that run across big cities, receives pollutants of complex composition from different sources (Wunderlin *et al.,* 2001; Nimptsch *et al.,* 2005; Contardo-Jara *et al.,* 2009). A significant drop in WQI has been previously informed and was directly related with the poorly treated sewage discharges from the municipal WWTP, the main source of water pollution in the Suquía River (Pesce & Wunderlin, 2000; Contardo-Jara *et al.,* 2009).

Table 1 - Water quality parameters of selected sampling sites in Suquía River basin. Values are expressed in mg L^{-1} if not indicated directly, total coliforms correspond to E^{+3} exponential values (i.e. 2.3=2300), and are expressed as MPN 100 m L^{-1} (most probable number per 100 mL). Different letters indicate significantly different values at different monitoring stations ($p<0.05$).

Parameters	Sampling sites				
	La Calera (LC)	Villa Corazón de María (CM)	Capilla de los Remedios (CR)	Río Primero (RI)	Santa Rosa de Río Primero (SR)
Ammonia-nitrogen	0.05 ± 0.04^{a}	11.07 ± 1.05^{b}	13.53 ± 2.55^{b}	0.60 ± 0.33^{a}	0.16 ± 0.04^{a}
Biological Oxygen demand after 5 days (BOD)	2.7 ± 0.5^{a}	20.0 ± 0.5^{e}	12.9 ± 0.2^{d}	6.9 ± 0.2^{c}	3.9 ± 0.1^{b}
Chemical oxygen demand (COD)	99 ± 1^{d}	202 ± 6^{e}	74 ± 2^{b}	54 ± 2^{a}	82 ± 1^{c}
Chloride	22.2 ± 0.1^{a}	89.9 ± 0.1^{c}	80.1 ± 0.2^{b}	89.9 ± 0.1^{d}	80.0 ± 0.2^{b}
Conductivity [µS cm^{-1}]*	405 ± 2^{a}	1474 ± 2^{e}	1354 ± 3^{c}	1387 ± 6^{d}	1340 ± 5^{b}
Dissolved oxygen *	13.1 ± 2.1^{b}	5.1 ± 1.6^{a}	7.1 ± 0.8^{a}	8.3 ± 1.4^{a}	10.4 ± 1.6^{b}
Nitrates-nitrogen	0.46 ± 0.04^{a}	3.74 ± 0.55^{b}	6.39 ± 0.78^{c}	7.96 ± 0.63^{d}	6.79 ± 0.45^{c}
Nitrites-nitrogen	0.03 ± 0.01^{b}	0.53 ± 0.01^{d}	0.23 ± 0.01^{c}	0.22 ± 0.01^{c}	0.01 ± 0.01^{a}
Orthophosphate Phosphorous	0.01 ± 0.01^{a}	0.37 ± 0.02^{b}	0.42 ± 0.03^{c}	0.35 ± 0.03^{b}	0.44 ± 0.05^{c}
pH *	8.0 ± 0.1^{b}	7.5 ± 0.3^{a}	7.6 ± 0.1^{a}	7.7 ± 0.1^{a}	8.3 ± 0.1^{b}
Solids: Total	168 ± 1^{a}	845 ± 2^{d}	819 ± 5^{c}	954 ± 6^{e}	786 ± 5^{b}
Sulfates	32 ± 1^{a}	244 ± 2^{d}	218 ± 2^{b}	232 ± 3^{c}	232 ± 2^{c}
Temperature * [°C]	8.3 ± 0.2^{a}	11.4 ± 0.2^{b}	10.3 ± 0.3^{b}	11.7 ± 0.4^{b}	13.5 ± 0.2^{b}
Total coliforms	5.0 ± 0.1^{a}	4400 ± 0.2^{e}	220 ± 0.1^{c}	750 ± 0.1^{d}	110 ± 0.1^{b}
WQI	73.7 ± 3.0^{e}	36.6 ± 3.4^{a}	42.6 ± 2.1^{b}	51.5 ± 0.5^{c}	60.1 ± 2.3^{d}

* Field measurements during the monitoring.

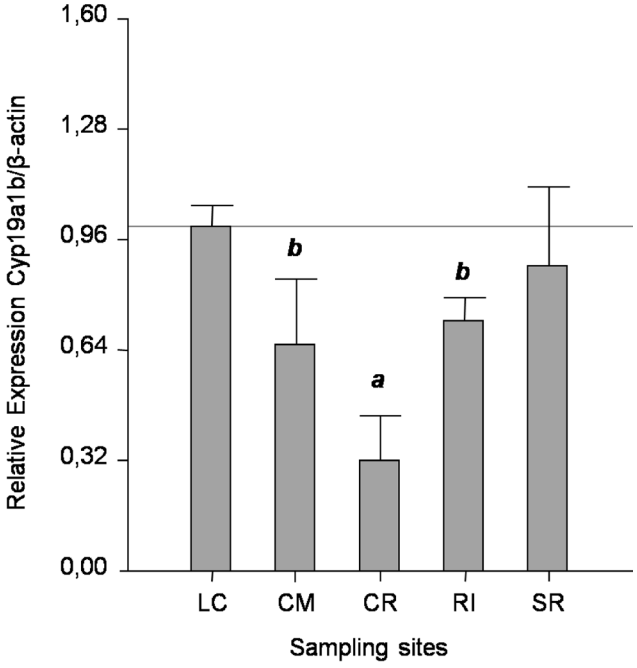

Figure 2 - Brain *cyp19a1b* expression in *J. multidentata* as fold change relative to *β-actin*, measured by qRT-PCR. Different letters indicate significant statistical differences to control ($p<0.05$). Horizontal line at 1 indicates the gene expression at the site located before Córdoba (LC).

The WQI presented here are lower than those registered before evidencing the progressive deterioration of this aquatic system. Moreover, in this study we have evidenced that the strong impact of Córdoba city persists even 110 km away from the pollution source.

Deterioration of water quality affects the aquatic life preservation. In particular, fish are sensitive to the impact of pollutants that can be found in the environment. Hued & Bistoni (2005) reported that in Suquía river fish assemblages changed with increasing water degradation, displaying a simpler structure in the most polluted sites.

Changes at community-level could be consequence of pollutants effects at lower biological levels (molecular, subcellular, cellular, tissue, organism and population, van der Oost *et al.,* 2003). Thus nowadays, different biological responses studies are advancing to propose new biomarkers that allow an early detection of toxic effects occurring due to pollution charges (Contardo-Jara *et al.,* 2008). In this way, the evaluation of changes in gene expression is emerging as the most promising molecular tool.

Several authors have used *J. multidentata* to investigate biomarkers at different organization levels such as histological, enzymatic and behavioral alterations (Cazenave *et al.,* 2005, 2008; Ballesteros *et al.,* 2007, 2009a, 2009b; Pesce *et al.,* 2008;

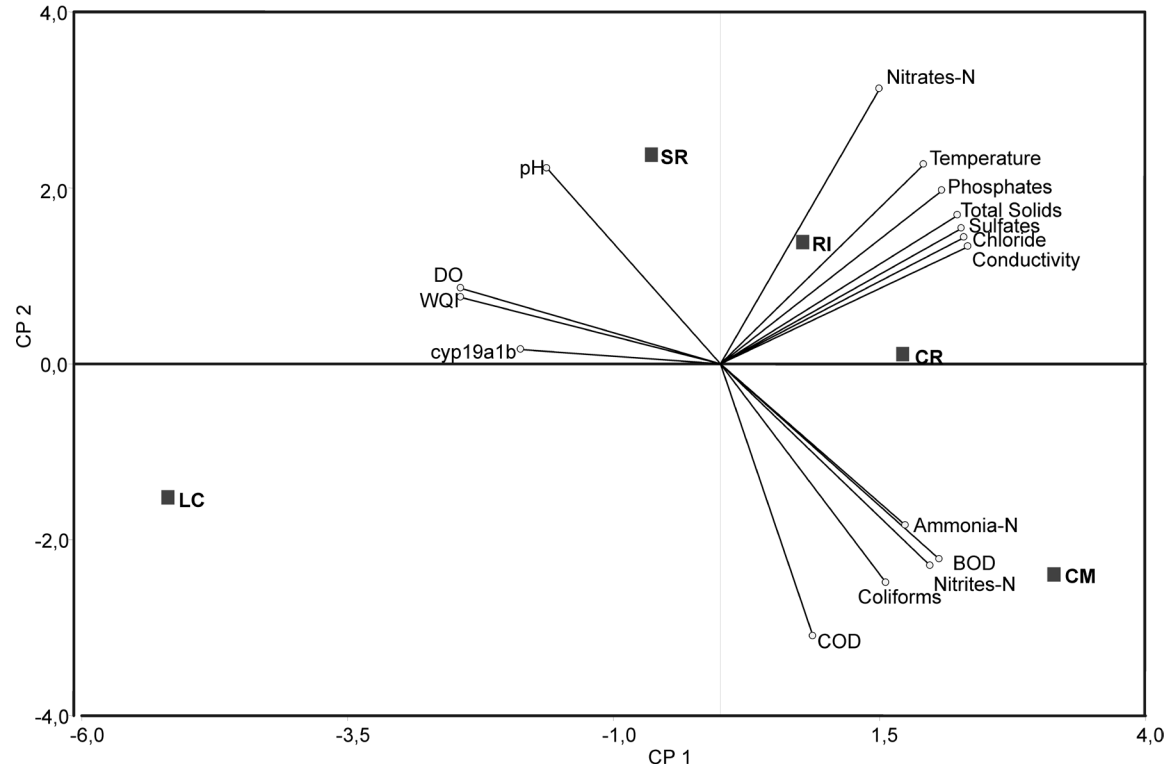

Figure 3 - Biplot of the two first principal components generated by the Principal Component Analysis showing the association between water quality parameters and *cyp19a1b* expression.

Table 2 - Results of the Principal Component Analysis (PCA) on the data set with Water Quality Index, chemical and physical parameters in water and cyp19a1b expression in brain of *J. multidentata*: coefficients of the eigenvectors (loadings of the original variables) in the linear combination of variables from which the PCA are computed.

	Principal Components		
	PC1 (64%)	PC2 (24%)	PC3 (9%)
WQI	-0,31	0,09	-0,09
Cyp19a1b	-0,23	0,02	-0,55
Ammonia-nitrogen	0,22	-0,23	0,37
5 days- BOD	0,26	-0,28	0,01
COD	0,11	-0,39	-0,42
Chloride	0,29	0,18	-0,11
Conductivity	0,29	0,17	-0,10
Dissolved oxygen	-0,31	0,11	-0,03
Nitrates-nitrogen	0,19	0,39	0,09
Nitrites-nitrogen	0,25	-0,29	-0,09
Phosphorous	0,26	0,25	-0,03
pH	-0,20	0,28	-0,31
Solids: Total	0,28	0,21	-0,05
Sulfates	0,28	-0,19	-0,14
Temperature	0,24	0,28	-0,25
Total coliforms	0,20	-0,31	-0,40

Amé *et al.,* 2009). The study of changes in gene expression needs the previous knowledge of DNA sequence. However, the information available for neotropical native fish species is very limited (Orlando *et al.,* 2002; Strobl-Mazzulla *et al.,* 2005). To the extent of our knowledge, this is the first study reporting the isolation and partial sequencing of *cyp19a1b* gene encoding brain aromatase of *J. multidentata*. With this information we were able to adjust the qRT-PCR method to assess if this biological response reflects changes in water quality.

Some studies have reported reproductive disorders and/or endocrine disruption in wild fishes exposed to environmental pollutants. Sometimes the results are discrepant, what could be due to many different factors, including the nature, concentration and duration of exposure to a given pollutant (Greytak *et al.,* 2005), and the stages of fish development when they are exposed. Orlando *et al.,* (2002) reported that brain aromatase activity was higher in female mosquitofish living downstream of a paper mill than in reference site. Greytak *et al.,* (2005) reported higher levels of *Cyp19a1b* in brain of killifish from highly polluted than unpolluted environments. On the contrary, Noaksson *et al.,* (2003b) found low brain aromatase activity in female perch from the leachate-contaminated Lake Molnbyggen, which is similar to our present qRT-PCR results revealing the influence of water quality on the expression of *cyp19a1b*. In our current study, we found aromatase inhibition at the most polluted areas of the basin (CM, CR and RI) (Fig. 1; Table I) while the highest mRNA levels were observed in females collected at the site situated before Córdoba (LC) and far downstream from WWTP

(SR), where there was some recovery of the water quality after the severe impact of the sewage exit.

The ecological significance of the *cyp19a1b* inhibition in the most polluted areas of Suquía River basin is yet unknown. However, it could be hypothesized that such inhibition cause or contribute to estrogen-related disorders, probably resulting in gradual loss of reproductive function upon long exposure.

There are evidences about the occurrence of EDCs in freshwaters receiving domestic and industrial effluents. Considering that most of streams receive some kind of effluents, endocrine disruption is probably more widespread than is currently documented. A more global assessment of endocrine disruption should include the more rare and vulnerable species, with different life stories and reproductive strategies (Jobling *et al.,* 2004). So, identifying biomarkers in native fish that could be used as sentinels is extremely necessary for a better understanding of how EDCs can affect fish sustainability.

As shown by the PCA analysis, changes of water quality along the pollution gradient studied in Suquia River is reflected in *cyp19a1b* expression. The impact on aromatase expression in wild fish seems to be less severe with increasing distance from the WWTP, where there is some partial recovery of water quality. So far, our results suggest that further studies of *cyp19a1b* expression in *J. multidentata* could be interesting in order to propose a new biomarker to evaluate changes in the water quality of South American rivers connected with the probable presence of endocrine disruptors.

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