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A multi-level approach using *Gambusia affinis* as a bioindicator of environmental pollution in the middle-lower basin of Suquía River

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ABSTRACT

The Suquía River middle-lower basin (Córdoba, Argentina) is subjected to a strong anthropogenic impact because it receives pollutants from different sources. The main goal of this study was to evaluate the introduced fish species *Gambusia affinis* as a bioindicator of environmental pollution in the middle-lower basin of the Suquía River. The assessment was performed by measuring biomarkers at different levels of biological organization, at two sampling sites (before and after Córdoba city), and at dry and wet seasons. Water quality evaluation was made through a water-quality index (based on physico-chemical parameters), heavy-metals and pesticides concentrations in water. The water quality varied between sampling sites, showing the most degraded conditions downstream Córdoba city. The same pattern of variation was detected in the biomarkers studied, mainly in: gill and liver histopathological indexes, copulatory organ (gonopodium) morphology and vitellogenin expression in males and females. The present study characterized the environmental conditions in the middle-lower basin of the Suquía River and revealed the low freshwater quality at the most polluted site. Although *G. affinis* is an introduced species, it could be considered a good sentinel of water resource quality of invaded Neotropical basins. Our results demonstrated the importance of addressing the environmental quality monitoring through an integrated analysis of water quality parameters together with histological, morphological and molecular parameters. Thus, our study provides a good model for application in other basins of South America.

1. Introduction

The increase in number and size of urban settlements increased significantly the amount of wastes and pollutants loadings into the aquatic resources. As a result, freshwater systems decrease their self-purification capacity and produce severe disturbances in water quality affecting the resident biota.

The Suquía River basin, considered as a key element of the central landscape of Córdoba province, runs through important urban areas and receives different environmental contaminants from point (sewage discharges and industrial effluents) and nonpoint pollution sources (urban runoff, automobile traffic, livestock wastes, fertilizer and pesticide applications) (Wunderlin et al., 2001; Nimptsch et al., 2005). At the same time, its scarce and seasonal flow, short length and endorheic condition complicates the output of xenobiotics compounds, contributing to their accumulation in the basin (Gaiero et al., 1997) and affects the resident aquatic biota (Hued and Bistoni, 2005; Contardo-Jara et al., 2009; Monferrán et al., 2011).

In aquatic environments, organisms are exposed to mixtures of pollutants, whose synergistic/antagonistic effects are hardly interpreted and predicted exclusively from chemical analyses. Some contaminants accumulate strongly in tissue without inducing toxic effects, while others are characterized by a high toxicity at low exposure levels. It is well known that biomarkers in fish could reflect the integrated effects of altered quality of a water

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body, and thus, they can be used to compare relative changes in water quality from site to site, or over a time of period (Van der Oost et al., 2003).

Fish are useful bioindicators to evidence environmental degradation (Fausch et al., 1990). Changes in water quality conditions can lead to different changes in fish, ranging from biochemical alterations in single cells up to changes at population level. Among the fish species used to evaluate the quality of freshwater systems, the mosquitofish, *Gambusia affinis* (Poeciliidae, Cyprinodontiformes) (Baird and Girard, 1853), a viviparous species with external sexual dimorphism, has been used as an excellent bioindicator in field studies and it is considered an important sentinel, due to its ability to establish in a wide range of habitats (Orlando et al., 2005). Mosquitofish was introduced into water bodies of Argentina in the 40s in order to avoid the proliferation of mosquito larvae (Haro and Bistoni, 1996).

In biomonitoring programs, the integrated analysis of both histopathological and biochemical endpoints facilitate the observation of physiological responses of the exposed individuals, thereby establishing a more realistic diagnosis for evaluating environmental health (Ayas et al., 2007). Histopathological alterations have been shown to be responsive and sensitive to a wide range of contaminants (Bernet et al., 1999). As an indicator of exposure to pollutants, histology represents a useful tool to assess the degree of pollution, particularly for sublethal and chronic effects (Van der Oost et al., 2003; Costa et al., 2009). In particular, the gills are considered primary markers for aquatic pollution as exhibit large surfaces which are in direct and permanent contact with the aquatic environment. In addition, measurements of gill dimensions (gill morphometrical indices) provide evidences of a physiological disturbances (Nero et al., 2006a, 2006b).

Another effective tool successfully used as a biomarker in fish is the expression of vitellogenin (yolk precursor protein). Under natural conditions, only mature females produce this protein. Although the vitellogenin gene is normally silent in males, it can be induced if male fish are treated with natural or synthetic estrogens (Sumpter and Jobling, 1995). Therefore, induction of vitellogenesis in males or variation in their expression levels are direct evidence of exposure to substances with estrogenic activity (Kime et al., 1999; Björkblom et al., 2008).

Exposure to endocrine disruptors can also be detected through changes in external sexual characteristics of a fish species. In this sense, *G. affinis* are especially useful for studying the effects of endocrine-disrupting chemicals on fish (Batty and Lim, 1999). Males have morphological traits that make them uniquely suited to study the effects of environmental endocrine disruptors, since the anal fin differentiates into a complex structure called gonopodium that is used for the transfer of sperm during copulation (Turner, 1941). Development of the gonopodium is androgen dependent and normally occurs in males of viviparous teleost at the time of sexual maturation, when the testis synthesizes androgens. However, its normal development can be affected by xenobiotic compounds. Therefore, the gonopodial morphology could be used as an excellent biomarker of estrogenic exposure (Angus et al., 2001; Doyle and Lim, 2002).

Given the current problems in the Suquía River basin and considering the advantages associated to the use of different biomarkers in freshwater quality assessment, the main goal of the present study was to determine whether a freshwater system polluted by anthropogenic activities (sewage, agricultural and industrial) causes alterations at different biological organization levels. We used the non-native fish, *G. affinis*, as a bioindicator of environmental pollution. Our work comprises a diversity of variables (biotic and abiotic parameters) that makes this investigation an integral study to evaluate the Suquía River watershed quality.

2. Materials and methods

2.1. Study area

The Suquía River basin is one of the most important freshwater systems of Córdoba province (Argentina) (Fig. 1). It includes the convergence of various rivers and streams that come from a mountainous area called “Sierras Grandes”. This endorheic basin flows from west to east in concordance with the altitudinal gradient. The Suquía River begins at the San Roque Dam (the main drinking water source for Córdoba city), runs for approximately 35 km before entering Córdoba city, flows for about 40 km across this city and then continues another 150 km downstream up to Mar Chiquita Lagoon. Its watershed covers approximately 7700 km^2, of which almost 900 km^2 correspond to the Córdoba city drainage area and represent the middle-lower basin (Vázquez et al., 1979).

The flow regime of Suquía River drainage network is exclusively pluvial origin, with a marked seasonality of the flow due to the irregular distribution of rainfall throughout the year (Pasquini et. al., 2011). The mean annual rainfall is in the range of 700–900 mm, with a dry season (from May to September) and a wet season (from October to April) with most of the rainfall occurring in January and

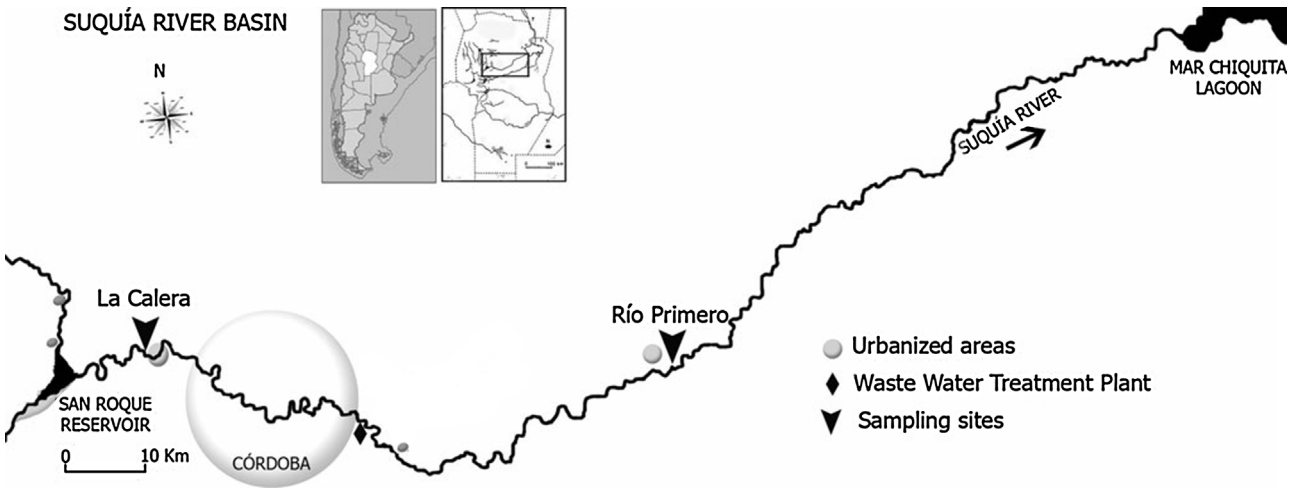

Fig. 1. Study area showing the location of sampling sites in the Suquía River middle-lower basin. From: Merlo et al. (2011).

February (Vázquez et al., 1979). Córdoba is the most important city of the basin with a population of approximately 1.5 million inhabitants (INDEC, 2010). Several studies have indicated this city has a negative influence on the basin, on both physico-chemical and biological characteristics, mainly by sewage pollution (Pesce and Wunderlin, 2000; Wunderlin et al., 2001; Hued and Bistoni, 2005; Monferrán et al., 2011).

We selected two sampling sites along the middle-lower basin of the Suquía River based on the contamination gradient showed by previous investigations (Merlo et al., 2011): (1) La Calera (LC) (31° 21′ S; 64° 21′ O): site located 15 km downstream San Roque Dam and 20 km upstream of Córdoba city west border, near to La Calera city. This site is representative of quasi-pristine conditions (Hued and Bistoni, 2005) and (2) Río Primero (RP) (31° 20′ 29″ S; 63° 36′ 58″ O): site located in an agricultural area, 55 km downstream Córdoba city. This site is in an agricultural area and the river is crossed by a high traffic route (Merlo et al., 2011; Pasquini et al., 2011).

2.2. Water quality assessment

Each study sites was sampled during 2010, once during the rainy season (March) and once during the dry season (August). In order to characterize the water quality conditions of each site, the following physico-chemical parameters were measured: pH, temperature (°C), conductivity (mS/cm), alkalinity (mg/L), dissolved oxygen (mg/L), carbon dioxide (mg/L), total and dissolved solids (mg/L), ammonia (mg/L), nitrites (mg/L), nitrates (mg/L), chemical oxygen demand (COD; mg/L), 5-day biological oxygen demand (5-DBO; mg/L), total phosphorus (mg/L), hardness (mg/L), calcium (mg/L), magnesium (mg/L), sulfates (mg/L), chlorides (mg/L), total iron (mg/L), total coliforms and faecal coliforms (MPN · 100 mL: most probable number per 100 mL). Analytical methods were standard (APHA, 1998). To characterize the conditions of sampling sites based on physico-chemical variables, the water quality index (WQI), proposed for the Suquía River basin by Pesce and Wunderlin (2000), was calculated. This index gives a percentage of water quality. A value of 100% represents the highest water quality, whereas values $\leq$50% are considered to have a negative impact on the biota.

Water samples were also taken to measure metals (aluminum, arsenic, barium, beryllium, boron, cadmium, calcium, cobalt, copper, chromium, strontium, gallium, iron, yttrium, lithium, magnesium, manganese, mercury, molybdenum, nickel, silver, lead, potassium, rubidium, selenium, vanadium and zinc) (Monferrán et al., 2011) and pesticide concentrations (atrazine, acetochlor, alpha-cypermethrin, alpha-endosulfan, beta-endosulfan, chlorpyrifos and endosulfan sulfate) (Bonansea et al., 2013).

2.3. Fish collection

Adult females and males of *G. affinis* were caught using standard backpack electrofisher equipment at each sampling site and at the same time that water samples were taken. The capture area covered 150 m stream length (approximately 500 m^2). Only individuals with a minimum body length of 20 mm were used in the study, since below this length sexing of the fish is uncertain (Batty and Lim 1999). They were transported alive to the laboratory, where they were killed by an overdose of tricaine methanolsulfonate (MS-222, Sigma–Aldrich, (500 mg/L)). The standard length (SL, mm) and weight (W_T, g) were taken from each individual and the Fulton condition factor was calculated as $K = (W_T/SL^3) \times 100000$.

Fish were dissected for the extraction of gills, liver and gonads. It was calculated the Hepatosomatic index as $HSI = (W_L/W_TM) \times 100\%$, (where W_L is the weight of liver) to estimate the relative size of the liver to body weight. Gonads were also weighted and the Gonadosomatic index was estimated as $GSI = (W_G/W_T) \times 100\%$ (where W_G is the weight of gonads) (Strange, 1996).

Gills and livers from 6 females and 6 males per site were fixed in buffered formalin for the histological analysis for wet and dry seasons. Another group of 6 females and 6 males per site for each season were sacrificed in order to obtain the liver to determine the vitellogenin (Vtg) gene expression. For this purpose the organs were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. The number of animals analyzed for each biomarker is detailed in results tables. All procedures are in compliance with the Guide for Care and Use of laboratory Animals (National Institutes of Health, 2011).

2.4. Histological analysis

Gill and liver tissue samples were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. Sections 4–6 μm thick were stained with hematoxylin and eosin (H&E). Tissue lesions were examined with a light microscope (Olimpus Cx-31) and photographed with a digital camera (Moticam®). For gill histological analysis, 5 filaments were examined per individual fish. On each filament 10 secondary lamellae on the middlemost section of the filament were selected. The number of secondary lamellae with a particular alteration was recorded and divided by the total number of gill secondary lamellae examined to give the percentage of filaments affected. To evaluate histological alterations of liver, tissue was divided into 5 equal areas (1.68 mm^2), at 10× power magnification. Then each area was further individually analyzed at 40×. The extension and number of each alteration at each area were recorded and divided by the total number of areas examined to give a percentage of liver areas affected. Histopathological lesions were evaluated following a standardized assessment tool by using a modified version of the protocol described by Bernet et al. (1999), which allows calculation of histopathological indices for every organ. Each histological alteration was classified into one of four reaction patterns: circulatory disturbances, regressive changes, progressive changes, and inflammation. Each reaction pattern includes alterations related to functional units of each organ. Circulatory disturbances (reaction pattern 1 = Rp1) correspond to pathological conditions of blood and tissue fluid flow (e.g., haemorrhage, blood congestion, aneurysm, etc.); regressive changes (reaction pattern 2 = Rp2) correspond to processes that cause functional decrease or loss of an organ (e.g., architectural and structural alterations of a tissue, atrophy, necrosis, etc.), progressive changes (reaction pattern 3 = Rp3) correspond to processes that lead to increased cell or tissue activity (e.g., hypertrophy and hyperplasia); and inflammation (reaction pattern 4 = Rp4) corresponds to inflammatory processes associated with other reaction patterns (e.g., oedema, infiltration of leucocytes, etc.). Table 1 lists the reaction patterns and their specific alterations for gills and liver.

Because the relevance of a lesion depends on its pathological importance and indicates how the lesion affects the function of an organ and the ability of the individual to survive, an importance factor (W) (range 1–3) was assigned to each alteration (Table 1). A high importance factor represents a greater potential of an alteration to impact fish health (Bernet et al., 1999). For both gill and liver, the percentage of gill filaments or liver areas affected were assessed using a score ranging from 0 to 8 according the degree and extension for a particular alteration: 0 = unchanged, 2 = mild occurrence, 4 = moderate occurrence, 6 = severe occurrence, and 8 = very severe occurrence. The importance factors and the score values were multiplied to give an index for a particular alteration. The indices for each alteration within a reaction pattern were summed to give an index for each reaction pattern

Table 1
Importance factor (*W*) assigned to gill and liver histological alterations for each reaction pattern according to Bernet et al., (1999).

Reaction pattern	Organ/Histological alteration	*W*
1. Circulatory disturbances	*Liver*	
	Haemorrhage	1
	Sinusoid dilation	1
	Blood congestion	1
	Gill	
	Blood congestion	1
	Lamellar aneurysm	1
2. Regressive changes	*Liver*	
	Hydropic degeneration	1
	Lipid degeneration	2
	Hepatocyte picnosis	2
	Fibrosis	3
	Necrosis	3
	Gill	
	Epithelial lifting	1
	Fusion of the distal end of secondary lamellae	2
	Secondary lamella shortening	2
3. Progressive changes	*Liver*	
	Hepatocyte hyperplasia	2
	Hepatocyte hypertrophy	2
	Intracellular eosinophilic bodies	2[a]
	Gill	
	Chloride cell hypertrophy	1
	Epithelial cell hypertrophy	1
	Mucous cell hyperplasia	2
	Chloride cell hyperplasia	2
	Epithelial cell hyperplasia	2
4. Inflammation	*Liver and Gill*	
	Leukocyte infiltration	2

[a] Importance factor (*W*) according to Costa et al. (2009).

($HI_{Org.\ Rp}$ = reaction pattern index for an organ). The indices for each reaction pattern of an organ were summed to give an overall organ index (HI_{Gill} = gill histopathological index; HI_{Liv} = liver histopathological index). To determine the overall health status based on the histological lesions, a total histopathological index (HI_T) was calculated by adding gills and liver indices of an individual fish (Bernet et al., 1999). The greater the index values, the more severely the organs were affected. To know which of these organs showed higher histological damage and to compare them, we calculated the proportion of changes in each of them, relativizing the individual value of each index with respect to the maximum value that the HI_T can reach.

2.5. Gill morphometric analysis

Morphometric analyses of gills were performed over the same filaments selected for gill histological analysis. On the medium part of each filament 10 secondary lamellae (5 lamellae per side of each filament) were selected to measure the following parameters: secondary lamellar length (SLL) and width (SLW), interlamellar distance (ID), and basal epithelial thickness (BET) (Fig. 2a). To measured SLW and ID, three measurements were taken on each secondary lamella (points where secondary lamellae met the basal lamina, the middle, and at the distal edge of the secondary lamellae) to give an average value for each variable. For BET, three measurements were made along each filament: measurements at both ends and in the middle on both sides of the cartilaginous support. The measurements of the mentioned parameters were obtained through the Image J software v. 1.47 and indicate changes in the gas diffusion distance in fish gills. The proportion of secondary lamellae available for gas exchange in function of lamellae length (PAGE) was calculated using the equation proposed by Nero et al. (2006a): PAGE (%) = 100 × [mean SLL/(mean BET + mean SLL)]. To offer more detailed information about the proportion of secondary lamellae available for gas exchange, two equations were calculated, according to Maggioni et al. (2012). The first one was a function of lamellae width: $PAGE_W$ (%) = 100 × [mean ID/(mean ID + mean SLW)]. The second one (total PAGE) was calculated as $PAGE_T$ (%) = (PAGE × $PAGE_W$)/100. These formulas give a more complete information than the generated by the solely application of the equation proposed by Nero et al. (2006b). For all the equations, lower percentages indicate fewer proportions of secondary lamellae available for gas exchange.

2.6. Gonopodial morphology assessment

Gonopodium of each male was examined with a stereoscopic microscope and photographed. To measure the gonopodium length (GL) each photographs was analyzed through Image J software v. 1.47 (Rasband, 2012). A Gonopodium Somatic Index [Gonop-SI = (GL × 100)/SL] was calculated to estimate the relative size of the gonopodium to standard length (Hued et al., 2013). It was also corroborated the presence or absence of hooks on the distal portion of the gonopodium (Doyle and Lim, 2002).

2.7. Vitellogenin gene expression

Total RNA was isolated from liver tissue by the guanidine thiocyanate–phenol–chloroform extraction method in according to Chomczynski and Sacchi (1987). The RNA pellet was resuspended in 100 μL nuclease-free water (Biodynamics S.R.L, Buenos Aires, Argentina). In order to reduce DNA contamination, RNA samples were treated for 30 min with 5 U RQ1 DNase I (Promega, Madison, USA). RNA was extracted again with the same protocol and resuspended in 20 μL nuclease-free water. The purity and concentration of RNA of each sample were validated using the absorbance of total RNA in water at 260 and 280 nm. The RNA integrity was checked running a non-denaturing gel electrophoresis. Non-specific reverse transcription was performed on 5 μg of RNA using a first strand complementary DNA synthesis method

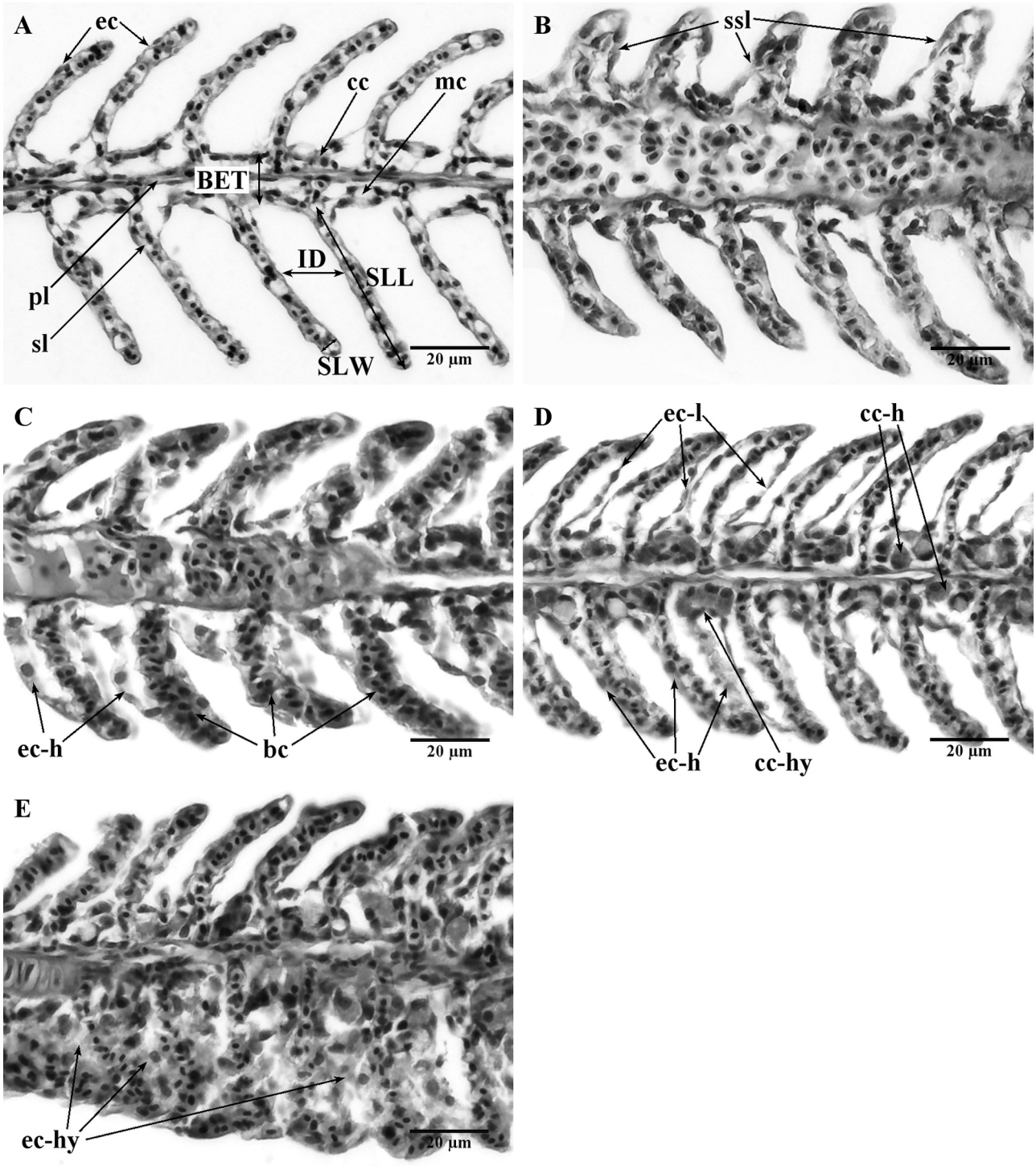

Fig. 2. Gill histological sections and morphometric parameters of *G. affinis* from the Suquía River basin. (a and b) Individuals from La Calera; (c–e) Individuals from Río Primero. References: bc – blood congestion, cc – chloride cell, cc-h – chloride cell hypertrophy, cc-hy – chloride cell hyperplasia, ec – epithelial cell, ec-h – epithelial cell hypertrophy, ec-hy – epithelial cell hyperplasia, ec-l – epithelial cell lifting, mc – mucous cell, pl – primary lamella, sl – secondary lamella, ssl – secondary lamellae shortening. BET – basal epithelial thickness, ID – interlamellar distance, SLL – secondary lamellar length, SLW – secondary lamellar width. H&E stain 400×.

catalyzed by the M-MLV Reverse Transcriptase (Invitrogen, Carls-bad, USA) using oligo $(dT)_{15}$ primer (Biodynamics S.R.L, Buenos Aires, Argentina) (Amé et al., 2009).

For mRNA relative expression levels assessment, real-time PCR was performed on a Bio-Rad iQ cycler (Bio-Rad Hercules, USA) 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, Foster city, USA) and 6 pmol primers in a final volume of 20 μL. Vtg primers for *G. affinis* designed by Raut and Angus (2010) and β-actin primers designed by Amé et al. (2009) were employed for this reaction. Cycling parameters were as follows: 95 °C for 13 min; 45 cycles of 15 s at 94 °C, 30s at 58,7 °C and 30 s at 72 °C. Real-time PCR reactions were run in duplicate for each cDNA sample and melting curves carried out to monitor the quality of amplicons and reactions. Amplifications were also performed on 25 ng of non-retrotranscribed RNA as negative

Table 2
Oligonucleotide primers of the genes used in real-time RT-PCR along with their NCBI accession numbers, annealing temperature (T_m) and the size of the amplicon.

Gene	Primers	Accession number	T_m (°C)	Product size (bp)
Vtg	Sense: 5′-ACCAGGGACCTGAACAACTG-3′	DQ190844.1	58.7	150
	Anti-sense: 5′-GATGGCATTAGCGACTGGTT-3′		58.7	
β-actin	Sense: 5′-AAGCCAACAGGGAGAAGATGAC-3′	EF362747	60.6	77
	Anti-sense: 5′-GCCTGGATGGCAACGTACA-3′		60.0	

control to check absence of genomic contaminants. DNA 100 bp ladder (Productos Biológicos, Bernal, Argentina) was used to estimate and verify PCR product length on a 2.5% agarose gel stained with ethidium bromide. Amplification products were quantified by comparison of experimental Ct (threshold cycle – defined as the PCR cycle where an increase in fluorescence over background levels first occurred) levels. Relative Vtg gene expression to *G. affinis* β-actin was calculated by using the standard curve method generated for each primer pair based on known quantities of cDNA (serial dilutions corresponding to cDNA transcribed from 40, 20, 10, 5 and 2.5 ng of total RNA) (Larionov et al., 2005). The use of *G. affinis* β-actin as housekeeping gene was previously tested. Primer sequences, NCBI accession numbers of sequences for *G. affinis*, annealing temperature and the expected size of each amplicon are listed in Table 2. PCR product sequence data were additionally obtained by DNA sequencing (Macrogen Inc., Korea) employing the same primers used for PCR amplification.

2.8. Statistical analysis

Data were checked for normality and homogeneity of variance. Because all of the biological and environmental parameters did not meet these assumptions, Kruskal–Wallis test (Sokal and Rohlf, 1995) was performed followed by Dunn's multiple-comparison test. Significant differences in the frequency of fish exhibiting gonopodial hooks were determined using the Chi-square analysis.

Linear discriminant analysis (LDA) was performed to determine which biomarker was the best predictor of effect between different water qualities. Generalized Procrustes analysis (GPA) was also applied to datasets. All the variables measured were used as descriptors for biotic group (biomarkers) and abiotic group (water quality parameters). GPA was applied for assessing the relationship among the descriptors. Specifically, GPA constructs the consensus configuration of a group of datasets by applying transforms in an attempt to superimpose them. Therefore, GPA theory and algorithms can be applied to match abiotic parameters to the corresponding biological data, and determine if the variables used were suitable for the characterization of the sampling sites studied.

All values are expressed as mean ± standard deviation. Statistical analyses were performed using the Infostat software package v. 2013 ($p < 0.05$) (Di Rienzo et al., 2013).

3. Results and discussion

3.1. Water quality assessment

3.1.1. Water-quality index

The WQI variation pattern was similar to that registered by (Pesce and Wunderlin, 2000). However, the values recorded in the present work were markedly lower than those obtained by the mentioned authors (Table 3). The index showed significant differences between sampling sites, showings spatial deterioration of water quality in the Suquía River basin. The WQI lowest value corresponds to RP during the dry season. Since Hued et al. (2010) has indicated a WQI value of approximately 50% are hardly compatible with aquatic life, a value of 57% at RP, showed the degraded condition of this site. These degraded conditions registered at RP exceed the river dilution and self-purification capacity. Even 55 km downstream Córdoba city, the water has not recovered the quality levels detected at LC. This result could be associated with the contribution of polluting human activities, population growth, the lack of urban planning along the basin, and specially, by the presence of the wastewater-treatment plant of Bajo Grande located upstream of RP (Monferrán et al., 2011; Maggioni et al., 2012). Another important factor that could alter the water quality is the influence of the crop area surrounding the eastern margin of the capital city which provides large amount of agrochemicals by run-off (Monferrán et al., 2011). The expansion of agricultural activity also implies an additional input of agrochemicals and sediments coming from erosive processes (Bonansea et al., 2013). In addition, the low water quality values registered during the dry season are related with the higher pollutant concentrations by reducing the river flow during this season.

3.1.2. Heavy metals

The additional load of metals to the Suquía River basin is strongly associated with sewage and domestic effluents, agricultural residues from the crop area, and industrial wastes (Contardo-Jara et al., 2009; Merlo et al., 2011). In general, the highest concentrations for most of the metals measured in water were detected during the dry season in relation with the low water volumes during the low-flow season (Table 3).

Among the measured metals at RP, chromium, copper, lead and zinc surpassed the guidelines for freshwater considered dangerous to the aquatic fauna health, and established by the Argentinean Environmental Water Quality Guidelines (SRHN, 2004) and the Canadian Water Quality Guidelines (CCME, 2013). Such metals exceeded their limits, 1 μg/L, 2 μg/L, 1 μg/L and 30 μg/L respectively, during the dry season. On the other hand, the chromium, copper, lead and aluminum (guideline value of 100 μg/L) were near the guidelines during the wet season. The presence of these metals as well as magnesium, nickel, lithium and cobalt, that were also elevated at RP, has been generally associated to anthropogenic activities in the basin (Contardo-Jara et al., 2009; Pasquini et al., 2011).

At LC, copper and mercury (guideline value of 0.03 μg/L) surpassed the guidelines for freshwater during the dry season, and chromium remained close to it. During the wet season only lead values were close to the limits. These results would indicate a slight degradation of water quality at LC, possibly by an increase of the contaminants from sewage and urban runoff from small towns located along the river. The presence of iron, nickel and magnesium in the Suquía River, specially at LC, reflects both the anthropogenic activities and the weathering of the fluvio-loessic sediments that cover the granitic and metamorphic rocks downstream the mountain range (Contardo-Jara et al., 2009; Pasquini et al., 2011).

The main threats to human health and to fish fauna exposed to heavy metals are associated with exposure to lead, cadmium, mercury, arsenic, manganese and zinc (Dyer, 2007; Velcheva et al., 2010). These metals are considered endocrine disruptors (Pait and Nelson, 2002; Choi et al., 2010), as they can mimic the biological activity of steroid hormones (androgens, estrogens and

Table 3
Water-quality index (%), heavy metals (μg/L) and pesticides (ng/L) measured in samples collected in Suquía River during the wet and dry seasons.

Parameter	Sampling sites			
	La Calera		Río Primero	
	Wet (n = 2)	Dry (n = 2)	Wet (n = 2)	Dry (n = 2)
WQI	76 ± 5[b]	75 ± 9[b]	62 ± 12[a]	57 ± 8[a]
Metals				
Aluminum	33.71 ± 0.47	9.58 ± 0.64	87.23 ± 1.26 *	44.23 ± 1.88
Arsenic	<LOD	<LOD	<LOD	1.91 ± 0.05
Barium	26.03 ± 3.88	31.68 ± 2.40	62.02 ± 9.42	54.00 ± 1.34
Beryllium	<LOD	0.22 ± 0.00	<LOD	0.22 ± 0.00
Boron	135.67 ± 68.66	<LOD	321.68 ± 94.13	181.15 ± 117.82
Cadmium	<LOD	<LOD	<LOD	<LOD
Calcium[wg]	21603.33 ± 569.04	31130.00 ± 630.24	85416.67 ± 1617.35	94120.00 ± 1215.03
Cobalt	<LOQ	<LOQ	0.50 ± 0.02	0.23 ± 0.03
Copper	<LOQ	2.39 ± 0.11**	1.99 ± 0.08 **	3.23 ± 0.11 **
Chromium	0.41 ± 0.01	0.87 ± 0.02*	0.53 ± 0.02	4.47 ± 0.06 **
Strontium[wg]	113.40 ± 2.78	154.75 ± 1.15	429.17 ± 6.35	518.60 ± 11.73
Gallium[wg]	3.37 ± 0.20	4.91 ± 0.21	6.06 ± 0.24	7.24 ± 0.09
Iron	50.55 ± 1.26	11.92 ± 0.73	75.48 ± 6.27	99.36 ± 1.61
Yttrium[wg]	<LOD	0.05 ± 0.00	0.14 ± 0.04	0.14 ± 0.01
Lithium[wg]	10.59 ± 2.46	7.14 ± 0.78	15.38 ± 2.78	15.68±1.27
Magnesium[wg]	3650.00 ± 65.57	5192.00 ± 133.84	11343.33 ± 5974.46	16041.00 ± 255.74
Manganese	25.56 ± 0.67	6.28 ± 0.17	49.02 ± 0.86	50.47 ± 1.07
Mercury	<LOD	0.33 ± 0.02 **	<LOD	<LOD
Molybdenum	<LOD	<LOD	<LOD	<LOD
Nickel	<LOD	2.46 ± 0.05	1.89 ± 0.03	9.04 ± 0.09
Silver	<LOD	<LOD ± <LOD	<LOD	<LOD
Lead	0.77 ± 0.21 *	0.67 ± 0.06	0.86 ± 0.24 *	2.11 ± 0.09 **
Potassium[wg]	2630.17 ± 67.35	2932.50 ± 74.15	9415.00 ± 205.37	9266.00 ± 182.15
Rubidium[wg]	2.05 ± 0.04	1.60 ± 0.04	5.03 ± 0.11	4.06 ± 0.24
Selenium	<LOD±	<LOD	<LOD	<LOD
Vanadium	3.25 ± 0.05	2.41 ± 0.09	5.84 ± 0.05	4.03 ± 0.03
Zinc	<LOQ	10.48 ± 0.39	<LOD	38.33 ± 1.31 **
Pesticides				
Atrazine	<LOD	<LOD	<LOD	118.70 ± 10.00
Acetochlor[wg]	<LOD	<LOD	4.70 ± 1.80	8.70 ± 2.60
Alpha-Cypermethrin	0.90 ± 0.10 **	30.4 ± 8.70 **	<LOD	23.40 ± 7.70 **
Alpha-Endosulfan	<LOD	<LOD	<LOD	<LOD
Beta-Endosulfan	<LOD	<LOD	<LOD	4.60 ± 1.80 **
Chlorpyrifos	<LOD	<LOD	3.30 ± 0.50 **	3.00 ± 0.50 **
Endosulfan-sulfate[wg]	1.20 ± 0.80	4.10 ± 0.60	5.10 ± 2.60	<LOD

Asterisks mean values close (*) or above (**) to the limit established for the protection of the aquatic biota according to Canadian Water Quality Guidelines (CCME, 2013) or according to Argentinean Environmental Water Quality Guidelines (Niveles Guía Nacionales de Calidad de Agua Ambiente) (SRHN, 2004). ([wg]) Compounds without established guideline values. LOD: lower than detection limit, LOQ: lower than quantification limit.
Values are expressed as means ± SDs. Different letters to WQI indicate significant differences among sampling sites and its corresponding seasons ($p < 0.05$).

glucocorticoids) and produce changes in physiological functions associated with development and growth processes in exposed individuals (Kime, 1998; Järup, 2003; Iavicoli et al., 2009). Excepting cadmium, these metals are present in the studied basin with a greater or lesser extent, some with levels approaching or already beyond the limits considered dangerous to aquatic life, and represent a risk to exposed organisms.

3.1.3. Pesticides

The most of the pesticides measured in water showed their highest concentrations at RP (Table 3), downstream Córdoba city. These results are associated with an intensive agricultural activity around this site, dedicated mainly to growing soybean and corn. Atrazine and alpha-cypermethrin were the pesticides that presented the highest concentration at RP. On the other hand, at LC, upstream Córdoba city, the pesticide concentrations were lower and the highest concentration registered corresponds to alpha-cypermethrin.

Organochlorine pesticides (OCPs), as endosulfan, are very toxic and persistent in the environment, and tend to accumulate in living organisms (El-Shahawi et al., 2010). Although most of them have been banned from use, they are still detected in natural ecosystems (Dong et al., 2005). The parental isomer beta endosulfan was found at RP during dry season and surpassed the Canadian Water Quality Guidelines (CCME, 2013) established for the protection of the aquatic biota in freshwater systems (3 ng/L). The presence of endosulfan sulfate at LC and RP, demonstrates the transformation suffered by the endosulfan in the environment. Endosulfan has been classified as a type II insecticide (moderately hazardous) by the IPCS-WHO (2010) and included in the Stockholm Convention of Persistent Organic Pollutants (POPRC, 2010). However, this pesticide was still allowed in Argentina at the time of sampling. From 2011, the Resolution 511/2011 (SENASA, 2011) gradually prohibits its manufacturing, commercialization, and use.

Organophosphorous pesticides (OPPs), as chlorpyrifos, and triazine herbicides, as atrazine, are widely applied and detected in freshwater systems around the world (Tankiewicz et al., 2010). In the Suquía River basin, chlorpyrifos was detected at RP during low and high flow periods, and its use is associated to both urban and agricultural uses (Wielgomas and Krechniak, 2007). Chlorpyrifos levels surpassed the Canadian Water Quality Guidelines (CCME, 2013) established for the protection of the aquatic biota (2 ng/L) during low and high flow periods. Atrazine presented the highest concentration (118.7 ng/L) at RP, during dry season in concordance with the high application period. The presence of this pesticide in water samples is mainly related to their extensive use as

agricultural herbicide, consumed at levels of millions of liters per year (CASAFE, 2011), and its relative persistence in surface waters, with a half-life of 159 days (Solomon et al., 2008).

Chloroacetamide herbicides are considered very safe for the environment. They can be found in high-quantities in soils and freshwaters (Gonçalves and Alpendurada, 2005). Acetochlor, which was detected at RP during low and high-application periods, is worldwide used for pre-emergence control of annual grasses and small seeded broadleaf weeds in corn and soybean. Due to its low adsorption coefficient, is a rather mobile pollutant of the soil, posing a potential danger to the aquatic environment (Lengyel and Földényi, 2003). Although Bonansea et al. (2013) confirmed their presence at LC and in nearby agricultural areas, in the present study this compound was not found at this site.

Pyrethroids, as alpha-cypermetrin, are considered of the group of the last generation pesticides. They are very stable to light and temperature although they may suffer rapid biological degradation in the aquatic environment (Albaseer et al., 2011). Alpha-cypermethrin was the second most concentrated pesticide in water samples from LC during wet season, with a maximum level of 30.4 ng/L. According to the Argentinean Environmental Water Quality Guidelines (SRHN, 2004), alpha-cypermetrin in the Suquía River exceeded the limit of 0.6 ng/L established for the protection of the aquatic biota at both sampling sites and during both seasons. The presence of this pyrethroid can be attributed to its use as an agricultural and urban insecticide (home and industry disinfection). This general pattern reveals the entrance of agrochemicals by the runoff coming from locations surrounding LC. These results agree with those obtained by Maggioni et al. (2012) for a different set of pesticides.

3.2. Body condition assessment

The relationship between the total body length and weight through the condition indices estimation in fish, provides indirect information of the growth, maturity, reproduction, nutrition and therefore health status of a population (Arismendi et al., 2011).

In the presents study, the condition factor (K) showed the highest values during wet season for both sexes, in all the sampling sites (Table 4). These results coincide with the breeding season. However, females showed significant differences between sampling sites, with lower values at RP, which coincides with the deterioration of river water quality. Moreover, GSI in males presented the same variation pattern as the registered for K, with high values during the wet season, at both sampling sites) (Table 4). During the dry season this index decreased indicating a resting period in the sexual activity of this species (Fraile et al., 1992).

HSI in females showed an opposite pattern of variation than K and GSI (Table 4). Their values were lower during wet season at both sites, consistent with the end of the parturition season for this species. The highest values obtained during the dry season, mainly in females, indicate the period prior to the breeding season, when females accumulate nutrients in livers to face the breeding season. In males, HSI varied between sites and hydrological seasons. Several studies have observed an increase in this index as a result of exposure to stressors or xenobiotics (Porter and Janz, 2003; Toft et al., 2003, 2004). In the present work significantly higher value of HSI was obtained during dry season in males from RP, which is in coincidence with the lower levels of water quality and the high pollutant concentration effects due to the reduce water flow during this season.

3.3. Histological analysis

Since the analysis of histological alterations did not show significant variations between males and females, we proceeded to analyze the histological data all together.

3.3.1. Gill histopathological analysis

The gill histological characteristics of *G. affinis* responded to the general pattern of teleost fish (Fig. 2a). However, gills of individuals from both sampling sites presented a wide range of histological alterations associated to different gill reaction patterns (Fig. 2b–e). There were a high prevalence of progressive changes (chloride cell hypertrophy, 77%; chloride cell hyperplasia, 36%; epithelial cell hypertrophy, 26%; epithelial cell hyperplasia, 11%) and regressive changes (mainly epithelial lifting, 48%, and secondary lamella shortening, 33%), followed by circulatory disturbances (blood congestion, 17%). No alterations were registered for the inflammatory reaction pattern. Contrary to our expectations, the gill lesions tended to be more frequent at LC where mercury and alpha-cypermethrin concentrations LC exceed the established limits (Table 3). Although it is difficult to relate gill damages to a particular toxic substance in the environment, different investigations have indicated several alterations in fish exposed to both contaminants. Similar histological disturbances registered in individuals of Suquía River basin have been recorded in other fish species exposed to cypermethrin (Singh and Singh, 2008; Velmurugan et al., 2009) and mercury (Adams and Sonne, 2013). Our results indicate the water quality deterioration of the basin at LC site.

Chloride cell hyperplasia and hypertrophy (Fig. 2d) were higher during the dry season at both sampling sites. The highest frequency of both changes occurred during this season at LC. Chloride cell hypertrophy is a nonspecific response, which tries to compensate the stress by toxic substances exposure and to restore equilibrium of ion concentrations lost by the entry of toxics into the cell (Lin and Randall, 1995). Therefore, there are a direct relationship between the chloride cell surface and the cell ability to performed the ion transport (Perry and Laurent, 1989). On the other hand, the increase in the number of cells would be a general response to stress (Nero et al., 2006a, 2006b). Furthermore, its positioning on

Table 4
Somatic indices for *G. affinis* males and females collected along the Suquía River basin.

Indices	Sampling sites			
	La Calera		Río Primero	
	Wet ($n_{♀}=12$; $n_{♂}=12$)	Dry ($n_{♀}=12$; $n_{♂}=12$)	Wet ($n_{♀}=12$; $n_{♂}=12$)	Dry ($n_{♀}=12$; $n_{♂}=12$)
$K_{♀}$	1.78 ± 0.16^{b}	1.73 ± 0.18^{b}	1.70 ± 0.15^{a}	1.61 ± 0.17^{a}
$K_{♂}$	1.68 ± 0.16^{b}	1.53 ± 0.16^{a}	1.75 ± 0.30^{b}	1.60 ± 0.22^{a}
$HSI_{♀}$ (%)	1.28 ± 0.52^{a}	1.98 ± 0.63^{b}	1.04 ± 0.89^{a}	1.72 ± 0.44^{b}
$HSI_{♂}$ (%)	0.94 ± 0.28^{ab}	0.79 ± 0.38^{a}	0.68 ± 0.50^{a}	1.28 ± 0.39^{b}
$GSI_{♀}$ (%)	0.94 ± 1.89	0.93 ± 0.62	1.82 ± 2.77	0.79 ± 0.46
$GSI_{♂}$ (%)	1.38 ± 0.80^{b}	0.78 ± 0.55^{a}	1.77 ± 1.05^{b}	0.84 ± 0.55^{a}

Values are expressed as means ± SDs. Different letters, when shown, indicate significant differences among sampling sites and hydrological seasons ($p < 0.05$). K: Fulton condition factor, HSI: hepatosomatic index, GSI: gonadosomatic index.

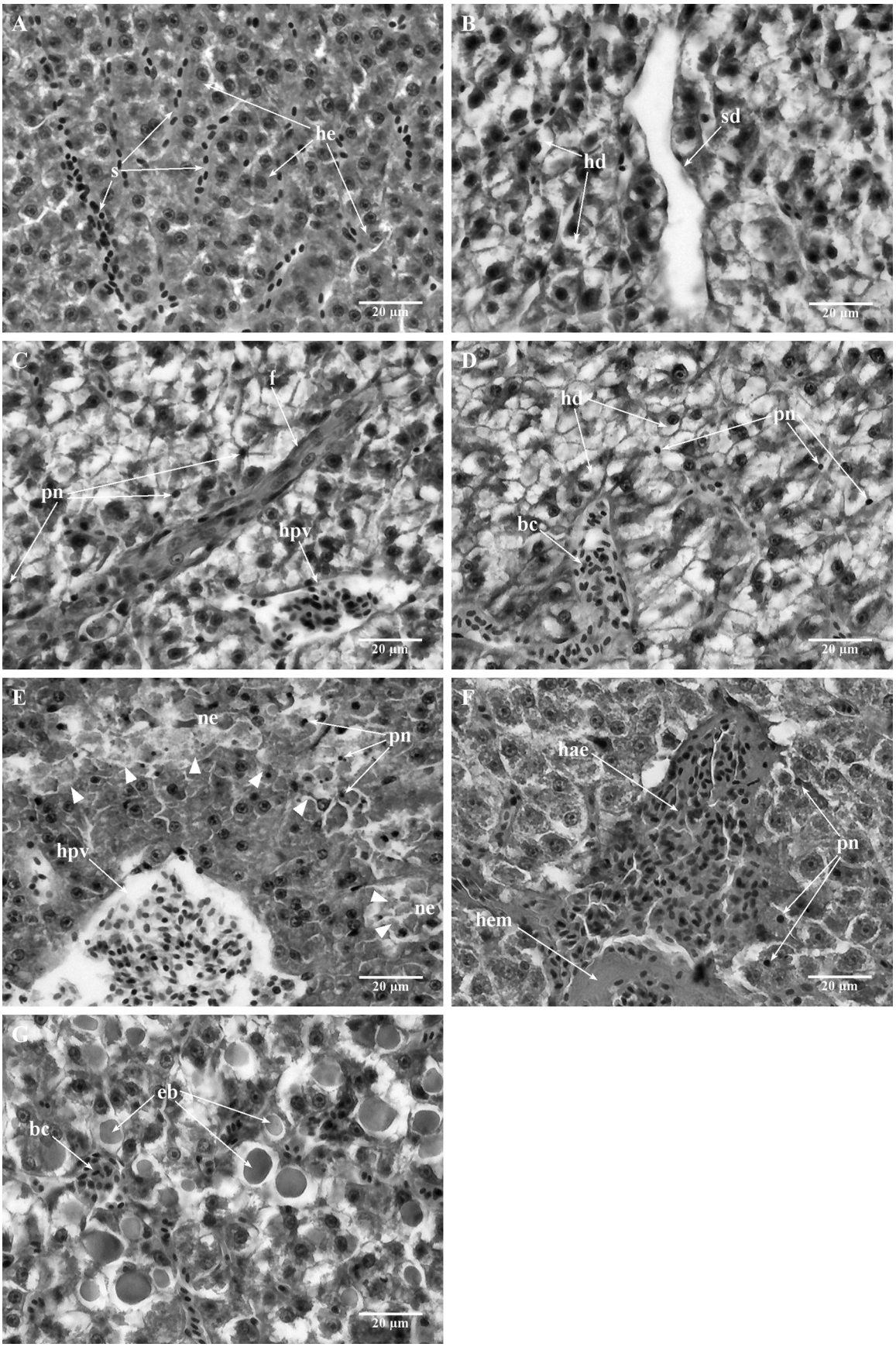

Fig. 3. Liver histological sections of *G. affinis* from the Suquía River basin. (a–d) Individuals from La Calera; (e–g) Individuals from Río Primero. References: bc – blood congestion, eb – eosinophilic bodies, f – fibrosis, hae – haemorrhage, hd – hydropic degeneration, he- hepatocyte, hem – hemolysis, hpv – hepatic portal vein branch with erythrocytes, ne – necrosis (white arrow heads), pn – picnotic nuclei, s – transversally sectioned sinusoid, sd – sinusoid dilation. H&E stain 400×.

the secondary lamellae basis constitutes a barrier of the respiratory area, preventing the entry of substances from the external environment into the circulatory system (Flores Quintana, 2009).

At LC site the secondary lamella shortening (Fig. 2b) and the epithelial cell hypertrophy (Fig. 2c and d) were high during both hydrological seasons. Bernet et al. (1999) described the secondary lamellae shortening as a regressive change of the tissue structure, which leads to a functional reduction or loss of the gill. On the other hand, Mallatt (1985) has indicated an association between the epithelial cell hypertrophy and heavy metal exposure.

Epithelial lifting (Fig. 2d) was one of the most frequent alterations; however, its frequency did not significantly differ between sampling sites and hydrological seasons. According to Wood (2001) epithelial lifting is the most frequently alteration reported in freshwater fish. As the epithelial cell hypertrophy and hyperplasia, epithelial lifting is also one of the first responses at tissue level to pesticides, (Cengiz and Ünlü, 2002; Pesce et al., 2008; Hued et al., 2012), heavy metals (Figueiredo-Fernandes et al., 2007) and wastewaters (Bernet et al., 2004; Maggioni et al., 2012). The diversity of potentially toxic substances measured along Suquía River, could be the responsible of the epithelial cell lifting, hypertrophy and hyperplasia (Fig. 2c–e) observed in the individuals sampled. Hyperplasia was also frequent in chloride and epithelial cells (Fig. 2d and e), reflecting chronic exposure to pollution at both sampling sites.

In general, these structural changes serve as a defense mechanism, leading to decreased respiratory surface and increased toxicant-blood diffusion distance (Cengiz and Ünlü, 2003). As a result, this could affect the normal functioning of the gill, decreasing its efficiency for gas exchange (lead to serious conditions of hypoxia), impacting on the overall health and this can lead eventually to death of the fish (Nero et al., 2006a).

3.3.2. Liver histopathological analysis

In general, all the fish livers analyzed presented a homogeneous parenchyma (Fig. 3a). However, a wide range of histological alterations associated to liver reaction patterns described by Bernet et al. (1999) were observed at both sampling sites (Fig. 3b–g).

There was a high prevalence of regressive changes (hydropic degeneration, 34%; hepatocyte picnosis, 20%; necrosis, 17%), suggesting that most of the alterations identified at field correspond to degenerative changes (processes causing reduction or loss of organ function). These alterations were followed by circulatory disturbances (blood congestion, 16%) and progressive changes (intracellular eosinophilic bodies, 8%). In very lower proportion it was registered fibrosis (3%), haemorrhage (2%) and sinusoid dilatation (1%) as well as hemolysis in some individuals (Fig. 3c, f and b, respectively). No alterations were registered for the inflammatory reaction pattern.

The highest frequency of hydropic degeneration and necrosis was registered at RP, during the wet and dry season respectively (Fig. 3e). These lesions are general responses associated to chronic exposure to a variety of pesticides, heavy metals and wastewaters (Pesce et al., 2008; Costa et al., 2011; Hued et al., 2012; Troncoso et al., 2012). Necrosis has been strongly associated with oxidative stress, enzyme inhibition, cell membrane damage, and impaired integrity protein synthesis and metabolism of carbohydrates (Mela et al., 2007). Associated to these necrotic processes, hepatocyte picnosis were observed (Fig. 3d and e).

Among the registered circulatory disturbances, blood congestion was the most frequent at both sites during the dry season (Fig. 3d and g), with a higher incidence in LC. This alteration involves a greater blood flow to the liver to ensure the biodetoxification activity of hepatocytes which could cause a slowing of the blood flow due to the increase blood spaces in the hepatic tissue (Fanta et al., 2003).

Intracellular eosinophilic bodies were the only lesion registered for the progressive reaction pattern, with similar frequency between sampling sites and hydrological seasons. This alteration consists in well-delimited cytoplasmic reddish inclusions (strong red pigmentation from eosin), commonly observed in association with a strongly damaged tissue (Fig. 3g). Costa et al. (2009) referred to the presence of these eosinoplilic bodies as hyaline degeneration. Eosinophilic bodies appeared to be membrane-delimited, ellipsoidal in shape and are present in small numbers inside the cells, usually one or two, with variable size. Considering the affinity of eosin to structural proteins such as actin, it is possible that eosinophilic bodies retain peptide material absorbed from the cytoplasm of degenerating cells (Koller, 1973; Costa et al., 2011). No specific information exists regarding the biological significance of the presence of eosinophilic bodies in fish cells but some biomedical and pathological research has linked this non-specific alteration to severe lesions such as hepatic neoplasms (Chedid et al., 1999). They have also been linked to structural damage in livers from fish exposed to xenobiotics (Camargo and Martinez, 2007; Van Dyk et al., 2007).

3.3.3. Histopathological indices

Among the liver histopathological indices calculated for fish collected at Suquía River basin, only the $HI_{Liv.Rp2}$ (regressive changes) was significantly different between sampling sites, showing the highest values at RP (Table 5).

The gill histopathological indices varied between sites and hydrological seasons (Table 5). $HI_{Gill.Rp1}$ values were higher at RP during the dry season whereas the $HI_{Gill.Rp2}$ showed the highest values at LC during the wet season. On the other hand, the highest values of $HI_{Gill.Rp3}$ correspond to LC for both hydrological seasons. Finally, the histological alterations of progressive and regressive patterns contributed most to HI_{Gill}, which presented the highest values at LC during the wet season.

To determine overall fish health status through histological analyses, a total pathological index (HI_T) was calculated by adding gill and liver indices of an individual fish. The HI_T showed a variation pattern similar to HI_{Gill}, with higher values at LC, during the wet season. The same pattern of variation indicates that gill damages further affect the overall health status of the fish (Table 5).

According to the water quality assessment through the WQI calculation, LC is characterized by a high water quality. However, histopathological indices estimated from *G. affinis* collected at this site showed a greater impact on the overall health status, primarily

Table 5
Histopathological indices for *G. affinis* collected in Suquía River.

HIs	Sampling sites			
	La Calera		Río Primero	
	Wet ($n = 12$)	Dry ($n = 12$)	Wet ($n = 12$)	Dry ($n = 12$)
$HI_{Liv.Rp1}$	1.11 ± 1.45	2.00 ± 0.85	1.27 ± 1.35	1.82 ± 1.08
$HI_{Liv.Rp2}$	7.78 ± 2.91^{a}	9.33 ± 5.80^{a}	13.09 ± 4.85^{b}	12.73 ± 6.08^{b}
$HI_{Liv.Rp3}$	4.00 ± 6.00	0.33 ± 1.15	0.36 ± 1.21	0.73 ± 1.62
$HI_{Liv.Rp4}$	–	–	–	–
$HI_{Gill.Rp1}$	–	1.00 ± 1.35^{a}	1.45 ± 1.81^{a}	2.91 ± 1.38^{b}
$HI_{Gill.Rp2}$	16.22 ± 5.24^{b}	8.67 ± 5.55^{a}	6.73 ± 5.00^{a}	5.09 ± 4.50^{a}
$HI_{Gill.Rp3}$	21.78 ± 7.24^{b}	21.17 ± 5.87^{b}	11.27 ± 5.53^{a}	17.27 ± 4.41^{ab}
$HI_{Gill.Rp4}$	–	–	–	–
HI_{Liv}	12.89 ± 6.94	11.67 ± 6.20	14.73 ± 5.82	15.27 ± 7.50
HI_{Gill}	38.00 ± 10.49^{c}	30.83 ± 9.08^{bc}	19.45 ± 6.93^{a}	25.27 ± 7.00^{ab}
HI_T	50.89 ± 8.67^{b}	42.50 ± 9.99^{ab}	34.18 ± 10.56^{a}	40.55 ± 10.92^{ab}

Values are expressed as means ± SDs. Different letters, when shown, indicate significant differences among sampling sites and hydrological seasons ($p < 0.05$).

Table 6
Gill morphometric parameters and PAGE indices for *G. affinis* collected in Suquía River.

Parameter	Sampling sites			
	La Calera		Río Primero	
	Wet ($n=12$)	Dry ($n=12$)	Wet ($n=12$)	Dry ($n=12$)
SLL	24.74 ± 5.06^{a}	30.83 ± 9.91^{b}	36.24 ± 9.69^{d}	33.24 ± 7.76^{c}
BET	26.75 ± 7.91^{b}	28.58 ± 7.41^{c}	23.35 ± 5.55^{a}	23.62 ± 7.61^{a}
SLW	8.17 ± 3.31^{a}	8.96 ± 3.34^{c}	9.55 ± 4.26^{c}	8.70 ± 3.57^{b}
ID	6.68 ± 3.23^{b}	7.12 ± 3.01^{c}	10.12 ± 4.19^{d}	6.16 ± 3.21^{a}
PAGE (%)	48.62 ± 7.95^{a}	51.46 ± 6.55^{a}	60.55 ± 5.62^{b}	58.51 ± 3.80^{b}
$PAGE_W$ (%)	44.71 ± 7.59^{ab}	43.88 ± 6.84^{ab}	51.33 ± 7.24^{b}	40.96 ± 7.49^{a}
$PAGE_T$ (%)	21.96 ± 5.89^{a}	22.89 ± 6.07^{a}	31.38 ± 6.71^{b}	24.13 ± 5.52^{a}

Values are expressed as means ± SDs. Different letters, when shown, indicate significant differences among sampling sites and hydrological seasons ($p<0.05$). SLL: secondary lamellar length, BET basal epithelial thickness, SLW: secondary lamellar width, ID: interlamellar distance.

at gill level. Although the water quality gradient has been demonstrated over the last decade, several studies have mentioned the deterioration of the quality throughout the basin, where river sections that were formerly classified as pristine now have some symptoms of degradation as occurs at LC site (Maggioni et al., 2012). The histological damages registered in the present work are due to the combined effects of several contaminants in the water, in addition to the measured heavy metals (chromium, copper, mercury, lead and alpha-cypermethrin) that were close to, or even exceeded, the limits considered dangerous to aquatic fauna. Thus, the biological and environmental results obtained from the present study point out the impossibility of considering the LC site as a quasi-pristine location.

Different authors has demonstrated that *G. affinis* is an effective biomarker that can reflect the water quality conditions (Batty and Lim 1999; Orlando et al., 2005). This species is considered by Hued and Bistoni (2005) as a tolerant species of the Suquía River basin, due to its high density, even in conditions of chronic hypoxia in highly contaminated sites. *G. affinis* is a species whose biology, genetics and evolution, make it adaptable, flexible and tolerant (both individual and population levels) and it can respond to a wide range of environmental conditions fluctuating in space and time (Pyke, 2005).

3.4. Gill morphometric analysis

Changes such as secondary lamellae shortening, lifting, epithelial and chloride cell hyperplasia and hypertrophy contribute to modify the indices of proportion of secondary lamellae available for gas exchange (PAGE). These indices showed high values at RP, during the wet season, suggesting that fish condition is better at this sampling site and hydrological season (Table 6). On the other hand, the lowest values registered at LC, indicate the degraded water quality conditions and the fish health deterioration as was mentioned in the above section.

3.5. Gonopodial morphology assessment

Gonopodium development in *G. affinis* males (Fig. 4a and c) occurs in the first 40–60 days of life (Angus et al., 2001). Sexual maturation in male mosquitofish is morphologically indicated by the production of a terminal complex, consisting of hooks and serrae, on the tip of the gonopodium, that act as holdfast devices during sperm transfer (Doyle and Lim, 2002). Once fully developed, as indicated by the appearance of terminal hooks, it remains as a permanent structure and does not grow or regress further under hormonal stimulation (Angus et al., 2001) (Fig. 4b).

Several authors had used the gonopodium relative length as an indicator of exposure to contaminants or substances with estrogenic activity (Doyle and Lim 2002; Toft et al., 2003). These authors showed that exposure to endocrine-disrupting chemicals reduced gonopodium length and inhibited the androgen-dependent development of the gonopodium. Similar results has been reported by Batty and Lim (1999),Wen-ting et al. (2011) and Hued et al. (2013), who demonstrated that males in sewage-contaminated waters showed a decreased in gonopodium length. Based on these studies, we initially assumed that fish from RP site would be influenced by the contribution of the sewage discharge from the wastewater treatment plant of Bajo Grande, located downstream Córdoba city. However, neither the Gonop-SI (Table 7) nor the percentage of fish with hooks and serrae (Figs. 4b and 5) differed significantly between sampling sites and hydrological seasons (Table 7). The absence of significant differences in both parameters, suggest that LC site has a similar degree of degradation than RP site, which is characterized by a low water quality. These results suggest that LC, considered as a reference site in the present study,

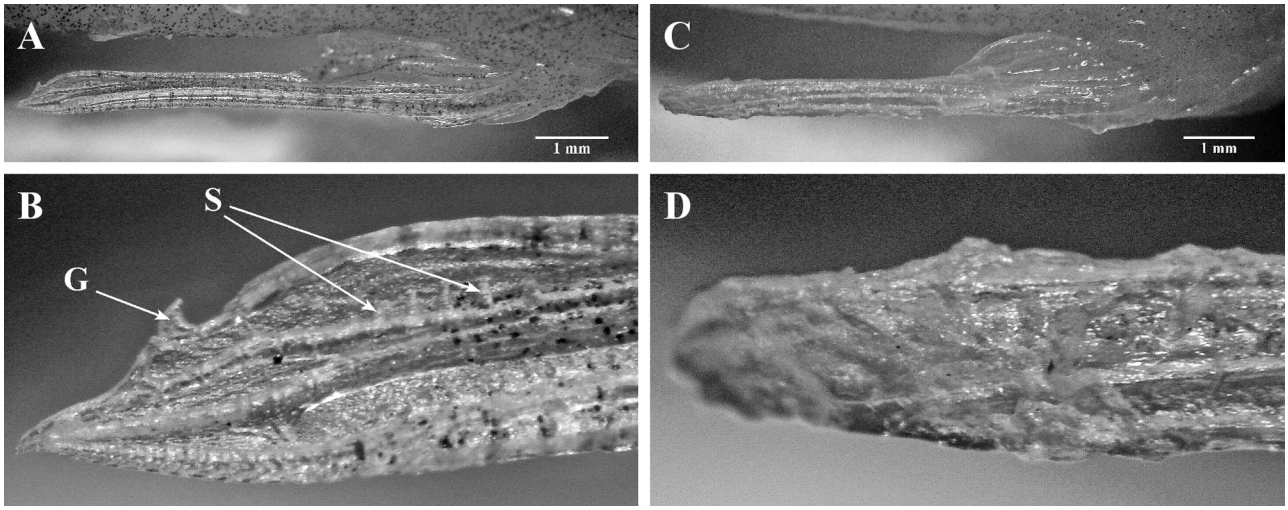

Fig. 4. Gonopodium morphology of *G. affinis* adult male. (a and b) Individuals from La Calera; (c and d) individuals from Río Primero (a) Fully developed gonopodium. (b) Gonopodial tip with hooks (G) and serrae (S). (c–d) Adult male gonopodium without hooks and serrae. (a and c = 20×, b and d = 40×).

Table 7
Descriptive statistics of somatic data for *G. affinis* males collected along the Suquía River basin.

Parameter	Sampling sites			
	La Calera		Río Primero	
	Wet ($n = 12$)	Dry ($n = 12$)	Wet ($n = 12$)	Dry ($n = 12$)
SL (mm)	23.45 ± 2.37^{b}	20.78 ± 1.74^{a}	23.70 ± 2.35^{b}	21.81 ± 2.20^{ab}
GL (mm)	7.73 ± 0.95^{b}	7.12 ± 0.40^{a}	8.06 ± 0.65^{b}	6.89 ± 0.93^{a}
Gonop-SI	33.02 ± 3.20	34.41 ± 2.26	34.15 ± 2.62	31.78 ± 4.77

Values are expressed as means ± SDs. Different letters, when shown, indicate significant differences among sampling sites and hydrological seasons ($p < 0.05$). SL: standard length. GL: gonopodium length. Gonop-SI: gonopodium somatic index.

presented a clear water quality deterioration respect to previous studies conducted at this site (Merlo et al., 2011; Maggioni et al., 2012; Hued et al., 2013). As was previously described, the LC site showed important levels of some metals (mercury, manganese, lead, zinc) and pesticides (beta-endosulfan, acetochlor and atrazine) that could act as endocrine disruptors and impact on the normal fish sexual development (Pait and Nelson, 2002; Järup, 2003; Iavicoli et al., 2009; Choi et al., 2010).

3.6. Vitellogenin gene expression

PCR in livers of *G. affinis* female produced a single amplicon of 150 bp, whereas in males two amplicons of 150 bp and 250 pb were obtained (Fig. 6). This reaction produced in both males and females a single amplification product of 77 pb for β-actin. The sequences of males PCR products were aligned with the sequence of Vtg previously described for *G. affinis* by Leusch et al., 2005 (GenBank accession number: DQ190844.1) and revealed a high identinty for both amplicoms. The 150 pb product showed 97% identity while the 250 pb product showed 100% identity with the sequence of Vtg described by Leusch et al. (2005). Further studies should be conducted in order to elucidate if the unexpected amplicom is a technical artifact or a different protein isoform. Consequently, the presence of the two PCR products allows us to confirm the expression of Vtg in male individuals but not to quantify the protein expression.

Considering the water contamination status at RP, the expression of Vtg in males collected at this site was not surprising. On the contrary, we thought that males from LC would not express this protein. However, all the males collected at this site expressed Vtg. Under normal conditions, only females express this liver protein. In males, estrogen levels are insufficient to initiate Vtg production; however, production of the protein can be induced by exposure to environmental estrogenic compounds (Björkblom et al., 2008). Therefore, Vtg expression in males provides a specific indicator of environmental estrogens. As described above for the

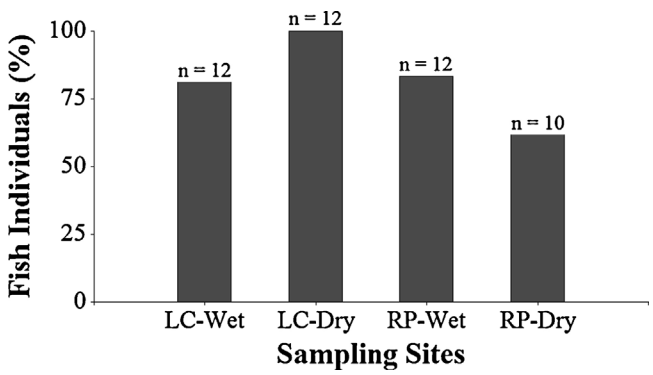

Fig. 5. Proportion of *G. affinis* males exhibiting hooks and serrae on the distal tip of the gonopodium. LC: La Calera, RP: Río Primero.

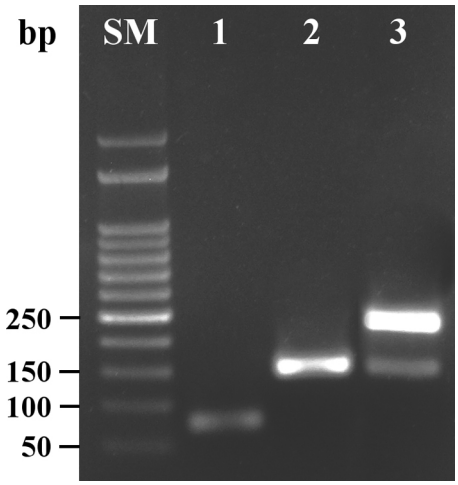

Fig. 6. Liver-specific expression of Vtg in *G. affinis*. Total RNA was analyzed by RT-PCR and amplified products of Vtg and β-actin visualized on ethidium bromide stained gels. SM size marker, lane 1 β-actin in liver 77 pb, lane 2 Vtg in liver females 150 pb, lane 3 Vtg in liver males 150 pb and 250 pb.

gonopodial morphology assessment, the presence of potential endocrine disruptors in both sampling sites affects the *G. affinis* male populations. The presence of Vtg in males of this species is the proof of the presence of substances with estrogenic activity in Suquía River basin.

The females showed significant variations in the Vtg expression (Table 8). The highest values were recorded during the dry season, at both sampling sites, which coincide with the pre-reproductive period of this species. On this season, females begin the process of vitellogenesis to address the breeding season, which takes place during the wet season. In particular, females from LC presented the highest expression values during the dry season, which directly corresponds to the breeding season. The Vtg expression registered in our work is also consistent with the HSI increase during the dry season at both sampling sites indicating an increase of liver function to synthesize Vtg.

3.7. Correlation between biotic and abiotic factors

Linear discriminant analysis (LDA) was carried out considering the "sampling site – hydrological season" combination as grouping variable (LC – wet season, LC – dry season, RP – wet season, RP – dry season). The biomarkers *K*, HSI, GSI, PAGE (%), $PAGE_W$ (%), $PAGE_T$ (%), $HI_{Liv.Rp1}$, $HI_{Liv.Rp2}$, $HI_{Liv.Rp3}$, HI_{Liv}, $HI_{Gill.Rp1}$, $HI_{Gill.Rp2}$, $HI_{Gill.Rp3}$, HI_{Gill}, HI_T were used as independent variables. Vtg gene expression was not used in LDA due to it could only be quantified in females. Classification matrix afforded 93.33% right assignations. The $PAGE_T$ (%) followed by PAGE (%), $HI_{Liv.Rp3}$ and $HI_{Gill.Rp3}$ were the most significant biomarkers to discriminate between sites and hydrological season, which means that these four parameters account for most of difference between environmental conditions.

Looking for evidences on the correspondence between biological parameters (biomarkers) and physico-chemical water parameters (WQI, metals and pesticides), we decided to apply Generalized Procrustes Analysis (GPA). This analysis produces a configuration of the different studied sites that reflects the consensus among the two matrixes (river and biota groups). The result is a consensus alignment that uses all the variables from the two data sets. The variables used as descriptors were the biomarkers *K*, HSI, GSI, $PAGE_T$ (%), HI_{Liv}, HI_{Gill}, and physico-chemical water quality parameters and those metals and pesticides that surpassed the guidelines for freshwater systems and considered dangerous to the aquatic fauna health (aluminum, chromium,

Table 8
Vtg expression by real-time RT-PCR and Hepatosomatic index in liver for *G. affinis* females collected in Suquía River.

Indices	Sampling sites			
	La Calera		Río Primero	
	Wet ($n=6$)	Dry ($n=6$)	Wet ($n=6$)	Dry ($n=6$)
$Ratio_{Vtg\ expression}$	1.19 ± 2.14^{a}	12.87 ± 9.06^{b}	4.29 ± 6.23^{a}	4.63 ± 5.01^{ab}
HSI	1.00 ± 0.42^{a}	1.93 ± 0.58^{b}	0.64 ± 0.43^{a}	1.81 ± 0.40^{b}

Values are expressed as means ± SDs. Different letters, when shown, indicate significant differences among sampling sites and hydrological seasons ($p < 0.05$).

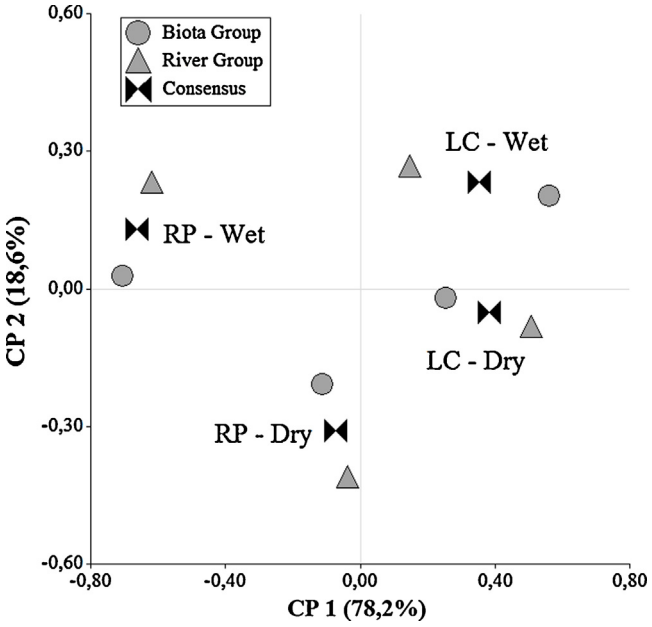

Fig. 7. Consensus space from Generalized Procrustes analysis: plot in the plane formed by the first two dimensions. Biota Group: biomarkers *K*, HSI, GSI, $PAGE_T$ (%), HI_{Liv} HI_{Gill}. River Group: WQI, aluminum, chromium, copper, mercury, lead, zinc, beta-endosulfan, alfa-cypermethrin, chlorpyrifos. LC: La Calera, RP: Río Primero.

copper, mercury, lead, zinc, beta-endosulfan, alfa-cypermethrin and chlorpyrifos). The biomarkers were named "biota group" and the environmental parameters were named "river group". The consensus configuration projected onto the plane defined by its first and second principal axis is shown in Fig. 7, explaining 96.9% of variability between samples. We can observe that the four "sampling site – hydrological season" combination are well separated on the basis of biotic and abiotic parameters. These results show that data obtained from biota has a significant consensus (91.3%) with those corresponding to river group, as the two data sets project the regions in the same way onto the plane defined by its first and second principal axis. Therefore, the results obtained through this analysis gives further indication on the connection between the biological and physicochemical parameters that leads to establish a close relationship and interaction between biotic and abiotic parameters that determine the particular conditions of each combination of sampling site and hydrological season.

4. Conclusions

Any effort to achieve rapid, inexpensive and early diagnosis towards preventing or minimizing the action of toxic substances in freshwater systems should be encouraged. Thus, the results obtained in this study contributes to strengthening the cause–effect relationship between the alterations observed in individuals of *G. affinis* and pollution levels in the aquatic environment of Suquía River middle-lower basin. This cause–effect relationship, together with its wide distribution in the basin, positioned *G. affinis* as an excellent sentinel species for water quality monitoring. This species and its closely related poeciliid species *G. holbrooki*, have been introduced into more than 50 countries and are considered among the most invasive fish (García-Berthou et al., 2005; Vidal et al., 2010). Furthermore, both species present similar life history traits and could hybridize. Therefore, they could be used as sentinels of environmental quality in different water bodies of the world.

Overall, biomarkers responded effectively over different environmental conditions, which was reflected in the somatic, histological and biochemical analyzes performed. These analyzes denote a gradient of environmental quality that positions RP as the most environmentally degraded sampling site. However, it should be noted that LC, which has been considered for over a decade as a reference site for its quasi-pristine conditions, currently shows a marked degradation. These conditions were revealed by biomarkers such as gill histology and Vtg gene expression in *G. affinis* males. The obtained results showed the negative impact that environmental degradation has on these species populations. Finally, multivariate analysis allows us to conclude the validity of integrating the study of biotic and abiotic parameters to determine the quality of water resources and thus able to provide the tools for evaluating the studied watershed.

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