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Reproductive Impairment of a Viviparous Fish Species Inhabiting a Freshwater System with Anthropogenic Impact

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Abstract The potential threat to animal reproduction by contaminated freshwater systems posed the necessity to identify and develop bioindicators and biomarkers to be used for screening and evaluation of the effects in organisms. The main goal of this work was to determine, through histological analyses and changes in gonopodium morphology, whether a freshwater system polluted by anthropogenic activities—sewage, agricultural, and industrial—could cause alterations at the organ level. We also propose the live-bearing fish, *Jenynsia multidentata*, as a species suitable to study the effects of contaminated aquatic environments. We compared male fish sampled at two different stations in Suquía River basin (Córdoba, Argentina), both differing in degree of pollution, through liver and testis histology and gonopodial morphometric parameters. The water quality, based on the physicochemical characteristics of the studied stations, varied markedly with a decrease in water quality at the downstream site (station 2). At the highest polluted area, detrimental effects on liver and testis were evidenced on histological analysis. Male individuals from station 2 also presented noticeable structural changes of the anal fin, such as a straight gonopodium and abnormal tip area. The present results demonstrate that a freshwater system polluted by the impacts of anthropogenic activities has detrimental effects to *J. multidentata*. The alterations registered in individuals from the polluted station indicate an impairment of male reproductive performance and imply a risk for other live-bearing species as well as the entire biodiversity. We consider *J. multidentata* a sentinel species that is useful to evaluate the potential risk present in the studied basin not only to itself but to other species as well.

Human influence from industry, agriculture, and urbanization are the main sources of chemical pollution of aquatic environments. Many of these activities have contaminated water supplies due to insecticide and herbicide runoff from agricultural land and industrial and sewage discharges into surface waters.

In the past few last decades, scientists have mainly been concerned about the increasing number of man-made chemicals that cause reproductive and developmental dysfunctions in fish (Viganó et al. 2001), amphibians (Reeder et al. 2005), reptiles (Guillette et al. 1996, 2000; Pickford et al. 2000), birds (Fry 1995), and mammals (Colborn and Clements 1992; De Guise et al. 1995). Reproduction, development, and growth of fish are often considered to be the most sensitive and ecologically relevant end points for environmental stressors (Goede and Barton 1990). Therefore, fish are excellent biomonitors of reproductive disorders produced by different chemicals (Kime 1999).

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Several field studies have provided direct evidence that water-quality degradation seriously impacts fish reproductive biology. Reported effects include changes in external sexual characteristics, delay in gonadal development and maturation, decrease in number and quality of eggs and sperm, inhibition of the response to pheromones, alterations of courtship patterns, decreased fertilization rates, and changes in aromatase activity and vitellogenin levels (Kime 1999; Jobling et al. 2002; Toft et al. 2003; Moncaut et al. 2003; Mills and Chichester 2005; Leusch et al. 2006; Jessica et al. 2007; Vázquez et al. 1979).

The impact of water pollution on fish assemblages and other aquatic organisms has been well documented in different watersheds of neotropical regions (Mangeaud 1998; de la Torre et al. 2002, 2005; Pompeu and Alves 2003; Cazenave et al. 2005a; Hued and Bistoni 2005; Hued et al. 2006; Kirschbaum et al. 2009). However, no studies regarding the effects of degraded water quality on fish reproduction have been published. The potential threat to animal reproduction by contaminated freshwater systems poses the necessity to identify and develop bioindicators and biomarkers to be used for screening and evaluation of their effects in organisms.

In the central region of Argentina, Córdoba Province, the water quality of the Suquía River Basin has become an important issue given that it flows into Mar Chiquita Lake, one of the largest salt lakes in the world. The mouths of the Suquía and Xanaes rivers on the southern shore, the swamps of the Dulce River on the northern shore, and the Mar Chiquita Lake itself are Ramsar sites (Ramsar Convention Bureau 2002). This area is one of the most important wetlands worldwide in terms of biodiversity and encompasses freshwater to saline environments. The presence of densely populated urban settlements and increasing environmental impact, particularly due to anthropogenic activities, characterize the central and lower sections of the Suquía River Basin (Gaiero et al. 1997; Pesce and Wunderlin 2000; Wunderlin et al. 2001; Hued et al. 2006).

The neotropical fish species *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes) is widely distributed through South America and has been found by Hued and Bistoni (2005) in pristine as well as severely degraded habitats in Argentina. *J. multidentata* has been successfully used as a sentinel species to evaluate the effects of different chemicals on different biological processes (Haro and Bistoni 1996; American Public Health Association [APHA] 1998; Malabarba et al. 1998; Hued and Bistoni 2005; Cazenave et al. 2005b; Hued et al. 2006; Ballesteros et al. 2007) prompting its inclusion as one of the suitable native fish species for the determination of the lethal acute toxicity of xenobiotics by the Argentinean Institute of Standardization and Certification (2008).

However, there is a lack of knowledge on the effect of sewage-, agricultural-, and industrial-contaminated freshwaters on the reproductive aspects of *J. multidentata*. This native fish is a sexually dimorphic live-bearing species in which the male anal fin differentiates into a complex structure called the "gonopodium," a tubular intromittent organ supported by anal fin rays that is used to transfer sperm during copulation (Jamieson 1991; Meisner 2005; Parenti et al. 2010).

Gonopodium development is an androgen-dependent process that normally occurs at the time of sexual maturation. However, normal development of the gonopodium can be affected by xenobiotic compounds. Batty and Lim (1999) reported the effects of sewage effluent exposure on gonopodial morphology in male fish of another cyprinodontiform species, *Gambusia holbrooki*. Doyle and Lim (2002, 2005) found decreased gonopodial length, anal fin elongation, and sexual activity in these male mosquitofish exposed to exogenous estrogen. Orlando et al. (2005) have proposed viviparous teleosts as excellent sentinels of exposure to environmental androgens and estrogens due to their several sexually dimorphic characteristics.

The main goal of this work was to determine, through histological analyses and changes in gonopodium morphology, whether a freshwater system polluted by anthropogenic activities—sewage, agricultural, and industrial—could cause alterations at the organ level. Given their reproductive strategies, we also propose *J. multidentata* as a fish species suited to study the effects of contaminated aquatic environments.

Materials and Methods

Study Area: Suquía River Basin

Córdoba city (Argentina) has approximately 1.4 million inhabitants, and nearly 0.7 million households are connected to the city sewage system; the rest of the sewage goes into the groundwater after home treatment (Pesce and Wunderlin 2000). During the last 20 years, the city has almost doubled its population, and the establishment of many industries has increased the risk of toxic effluents flowing into the river. Near the eastern edge of Córdoba city, the Suquía River receives sewage discharge from the wastewater treatment plant (WWTP) of Bajo Grande.

The Suquía River originates at the San Roque Dam, flows mainly from west to east for approximately 40 km across Córdoba city, and then continues down to Mar Chiquita Lake (150 km downstream from Córdoba city). This basin is located in a semiarid region of Córdoba Province, Argentina. The mean annual rainfall is in the range of 700 to 900 mm (Vázquez et al. 1979). The watershed covers approximately 7700 km^2, of which almost 900 km^2 correspond to the Córdoba city drainage area.

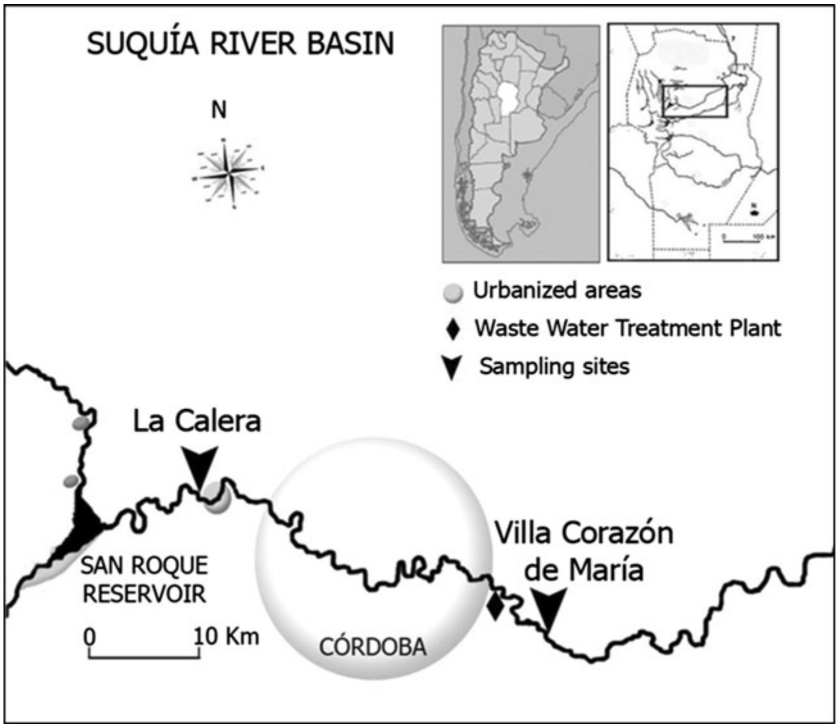

Fig.1 Studied area showing the location of sampling sites on the Suquía River basin. La Calera 526. (Station 1, non-polluted area), and Villa Corazón de María (Station 2, polluted area)

Two sampling stations were selected on the Suquía River Basin (Fig. 1). Station 1 is situated 20 km upstream of Córdoba city, near La Calera city (31°21′S; 64°21′W), and has been described as having nearly pristine conditions by Hued and Bistoni (2005). Station 2 is located 30 km downstream from Córdoba city, close to Villa Corazón de María city (31°27′S; 63°59′W), after the area where both agricultural runoff and industrial and city sewage discharges occur. Station 2 is classified as a highly polluted site (Pesce and Wunderlin 2000; Wunderlin et al. 2001; Hued and Bistoni 2005). An important decrease in water quality downstream from Córdoba city has been described, and the occurrence of diverse pollution sources, mainly associated with city sewage, has been shown (Wunderlin et al. 2001; Hued et al. 2006; Guzmán et al. 2004; Hued and Bistoni 2005, 2007).

Water-Quality Assessment

To evaluate water-quality conditions, water samples were collected at each site during 2007 totaling 12 samples (1/month) for each sampling site. The following physicochemical parameters were measured: pH, temperature (°C), conductivity (mS cm^{-1}), alkalinity (mg L^{-1}), dissolved oxygen (mg L^{-1}), carbon dioxide (mg L^{-1}), total solids (mg L^{-1}), ammonia (mg L^{-1}), nitrites (mg L^{-1}), nitrates (mg L^{-1}), chemical oxygen demand (COD; mg L^{-1}), 5-day biological oxygen demand (5-DBO; mg L^{-1}), total phosphorus (mg L^{-1}), hardness (mg L^{-1}), calcium (mg L^{-1}), magnesium (mg L^{-1}), sulfates (mg L^{-1}), chlorides (mg L^{-1}), total iron, 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 physicochemical variables, the water quality index (WQI), which was 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.

Fish Collection

Twenty adult male fish were captured at each sampling station with standard backpack electrofisher equipment in December 2009. Individuals were transported to the laboratory within 2 h of capture and placed into tanks containing 20 L of water from the capture area. At the laboratory, fish were individually killed by overdose of tricaine methanolsulfonate (MS222 [500 mg/L]). All procedures are in compliance with the Guide for Care and Use of laboratory Animals (National Institutes of Health 2011).

Histological Analysis

Liver and gonads were quickly removed and fixed in 5 % neutral buffered formalin, dehydrated through graded series of ethanol, cleared in xylene, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin for histological examinations. Sections were examined and photographed under a light microscope (Arcano XSZ-107BN).

Morphometric Parameters

Standard length (SL), gonopodium length (GL), and the gonopodium somatic index (Gonop-SI = GL × 100/SL) were calculated. The gonopodium angle (GA) and the area of the tip of the sixth anal-fin ray (GTA) were also measured. In adult male fish, this ray is an unbranched structure that is displaced forward during copulation. Gonopodia were examined with a stereoscopic microscope and photographed. To measure GL, GA and GTA, digital photographs were analyzed through Image J software v. 1.3 (Rasband 2004).

Statistical Analysis

To evaluate changes in physicochemical (WQI) and biological parameters between sampling sites, Student *t* test was performed (InfoStat 2011). Differences were considered to be statistically significant when $p < 0.05$. Discriminant analysis (DA) was performed to determine which somatic characteristics were the best predictors of effect between sampling stations.

Results

Water Quality

The physicochemical characteristics of the studied stations on the Suquía River basin varied markedly with a decrease in water quality at the downstream site (station 2) ($F = 175.94$; $p < 0.0001$). Upstream of Córdoba city (station 1), the mean WQI value was high (83.99 %), indicating nearly pristine conditions (Table 1), whereas station 2 presented a low mean WQI value (52.21 %) indicating heavily degraded conditions.

Histological Analysis

Under light microscopy, liver tissue of male fish from station 1 was composed of homogeneous parenchymal structure. Hepatocytes were supported by lattice fibers, located around blood sinusoids, and organized in poorly defined cord-like structures. This species has a hepatopancreas, with pancreatic acinar tissue interspersed with the hepatic tissue, mainly around the major blood vessels, which is a common feature among certain teleost families. Hepatic cells had a polyhedric shape with a basal nucleus and usually one nucleolus. The nuclear membrane was

Table 1 Mean values and standard deviation of each physicochemical parameters and WQI from the control and polluted sites (stations 1 and 2, respectively)

Parameters	Station nos.	
	1 ($n = 12$)	2 ($n = 12$)
pH	8.32 ± 0.57	7.49 ± 0.23
Temperature (°C)	18.19 ± 5.52	17.31 ± 6.48
Conductivity (mS cm^{-1})	0.19 ± 0.03	0.77 ± 0.24
Alkalinity (mg L^{-1})	91.75 ± 7.57	168.25 ± 11.03
Dissolved oxygen (mg L^{-1})	11.04 ± 2.31	4.06 ± 1.15
Carbon dioxide (mg L^{-1})	1.34 ± 1.13	10.65 ± 6.24
Total solids (mg L^{-1})	122.23 ± 31.70	589.38 ± 252.50
Ammonia (mg L^{-1})	0.32 ± 0.10	2.45 ± 3.24
Nitrites (mg L^{-1})	0.05 ± 0.12	0.42 ± 0.25
Nitrates (mg L^{-1})	1.23 ± 1.54	2.49 ± 1.08
Chemical oxygen demand (COD) (mg L^{-1})	25.88 ± 16.59	59.71 ± 37.35
5-DBO (mg L^{-1})	1.58 ± 0.93	3.73 ± 2.06
Total phosphorus (mg L^{-1})	0.03 ± 0.05	0.99 ± 0.38
Hardness (mg L^{-1})	82.42 ± 19.88	228.04 ± 48.51
Calcium (mg L^{-1})	22.24 ± 4.22	63.59 ± 13.36
Magnesium (mg L^{-1})	6.54 ± 2.43	16.55 ± 4.10
Sulfates (mg L^{-1})	18.62 ± 4.25	141.94 ± 41.28
Chlorides (mg L^{-1})	7.20 ± 0.99	62.58 ± 21.98
Total iron	0.05 ± 0.07	0.12 ± 0.15
Total coliforms (MPN 100 ml)	1475.69 ± 2139.44	666750.00 ± 845512.65
Faecal coliforms (MPN 100 ml)	434.21 ± 581.01	170937.50 ± 132363.79
WQI	83.99 ± 3.39	52.21 ± 5.87

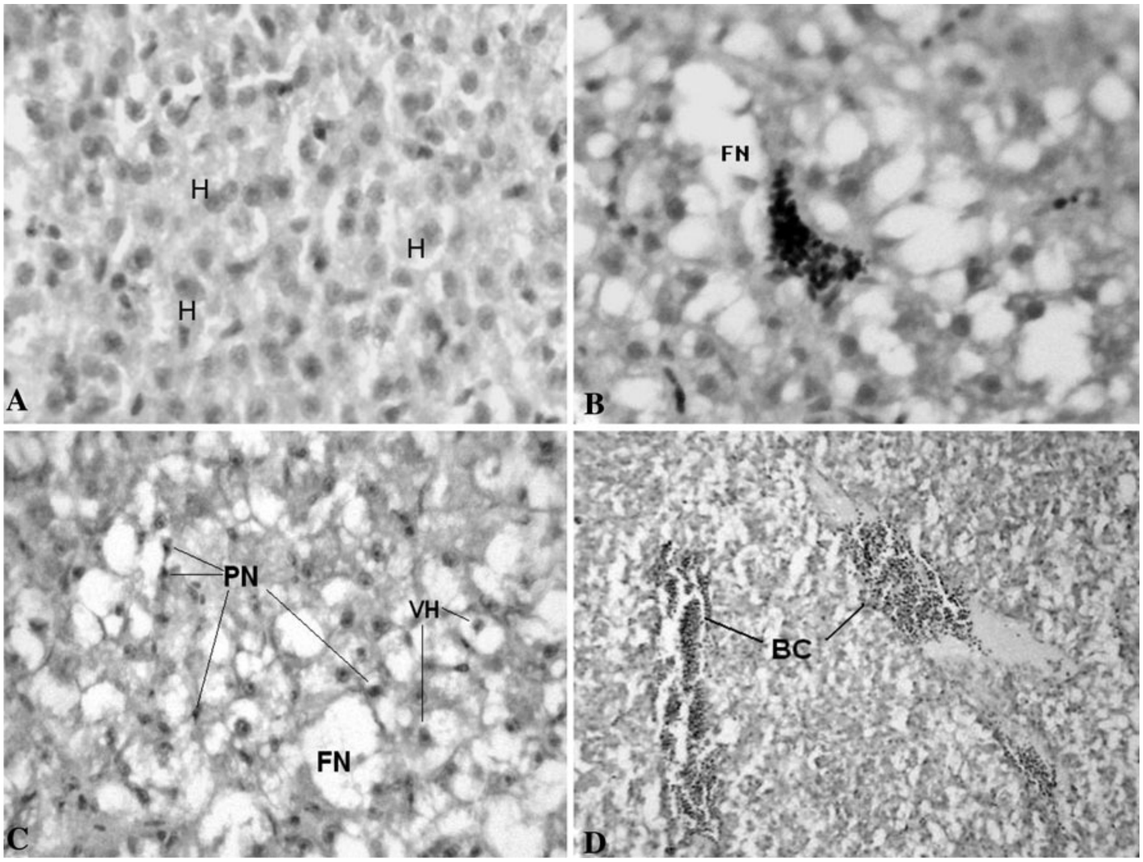

Fig. 2 Liver section of *J. multidentata*. **a** Normal aspect of a male liver from the nonpolluted area (station 1). **b–d** Liver of male fish from the polluted site (station 2). *FN* focal necrosis, *H* hepatocytes, *PN* picnotic nucleus, *VH* vacuolated hepatocytes, *BC* blood vessel congestion. Magnification: **a**, **b** = 400×, **c** = 200×, and **d** = 100×

weakly basophilic, and the cytoplasm contained eosinophilic material (Fig. 2a).

Fish liver from the polluted sampling site (station 2) showed a loss of hepatic structure with cellular swelling where hepatocytes appeared cloudy and enlarged due to water influx (hydropic degeneration) (Fig. 2b, c). Hepatocytes showed picnotic nuclei and highly vacuolated cytoplasm leading to a foamy aspect. Together these cellular characteristics indicate a necrotic process (Fig. 2c). The liver tissue also showed severely congested hepatic blood vessels (Fig. 2c, d).

Testes of *J. multidentata* were a pair of elongated structures and corresponded to the lobular restricted testis type according to the arrangement of the germinal compartment. Under light microscopy analysis, lobules contained isogenic germ cells enclosed by Sertoli cells forming cysts organized (from the periphery to efferent ducts) from immature to mature germ cell cysts; spermatogonia were restricted to the distal termini of lobules. In testes of fish from station 1, all of the stages of spermatogenesis were present. Testicular lobules contained spermatogonia as well as numerous cysts with early (spermatocytes) and late (spermatids) stages of spermatogenesis (Fig. 3a, b). Mature sperm was observed only near the efferent ducts. Interstitial compartment consisted of connective tissue, blood vessels, and Leydig cells (data not shown).

Histological examination of fish testes from station 2 showed disarrangement within the interstitial and germinal compartment and, consequently, damage of testicular structure. Although different stages of spermatogenesis were observed, cysts with immature germ cells were located near the efferent ducts indicating disorganization of testicular tissue (data not shown). Some spermatogonia presented variable amounts of cytoplasmatic vacuolization that did not displace the nucleus, whereas others presented pale and foamy cytoplasm and a peripheral and condensed nucleus (Fig. 3c). Degenerative changes also included occasional germ cell syncytia (Fig. 3d).

Morphometric Parameters and Gonopodial Morphology

The adult male fish collected from station 2 presented shorter length and lower weight compared with those collected from station 1 (Table 2). GL, Gonop-SI, and GTA were significantly lower in fish from station 2, but GA was greater, thus indicating impairment of secondary sexual characteristics.

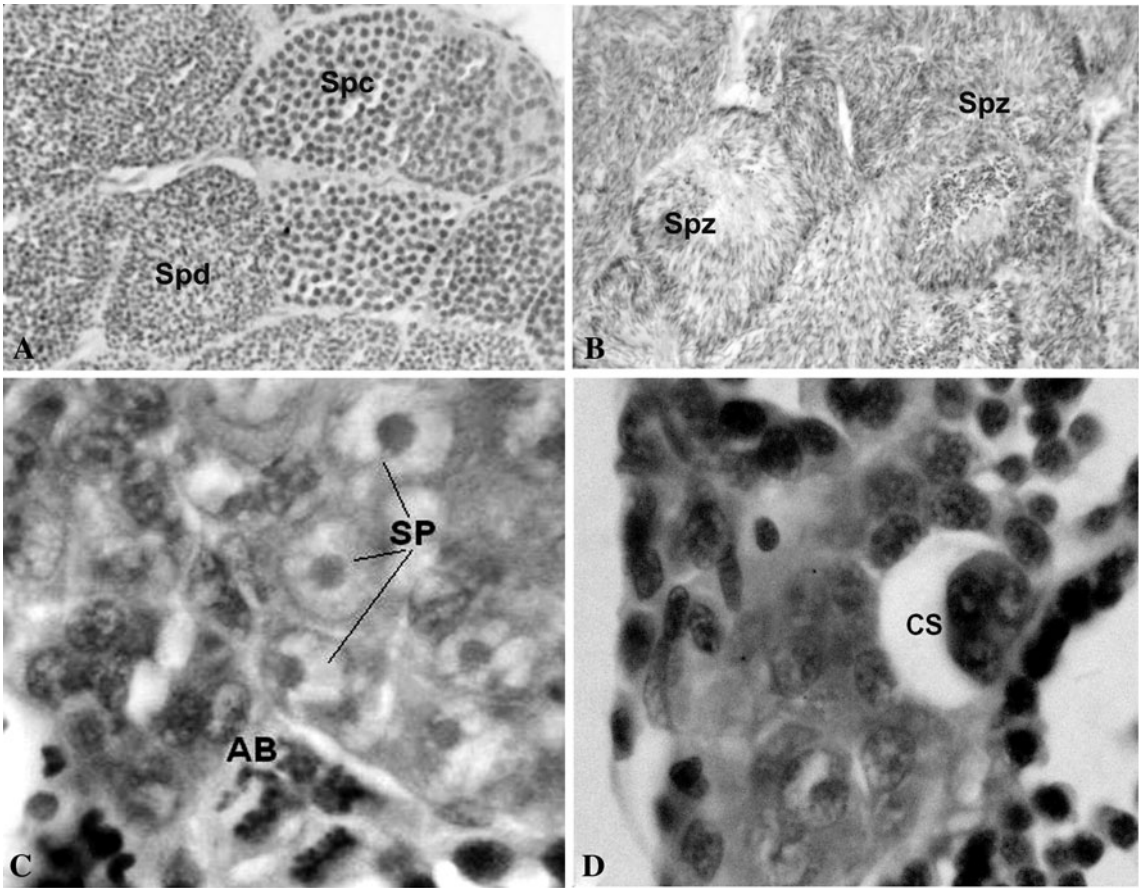

Fig. 3 Testes section of *J. multidentata*. **a**, **b** Normal spermatogenesis stages within spermatocysts (spermatocytes and spermatids) of a testis from the control site (station 1). **c**, **d** Testes from male fish in the polluted site (station 2). **b** Cysts full of spermatozoa. **c** Apoptotic cell bodies and citoplasmatic vacuolization of spermatogonias. **d** Germ cell syncytia. *AB* apoptotic bodies, *GC* germ cell syncytia, *SP* spermatogonia, *Spc* spermatocytes, *Spd* spermatids, *Spz* spermatozoa. Magnification: **a**, **b** = 100×; **c**, **d** = 600×

Table 2 Descriptive statistics of somatic data for male *J. multidentata* and total abundance from the control and polluted sites (stations 1 and 2, respectively)

Somatic variables	Stations	
	1 ($n = 20$)	2 ($n = 20$)
SL (mm)	32.37 ± 1.96	24.10 ± 4.86*
Weight (g)	0.528 ± 0.140	0.214 ± 0.093*
Fulton condition factor	1.98 ± 0.25	1.98 ± 0.27
GL (mm)	7.41 ± 0.64	5.45 ± 1.07*
Gonop-SI (%)	24.68 ± 2.13	21.81 ± 3.96*
GTA (mm^2)	0.34 ± 0.15	0.19 ± 0.24*
GA	21.96 ± 5.09	50.19 ± 56.55*

* Significant differences at $p < 0.05$

SL Standard Length, *W* Weight, *K* Fulton Condition Factor, *GL* Gonopodium Length, *Gonop-SI* Gonopodium Somatic Index, *GTA* Gonopodium Tip Area, *GA* Gonopodium Angle

The gonopodia of fish collected from station 1 showed normal external morphology. The tubular intromittent organ, supported by the anal fin rays, presented an inclination that determines an angle on the distal part of the gonopodium (Fig. 4a, b).

Male individuals from station 2 presented noticeable structural changes of the anal fin. Figure 4c, d show a straight gonopodium and an abnormal tip area, respectively. The abnormalities observed correlated with the variations in morphological parameters.

DA based on morphological characteristics also defined the two same working groups: one corresponding to fish from station 1 (nonpolluted), whereas the other corresponded to fish from station 2 (polluted). Stepwise DA affords a classification matrix showing 100 % right assignation (Table 3). Discriminant functions showed that SL and Gonop-SI are the main parameters to differentiate between male fish from both site samplings.

Discussion

The present results demonstrate that sewage-, agricultural-, and industrial-contaminated freshwater impact the liver

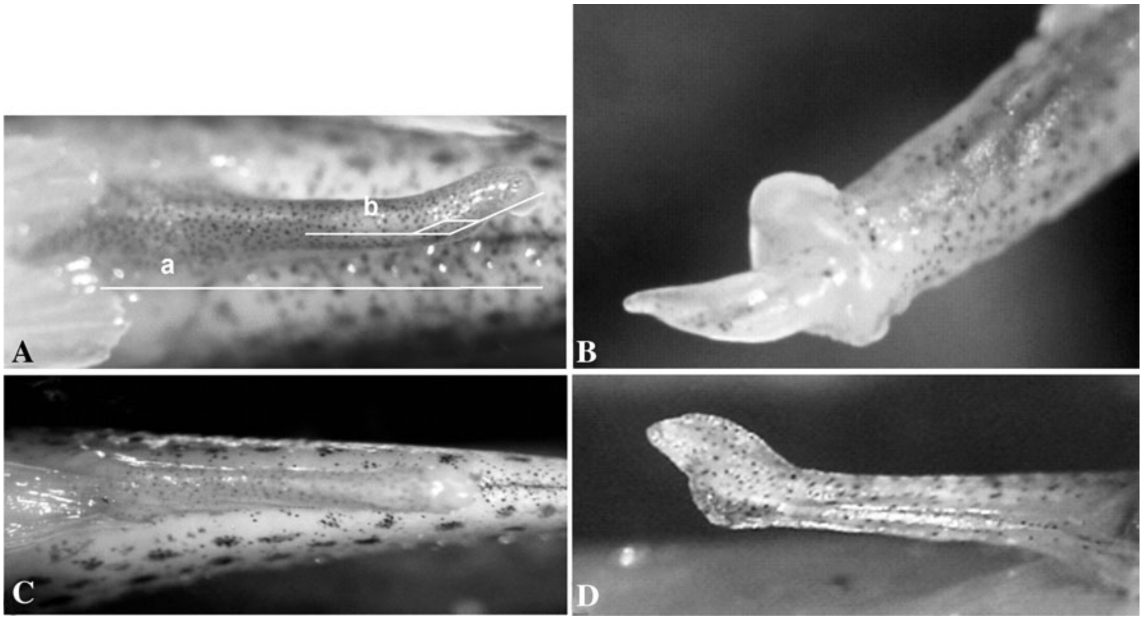

Fig. 4 Normal and altered external morphology of *J. multidentata* gonopodium from stations 1 and 2, respectively. Note **a** normal gonopodium angle, **b** area of the tip of the sixth anal-fin ray in a fish from station 1, **c** straight gonopodium, and **d** abnormal gonopodium tip in a fish from station 2. **a** gonopodium length; **b** gonopodium angle. Magnification: **a**, **c**, and **d** = 20×; **b** = 40×

Table 3 Discriminant function coefficients of somatic variables between sampling stations on the Suquía River Basin

Somatic variables	Discriminant function coefficients[a]
SL	–2.00
Gonop-SI	–1.20
GL	0.81
GTA	0.14
GA	0.29

[a] Coefficients are ordered by their relevance

and testis histology and gonopodial morphology of *J. multidentata*.

Most toxic compounds are biotransformed in metabolites by the liver. Therefore, this organ undergoes different levels of damage as a consequence of this process (Fanta et al. 2003). Histological analysis showed a loss of hepatic structure, hydropic degeneration, and necrotic areas. The registered alterations indicate that wild *J. multidentata* from the polluted site have been affected by the poor water quality (WQI) registered in the present work.

According to Martínez and Monasterio de Gonzo (2002), and Parenti and Grier (2004), testes of *J. multidentata* correspond to the lobular restricted type. The histological analysis of fish testes also suggests an impact of water pollution on fish gonads. Fish testes presented structural changes in gonadal tissue. The common lesions observed included disorganization of testicular tissue, spermatogonia with different degrees of cytoplasmatic vacuolation, and germ cell syncytia. The alterations observed have been indicated as common changes occurring in fish exposed to different chemicals with hormonal activity (Miles-Richardson et al. 1999; Kwak et al. 2001). These alterations could decrease the reproductive success of fish living in degraded water-quality conditions.

Recent research in the Suquía River basin has shown 39 contaminants belonging to the following families: chlordanes, DDTs, drines, endosulphanes, heptachlor-hexanes, and polychlorinated biphenyls (Ballesteros 2010). Monferrán et al. (2011) pointed out that some metals exceed the risk levels at the same stations where the studied fish were captured. For instance, values registered for chromium at station 2 exceed the threshold regulated value of 2.5 mg L^{-1} (Subsecretaria de Recursos Hídricos de la Nación [SRHN] 2004). A similar situation was observed with lead, which exceeded the threshold value (1.6 mg L^{-1}) for aquatic life preservation in the Suquía River (Merlo et al. 2011; Monferrán et al. 2011).

These findings, together with water-quality deterioration due to sewage discharges registered through physicochemical parameters from 1998 (Hued and Bistoni 2005) to the present, indicate the potential risk of fish species of suffering different behavioral and physiological alterations. The presence of several xenobiotics and the variations of natural chemical parameters, such as decreased dissolved oxygen or increased nitrates due to anthropogenic activities, have been related to alterations in different aspects of fish reproduction (Edwards et al. 2006; Edwards and Guillette 2007; Wu 2009).

The xenobiotics reported, as well as the degraded water-quality conditions along the Suquía River basin, could affect the reproductive characteristics of male *J. multidentata* as evidenced by gonopodium shortening presumably by way of disruption of gonopodium growth and development during sexual maturation. Gonopodium development is androgen-dependent and normally occurs at the time of sexual maturation (Ogino et al. 2004). Once developed, this reproductive bony structure requires no further androgenic stimulation to be maintained. Toft et al. (2003) pointed out that gonopodium development is sensitive to chemicals in the environment possibly through disruption of endocrine function. Our results suggest that male fish were under the effects of xenobiotics that could disrupt normal development of gonopodial structure. Similar results were reported by Batty and Lim (1999), who showed that male *Gambusia affinis* in sewage-contaminated waters showed decreased gonopodium length. The size of *J. multidentata* is a sexually dimorphic trait with male fish being smaller and displaying secondary sex ornamentation. During development, the male gonopodium is brought forward on one side only resulting in "left" and "right" male fish (Chambers 1987). This inclination determines the appearance of an angle on the distal gonopodium tip and the copulatory position during copulation. Mating in *J. multidentata* is coercive: Male fish approach female fish from behind and attempt to thrust their copulatory organ into the female genital pore (Bisazza et al. 2000). The decrease in the gonopodium angle or the absence of an angle (straight gonopodium), in addition to the gonopodium shortening registered in the present work, indicate that male fish may not be able to bring forward the gonopodium in the correct direction to contact the female gonopore during copulation. Moreover, Kahn et al. (2010) showed that female mosquitofish displayed a preferential behaviour to associate with male fish with longer intromittent organs. Thus, we suggest that all of these alterations are likely to decrease successful mating.

According to DA, SL and Gonop-SI were the main parameters differentiating male fish between sites with different water qualities, thus indicating potential dysfunctional reproduction. The observed histological alterations and the gonopodium modifications in *J. multidentata* captured at the polluted station indicate impairment of male reproductive performance and imply a risk for other live-bearing species as well as the entire biodiversity.

The widespread distribution of *J. multidentata*, its persistence in highly polluted waters, and the observed alterations (liver, testes, and gonopodium) make this fish species an interesting model to assess the effects of contaminated of freshwater systems in South America. In addition, we consider *J. multidentata* a sentinel species, *i.e.*, useful to evaluate the potential risk present in the studied basin not only to itself but to other species as well.

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