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Female masculinization and reproductive success in *Cnesterodon decemmaculatus* (Jenyns, 1842) (Cyprinodontiforme: Poeciliidae) under anthropogenic impact

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Abstract

Aquatic organisms are exposed to a myriad of chemical compounds, with particular concerns focused on endocrine disruptors. Growing scientific evidence indicates that these compounds interfere with normal endocrine function and could affect the reproductive system of humans and wildlife. We analyzed the proportion of masculinized females, defined by elongation and fusion of the anal fin rays, and the extent of masculinization, masculinization index, defined by anal fin length divided by the standard length, of *Cnesterodon decemmaculatus* resident to areas of agricultural and urban-industrial activities in the Arroyo Colorado basin. Additionally, a bioassay was carried out to assess the potential effects of masculinization on reproductive success, measured as the number of viable progenies, using pregnant females from the site downstream of the urban–industrial zone. Masculinized females were observed in all sampling sites, particularly downstream of the urban–industrial area, where over 80% of females presented abnormal sexual characteristics and the highest masculinization index was registered. In the laboratory, masculinized adult females showed male mating behavior, and survival of their progeny was lower than those of normal females. To our knowledge, this is the first report of endocrine disruption in field-collected *C. decemmaculatus*, and the first evaluation of the reproductive success of masculinized females. Finally, our results support *C. decemmaculatus* as an excellent sentinel species due to its wide distribution, easy culture in laboratory conditions, and its potential capability to respond to sources of pollution, particularly endocrine disruptors.

Keywords Masculinization · *Cnesterodon decemmaculatus* · Urban-industrial effluents

Introduction

The release of contaminants into the environment has increased dramatically due to human population and urbanization expansion, among other factors. Contaminant exposures in the aquatic environment depends on the type and intensity of land use (residential/industrial), and sources of pollutants, such as industrial and wastewater treatment plant effluents, and storm water drainage. Particularly in developing countries, urban areas are often not properly planned and controlled, and consequently sewage and industrial effluents are frequently discharged without proper treatment (FAO-AQUASTAT 2012, Sato et al. 2013). In addition, agricultural land use is becoming more intensive worldwide because of increased demand for food, fiber and fuel (Paruelo et al. 2006). Consequently, aquatic ecosystems receive different pollutants that affect the resident biota. In recent years, particular emphasis has been focused on endocrine disruptor chemicals (EDCs) and their effects on

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wildlife (WHO/UNEP 2013). These substances interact with the normal endocrine system functioning and cause reproductive and developmental disorders. A large numbers of compounds (heavy metals, pesticides, pharmaceutical products) (Colborn et al. 1993; Tyler et al. 1998) and anthropic sources (industrial and urban effluents) (Parks et al. 2001; Liney et al. 2006) have been recognized as EDCs.

Aquatic organisms, particularly fish, are vulnerable to EDCs because they are directly exposed via specialized permeable transport tissue such as gills and skin, and through their diet (Kwong et al. 2008). Adverse effects on the reproductive system and functioning of fish exposed to EDCs have been reported, including changes in sex ratio and plasma steroids levels (Folmar et al. 1996; Parks et al. 2001), alteration of gonadal tissue (Devlin and Nagahama 2002; Hued et al. 2012) and modification of the secondary sexual characters (Orlando et al. 2002; Young et al. 2016).

For these reasons, fish populations and communities have been proposed as indicators of aquatic quality (Karr 1981; Karr 1991; Peterson et al. 2011). In recent years, different biological aspects of the fish species *Cnesterodon decemmaculatus* (Jenyns, 1842) (Cyprinodontiforme: Poeciliidae) have been evaluated as environmental biomarkers in the extensive Pampean region that covers three South American countries (Uruguay, Brazil and Argentina) (Maggioni et al. 2012; Chalar et al. 2013; Benejam et al. 2015; Bonifacio et al. 2016). Based on their findings these authors have proposed *C. decemmaculatus* as a local sentinel species and as an excellent laboratory model to be used in ecotoxicological evaluations. *Cnesterodon decemmaculatus* is a lecitotrophic viviparous fish, with internal fertilization and sexual size dimorphism, with females being larger and more robust than males. During maturation, anal fin rays of males are fused to form the *gonopodium* involved in the internal fertilization (Chambers 1990). *Gonopodium* development is modulated by the correct androgenic stimulation during sexual maturation (Angus et al. 2001; Angus et al. 2005; Brockmeier et al. 2013). Other fish species with similar hormone-dependent sexual dimorphism, such as *Gambusia affinis* and *Poecilia reticulata* (Poecilidae), have been proposed as sentinel organisms for monitoring EDCs exposure (Parks et al. 2001, Orlando et al. 2002; Larsson et al. 2002). A remarkable feature is that under androgenic contamination, the anal fin of the females of these species could be modified to develop a *gonopodium* that is identical to normal males, a phenomenon called female masculinization. This modification indicates that the females have been exposed to and have been affected by environmental substances with androgenic activity. Female masculinization has been described in different fish species exposed to complex environmental mixtures such as urban-industrial effluents (Xie et al. 2010; Wen et al. 2013), and particularly paper mill effluents (Bortone et al. 1989; Parks et al. 2001; Larsson et al. 2002; Orlando et al. 2002; Hou et al. 2011).

Since we found females of *C. decemmaculatus* with male sexual secondary characters, (*gonopodium*) in the Colorado stream (La Plata River basin, Uruguay), we decided to evaluate this response as an indicator of responses to anthropogenic activities. For this purpose, we analyzed the proportion of masculinized females, and compared the masculinization index in four sites of the Colorado stream basin with contrasting anthropogenic activities. Additionally, we assessed the effect of masculinization on reproductive success through the survival analysis of normal and masculinized females and their progeny in a laboratory assay.

Methods

Study area and sampling design

Fish were collected in the Colorado stream basin (62.2 km^2), a small tributary of the Santa Lucia river located in southern Uruguay (Fig. 1a). The main land uses in the Colorado stream basin include agriculture (deciduous fruit trees and vegetable crops) and two urban areas (Progreso and Las Piedras), with around 85,000 inhabitants (INE, 2006). The urban area also includes industrial activities such as tanneries (Fig. 1a).

Samplings were conducted in four sites along the basin, three located on Colorado stream: Site A (34°41'54.29"S; 56°11'47.38"W) upstream the urban-industrial zone, site B (34°41'36.63"S; 56°14'14.38"W) immediately downstream the urban-industrial zone and site C (34°41'54.29"S; 56° 11'47.38"W) eight kilometers downstream to the urban-industrial zone and one site on Cañada del Colorado stream site D (34°40'26.77"S; 56°15'32.39"W) (Fig. 1a). In order to study the seasonal variability, samplings were performed in: autumn (April 2004), winter (August 2004), spring (early November 2004) and summer (February 2005).

Fish were captured using electrofishing (Sachs Elektrofischfanggeräte GmbH, Type FEG 1000) (Garner 1996) with a sampling effort in each site of 50 electric pulses along 100 meters, grouped in 5 replicates of ten pulses each. Collected fish were euthanized at field immediately after the capture with an overdose of 2-phenoxi-ethanol (1 ml L^{-1}) and then were fixed in 4% formalin solution. The genus *Cnesterodon* is widespread in the pampean region (Lucinda 2005); particularly, this species is ubiquitous in all the sampled basins in the Uruguay (see data from the Fish Collection of Facultad de Ciencias, Montevideo Uruguay, ZVCP) (Fig. 1b). This study was conducted outside protected areas and since *C. decemmaculatus* is a non-

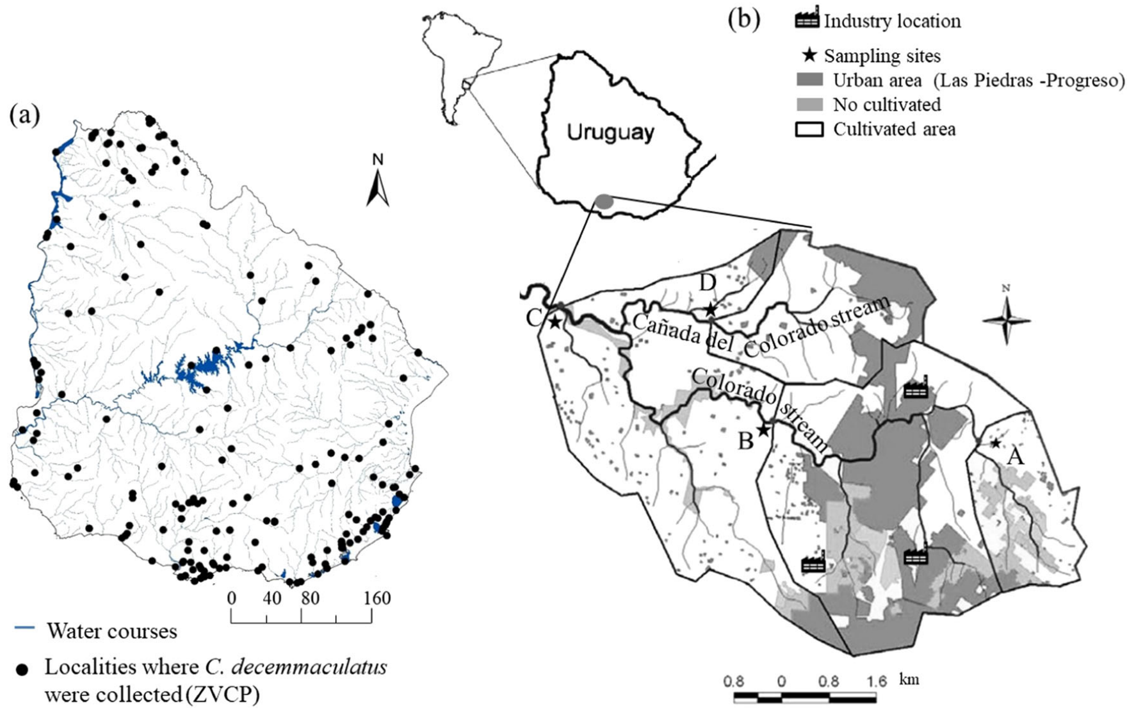

Fig. 1 **a** Distribution of *C. decemmaculatus* in the different basins of Uruguay. Dots represents localities where individuals of *C. decemmaculatus* were historically collected (fish collection of Facultad de Ciencias, Montevideo Uruguay, ZVCP). **b** Colorado stream basin and land uses, sampling sites and industries location. Site A and B are located on Colorado upstream and downstream respectively of Las Piedras-Progreso (urban-industrial) and site C, 8.5 km downstream the urban zone. Site D, is located on the Cañada del Colorado stream, which receives contributions from a largely agricultural area with an urban area without industrial activity

endangered species, permissions for fish collection were not required. In addition, this sampling protocol was developed to minimize the number of collected individuals and suffering of the animals and it was approved by an ethic committee (Comisión Honoraria de Experimentación Animal, Universidad de la República, Uruguay https://chea.edu.uy/) which regulates the use of animal for investigation in Uruguay.

Land use and water quality

Because we couldn't measure contaminants directly, we inferred contaminant exposures by interpreting land use and general water quality parameters. From satellite images from year 2000, and digital topographic charts (1:50,000), land uses were classified in cultivated, non-cultivated, and urban areas, and were measured using the QGIS (Quantum GIS), a free geographic information system. The industries location was obtained from the public list of industrial effluent discharge available in http://www.dinama.gub.uy/.

To characterize the water quality at each site before fish capture, we measured at five points along 100 meters of the stream reach, the following parameters: conductivity and pH using a Tracer Pocket tester Code 1766 (0.1 μScm^{-1} and 0.01 acid units); temperature and dissolved oxygen (YSI Model 58, 0.01 $mg\,L^{-1}$). Additionally, we collected five water samples from each site in 500 ml plastic bottles, which were transported to the laboratory at 4 °C, and measured for alkalinity (mg $CaCO_3$ L^{-1}), total solids (mg L^{-1}) (STS) suspended organic matter (mg L^{-1}) (SOM), (APHA 1995), total phosphorus (µg L^{-1}) (TP) (Valderrama 1981) and nitrate (µg L^{-1}) (Müller and Widemann 1955).

Presence of masculinized females, masculinization index and reproductive success

Standard length (SL) and anal fin length (AL) of all the individuals of *C. decemmaculatus* were measured in the laboratory using a caliper, accurate to the nearest 0.1 mm. Sex was determined by visual inspection of the anal fin; individuals were classified into four categories: (i) normal female (anal fin without enlarged fused rays) (Fig. 2a); (ii) masculinized females (elongated and occasionally fused anal fin rays) (Fig. 2b); (iii) normal males (fully developed *gonopodium*) (Fig. 2c); and (iv) immature individual less than 1.5 cm of SL. To examine the bone structure of the *gonopodium* and anal fin, a subsample of adults was cleared and stained following the Dingerkus and Ulher (1977) technique (Fig. 2).

To compare the masculinization degree of females between sampling sites, we calculated a masculinization index (MI), which is the anal fin length divided by the standard length for each individual.

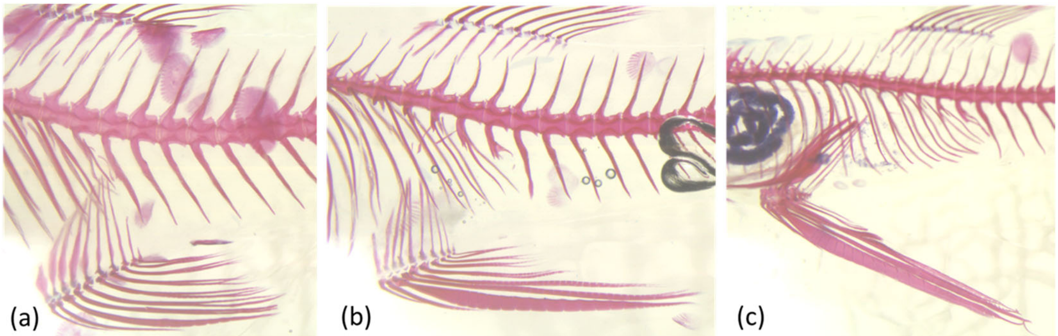

Fig. 2 Cleared and stained bony tissues of midsections of adults of *C. decemmaculatus*: **a** normal female, **b** masculinised female showing elongated anal fin rays and **c** normal male

The reproductive success (measured as the number of viable progeny) of masculinized and normal females was compared in a 30 days laboratory assay using 15 masculinized and 14 normal pregnant females, collected during austral spring at the beginning of the reproductive period (Lorier and Berois 1995). Masculinized pregnant females were collected from Colorado stream downstream from the urban- industrial location (site B). Normal pregnant females were collected from another site located in Santa Lucía river basin without apparent sources of pollution. The individuals were transported to the laboratory and kept in a common aquarium with tap water and aerators for acclimation for two days. Then pregnant females were placed separately in individual aerated aquariums, 2 L capacity, filled with dechlorinated tap water. We measured temperature, dissolved oxygen, and pH in each aquarium using portable instruments. During the experimental period, the water temperature was 24.4 ± 1.6 °C SD; dissolved oxygen 8.8 ± 0.9 SD mg L^{-1} and pH 7.0 ± 1.7 SD. Adults were fed using commercial balanced fish food (Marplatense S.A). Every day, the newborns were separated from adults after birth and counted; they were fed with algae (*Senedesmus sp.*) during the first two days and then fed commercial balanced feed. Data obtained were grouped into ten days intervals (day 0–10, 11–20, 21–30) and cumulative percent mortality within these intervals was calculated for adult females and their progeny.

We also performed a behavior assay: masculinized and normal females were placed in the same aquaria for 10 min and male mating behavior of masculinized females was recorded. The presence-absence of male mating behavior (approach, chase, display, thrust or penetration) was recorded following Bortone et al. (1989).

Statistical analysis

To test differences in the physicochemical characteristics between sites and time we performed a non-parametric Kruskal-Wallis test (KW) followed by multiple comparison test ($p < 0.05$) using as categorical predictor sites and time, respectively. To test differences in female (normal and masculinized) characteristics between sites, we selected the same range of sizes of females: larger than 1.5 cm and smaller than 3.0 cm for the three sites. In order to compare the anal fin length and the proportion of masculinized females between sites, ANOVA and ANCOVA were applied, with standard length as co-variate for the latter. To evaluate changes in the allometric characterization of secondary sexual characters, a Student *t* test was applied. The relationship between the number of fry of masculinized females and the masculinized index and standard length were described using regression analysis. For all statistical procedures we used Past Software Package (Hammer et al. 2001).

Results

Land use and water quality

Land use was classified as: agriculture areas, (67.0 %), urban areas (19.9 %), areas without agriculture (10.8%) and areas occupied by abandoned sand and gravel pits (2.3%) Site A drains 4 km and the most important land use was agriculture (71.2%), followed by urban areas (3.0%) without industries. Site B drains 19.4 km^2 and includes agriculture (52.9%) and a large urban area (33.1%) with three industries (tanneries). Site C is located downstream, drains 41.1 km^2, including agriculture and urban area (72.8 and 17.2%, respectively) with the same three industries (tanneries). Finally, site D drains 6.3 km^2 and land use included agriculture and urban area (71.0 and 26.1%, respectively) without industries (Table 1).

We detected significant differences between sites for organic matter, conductivity, dissolved oxygen, alkalinity, total phosphorous and nitrate (Table 2). The highest average

Table 1 Description of the area and land use registered in each study sites

Land use	A	B	C	D
Area (Km^2)	4.0	19.4	41.1	6.3
% Urban area	3.0	33.1	17.2	26.1
% Cultivated area	71.2	52.9	72.8	71.0
% Not cultivated area	20.5	11.2	8.6	3.0
Number of tanneries	0	3	3	0

value for organic matter was registered at site B (11.66 ± 1.67 mg. L^{-1}), whereas the highest conductivity value corresponded to site C (965 ± 30.6 μS.cm^{-1}). The lowest value for dissolved oxygen was recorded at site C (4.69 ± 0.22 mg L^{-1}), and the highest value for alkalinity at site D (303±17.3mg $CaCO_3$ $L^{-1)}$ (Table 2). The highest value of phosphorous was registered at site B (1197 ± 50.4 μg L^{-1}), while the nitrate value was highest at site A (1848.7 ± 216.7 μg L^{-1}). On the other hand, significant differences between sampling times were registered for all the environmental variables except for TP (Table 3). The highest values of total suspended solids and organic matter were observed during spring (56.1 ± 9.2 and 13.4 ± 1.8 mg. L^{-1} respectively); the values of pH, conductivity and oxygen were higher during autumn (7.8 ± 0.1, 966.2 ± 26 μS.cm^{-1} and 9.9 ± 0.7 mg L^{-1} respectively). Finally for alkalinity and nitrate, the highest values were recorded during winter (270.0±7.4 mg $CaCO_3$ L^{-1} and 2127.4±175.1μgL^{-1} respectively) $^($Table 3$^)$.

Proportion of masculinized females and masculinization index, in field

A total of 1295 individuals of *C. decemmaculatus* were collected at the four sites during the seasonal sampling (Table 4). The highest proportion of masculinized females (87.5 %) (ANOVA $F_{(2, 11)} = 42.5$, p<0.001) was observed at site B, located immediately downstream of the urban and industrial zone. However, some females of *C.decemmaculatus* with some degree of masculinization were collected at the other sites located on Colorado stream, but at lower percentages: 14.0% at site A and 6.3 % at site C. Masculinized female were not registered at site D, located on the Cañada del Colorado stream, immediately downstream of an urban zone without industries. The masculinization index (MI) was significantly different between the three sites (K-W, $H_{(2, N=636)} = 264.3$; $p < 0.001$, M-W $^{\text{pairwise comparison)}}$. The highest MI values was observed at site B (0.27 ± 0.05 SD), located downstream of the urban industrial zone, whereas site C showed intermediate values of MI (0.23 ± 0.05 SD), followed by site A (0.21 ± 0.03 SD) located upstream of the urban industrial area (Fig. 3). The MI was not calculated for site D because masculinized females were not captured.

The interaction model using the standard length as a co-variable and the site as categorical predictor (ANCOVA, Lanal~Lst*site), was significant ($F_{(2599)} = 15.2$; p < 0.0001), $^{\text{consequently}}$ the effect on the anal fin was different between sites. Individuals from site B showed longer anal fin length (0.625 ± 0.098 cm), compared to site C (0.533 ± 0.078 cm) and site A (0.486 ± 0.065 cm).

The standard length and anal fin length of normal and masculinized females showed a negative allometric relationship, whereas an isometric relationship was registered in males (Table 5). Morphological differences among males, normal and masculinized females of *C. decemmaculatus* are shown in Fig. 2 and the regression parameters in Table 5. The maximum standard length reached by normal females was higher than masculinized females (3.41 and 2.83 cm, respectively) ($F_{(1636)} = 4.8$, p=0.03). However, no differences were observed among sites in female size (normal and masculinized) ($H_{(2, N=636)= 1.8}$p=0.4) or male size ($H_{(2, N=428)} = 2.2$, p= 0.03).

Reproductive success in C. decemmaculatus in the laboratory

A total of 29 parturitions (487 fry) were obtained from the laboratory assay. Fifteen of them corresponded to masculinized females (359 fry), and 14 to normal females (128 fry). Mortalities of masculinized female progeny (47%) and masculinized females (27.3%) were recorded during the birth. In contrast, mortality of normal adult females and their progeny during birth never occurred. The accumulated progeny mortality for masculinized females reached 96% at day 30 of the experiment, whereas only 22% mortality of the normal female progeny was recorded at the same time (Fig. 4). The total progeny number of masculinized females was negatively correlated with the masculinization index (MI) ($R^2 = 0.61$; $p < 0.001$), while the relationship with the standard length (cm) was not significant (Fig. 5).

During the behavioral observations, all the masculinized females showed some degree of male mating behaviors when they were placed in the same aquaria with normal females. Masculinized female chased normal females with erect *gonopodium* and thrusting, however penetration was never observed. In contrast, normal females did not show male display when they were placed together.

Discussion

The Colorado stream basin is strongly influenced by the combination of intensive agricultural land use, and urban and industrial activities without proper treatment of

Table 2 Mean and standard error of physicochemical parameters (n = 20) for the different sampling sites

Physicochemical parameter (n = 20)	A	B	C	D
Total suspended solid (mg L^{-1})	35.5 ± 4.2	36.8 ± 8.7	33.5 ± 5.6	38.93 ± 4.19
Suspended particulate organic matter (mg L^{-1})	7 ± 0.9	11.7 ± 1.7	7.8 ± 1.3	9.79 ± 0.76
pH	7.4 ± 0.1	7.6 ± 0.1	7.3 ± 0.1	7.5 ± 0.1
Conductivity (µS.cm^{-1})	799 ± 34^{a}	961 ± 12 ^{b}	965 ± 30.6^{b}	893 ± 43 ^{ab}
Dissolved oxygen (mg L^{-1})	7.8 ± 0.3^{a}	7.6 ± 0.9^{ac}	4.7 ± 0.2^{b}	7.0 ± 0.4^{c}
Alkalinity (mg $CaCO_3$ L^{-1})	237.6 ± 11.3^{ac}	251.6 ± 4.0^{ab}	225.6 ± 12.2^{c}	303.1 ± 17.3^{b}
Total phosphorus (µg L^{-1})	368.7 ± 46.1^{a}	1197.2 ± 50.4^{b}	956.3 ± 32.8^{c}	754.7 ± 31.2^{d}
Nitrate (µg L^{-1})	1848.7 ± 216.7^{ac}	1156.6 ± 141.6^{b}	1616.0 ± 67.5^{a}	1515.8 ± 79.3^{c}

Statistical differences between sampling sites, are shown as different letters (*H*, Kruskal-Wallis) ($p < 0.05$). Values are shown as mean ± SE

Table 3 Mean and standard error of physicochemical parameters (n = 20) for the different sampling times

Physicochemical parameter (n = 20)	Autumn	Winter	Spring	Summer
Total solid (mg L^{-1})	19.1 ± 2.0^{a}	40.5 ± 3.0^{b}	56.1 ± 9.2^{b}	29.1 ± 2.2^{ab}
Organic matter (mg L^{-1})	5.0 ± 0.6^{a}	9.3 ± 0.8^{b}	13.4 ± 1.8^{b}	8.4 ± 0.6^{b}
pH	7.8 ± 0.1^{a}	7.5 ± 0.03^{a}	7.1 ± 0.04^{b}	7.2 ± 0.03^{b}
Conductivity (µS.cm^{-1})	966.2 ± 26^{a}	950.7 ± 45.5^{a}	918.2 ± 13.4^{a}	783.9 ± 32.1^{b}
Dissolved oxygen (mg L^{-1})	9.9 ± 0.7^{a}	6.2 ± 0.4^{b}	6.2 ± 0.2^{b}	4.8±2.0^{b}
Alkalinity (mg $CaCO_3$ L^{-1})	282.4 ± 19.6^{a}	270.0 ± 7.4^{a}	254.4 ± 11.0^{ab}	211.2 ± 7.8^{b}
Total phosphorous (mg L^{-1})	897.8 ± 83.5	836.9 ± 77.1	780.1 ± 92.6	762.0 ± 63.5
Nitrate (mg L^{-1})	1483.3 ± 108.9^{ab}	2127.4 ± 175.1^{a}	1183.3 ± 109.1^{b}	1252.1 ± 90.9^{b}

Statistical differences between sampling sites, are shown as different letters (*H*, Kruskal-Wallis) ($p < 0.05$). Values are shown as mean ± SE

Table 4 Number of individuals of each category captured in each site during the four seasons

		Number of individuals			
Season	Sex	Site A	Site B	Site C	Site D
Autumn	Normal female	99	29	21	5
Autumn	Masculinized	1	181	0	0
Autumn	Male	94	179	44	2
Autumn	Immature	21	71	17	0
Winter	Normal female	47	15	7	1
Winter	Masculinized	9	88	0	0
Winter	Male	27	39	13	2
Winter	Immature	9	11	1	0
Spring	Normal female	48	0	23	5
Spring	Masculinized	0	5	0	0
Spring	Male	18	4	6	0
Spring	Immature	2	0	0	0
Summer	Normal female	11	3	30	4
Summer	Masculinized	7	11	10	0
Summer	Male	8	6	13	0
Summer	Immature	22	1	23	2
	Total	423	643	208	21

effluents. Consequently, the water quality in the basin is poor (MVOTMA-JICA 2011), particularly the reach downstream of the urban area Las Piedras-Progreso. Although fish assemblages from each site were not surveyed in our study, it is important to note that *C. decemmaculatus* was the dominant species, particularly in the polluted site. This species has been recognized as an aquatic pollution tolerant species, with high abundance in polluted areas (Teixeira-de Mello et al. 2012; Chalar et al. 2013, Benejam et al. 2015). Despite the tolerance showed by *C. decemmaculatus*, at field or under laboratory conditions, the process of masculinization due to certain types of contaminants (not explored in this work), could affect population dynamics. The mortality of masculinized females and their progeny observed in the laboratory suggests a potential impact on the population structure in the field. In addition, the recorded difference in the total progeny number of normal and masculinized females is potentially important for population sustainability. However, it is also important to note that normal and masculinized females were collected from different sites, and site factors independent of those directly related to masculinization per se could have affected the survival of females and their progeny. Thus, our results highlight the need for further research to identify and

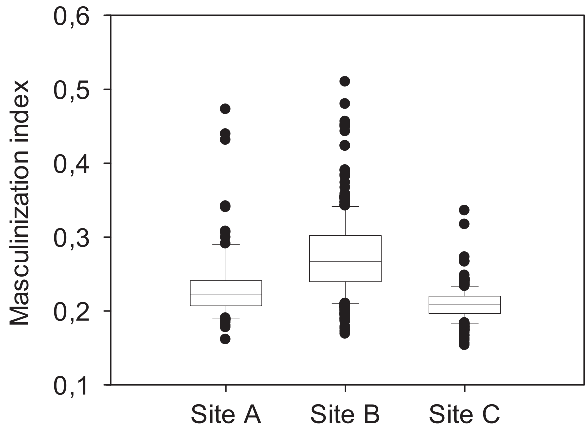

Fig. 3 Box plot of masculinization index (MI: anal fin length standardized by standard length) of normal and masculinized females including all sampling period in the three sampling locations where masculinized females were detected. Line represent the median, bars represent maximum and minimum

Table 5 Parameters of the linear regression between the standard length and anal fin length of males, females and masculinized females

Sex	N	*b*	*a*	r2	Error *a*	Error *b*	*p*-value
Male	427	0.83	−0.34	0.66	0.03	0.01	<0.001
Female	325	0.99	−0.68	0.79	0.03	0.01	<0.001
Masculinised	291	0.96	−0.55	0.45	0.06	0.02	<0.001

N number of individuals, r^2 regression coefficient, *b* slope of the curve, *a* the intercept, *p* statistical significance

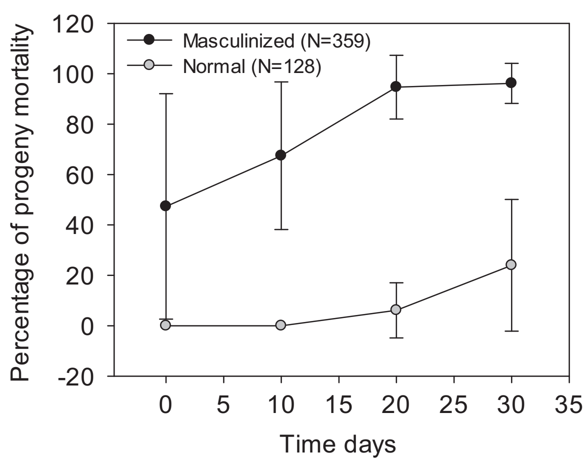

Fig. 4 Cumulative percentage of progeny mortality for masculinized (grey dots) and normal females (black dots) grouped in ten-days intervals (day 0-10, 11-20, 21-30). It is indicated the total number of progeny obtained during the experimental assay (n)

characterize the effects and mechanisms of environmental EDCs on fish populations.

In this study, we reported for the first time male secondary sexual characters (elongation of the anal fin rays) of field-collected *C. decemmaculatus* females. These abnormalities were present with different frequencies and degrees in the studied sites. The higher frequency of masculinized female and the larger anal fin length and masculinization index (MI) occurred at the Site B located downstream of Las Piedras-Progreso urban area. The stream at Site C, located 8.5 km downstream, showed a reduction of the proportion of masculinized female, anal fin length and MI, whereas Site A showed the lowest MI values. The gradient observed in the biological responses, suggests the potential of *C. decemmaculatus* and particularly, the masculinization phenomenon, as indicators of urban-industrial activities. The expression of the masculinization characteristics could be attributed to a different degree of exposure to masculinization agents. High level exposure of the individuals inhabiting the site located downstream of the source of pollution (site B), lower exposures, caused by dilution, in the case of the site located far from the source (C), and no effect level exposures when the sampling site was located upstream of the contamination sources (A) or located in other unpolluted stream (D). Still, from our results is not possible to attribute causality to one particular pollutant, since the discharge at the urban-industrial zone constitutes a mixture of pollutants from agriculture and releases of domestic and industrial effluents. The commonality between these sites is the presence of tanneries immediately upstream of site B. The tanning process involves the use of several chemicals, as surfactants and emulsifiers (Guillén et al. 2011). In particular,nonylfenol ethoxilates (NPEs) exhibit excellent properties as surfactants, and under anoxic conditions are degraded to several isomers of nonylphenol (NP) (Lee Ferguson and Brownawell 2003). Nonylphenol isomers have been detected in surface waters exposed to leather tanneries effluents (Azevedo et al. 2001), and their use has been restricted in Europe due its endocrine disrupting properties (EU Directive 2003/53/EC). Schwaiger et al. (2002) detected an androgenic effect on the second generation of rainbow trout, causing masculinization of female progeny exposed to NP. Thus we reiterate that more investigations are needed to elucidate the cause of the masculinization in field-collected *C. decemmaculatus* as well as the mechanisms of action of the endocrine disruption.

The male sexual behavior showed by adult masculinized females during the laboratory assay indicates that individuals are not only affected at the morphological level but also at the reproductive behavioral level. Similar results were observed in other poeciliids such as *G. affinis* exposed to pulp mill effluents (Bortone et al. 1989; Rodriguez-Sierra and Rosa-Molinar 1990; Ellis et al. 2003). Ellis et al. (2003), proposed that behavioral alterations could affect the population. However, in our study we reported pregnant masculinized females that produced offspring, in accordance to Howell et al. (1980). Our results suggest that the

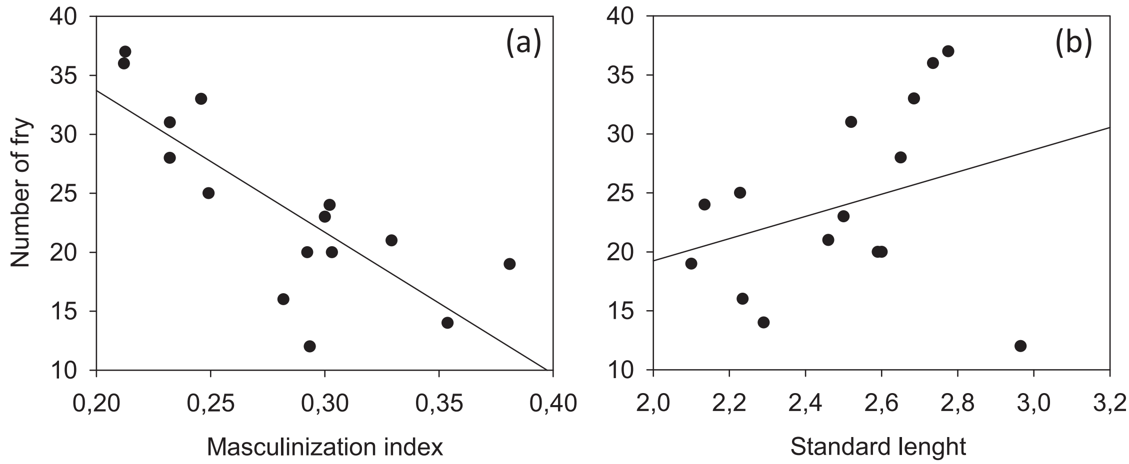

Fig. 5 Relationship between number of progeny and masculinization index (y = −120.1 × + 57.7; $R^2 = 0.61$; $p < 0.001$) **a** and standard length of masculinized females (y = 9.4 × + 0.4; $R^2 = 0.10$; $p > 0.05$) **b** obtained during the laboratory assay

behavior per se does not necessarily represent a direct impact on reproduction with consequences at the population level. However, the higher mortality of masculinized females and their progeny observed in the laboratory may have consequences at the population level. The mechanisms that explain the new-born mortality is not clear, but *gonopodium* development during the sexual differentiation in normal males produces modifications of the internal bone structure of the anal fin (Hou et al. 2011). We suggest that when these changes occur in masculinized females, the birth canal might be constricted and consequently the passage of the new-born might be hindered. In agreement with this statement, during the experiments, masculinized females were observed with fry "stuck" during birth, but this was not observed for normal females. This obstruction during birth may cause the death of both the masculinized female and the new-borns. However, masculinized female also had lower body size compared to normal females. The exposure to pollution may produce a reduction in size due to reallocation of energy to detoxification instead of growth (Adams et al. 1992). However, this effect should be observed for both males and normal females, and we did not find differences among sites for male size or females (including normal and masculinized females). Consequently, our results are consistent with other EDCs effects on masculinized females. Specifically, Hou et al. (2011) reported reduced size of masculinized females of *Gambusia affinis* exposed to paper mill effluent. These authors suggested that the reduced body size might be interpreted as consequence of the masculinization process, since poeciliids have a markedly sexual size dimorphism, with adult males smaller than adult females.

The use of sentinel fish species is a valuable strategy for monitoring and assessment of aquatic systems affected by different sources of pollution. Specifically, poeciliids such as *G. affinis* and *Poecilia reticulata* have been proposed as sentinel species to endocrine disruptor exposure (Parks et al. 2001; Orlando et al. 2002; Larsson et al. 2002). *Cnesterodon decemmaculatus* meets the assumptions of a good bioindicator to be used as sentinel species for biomonitoring environmental contamination levels caused by anthropogenic pollution because: (i) it is a very common and abundant species, easy to capture at field; (ii) it has a widespread distribution in pampean basins (Menni et al. 1996; Lucinda 2005); (iii) it is tolerant to altered aquatic resources contaminant, inhabiting lakes and streams with contrasting pollution levels (Teixeira-de Mello et al. 2009; Teixeira-de Mello et al. 2012; Chalar et al. 2013; Hued et al. 2013; Benejam et al. 2015); (iv) it is a relatively sedentary species, with no large-distance migration; (v) it has a size large enough to allow the study of different tissues; (vi) it is very easy to handle and maintain in laboratory conditions. In addition, the masculinization of *C. decemmaculatus* females is easy to detect and to quantify. For these reasons, we propose *C. decemmaculatus* as a bio-monitor with potential use as a sentinel species of aquatic pollution with EDCs, particularly those with androgenic activity, in Uruguay and other Pampean regions.

Conclusions and perspectives

In this study we reported for the first-time masculinization of *C. decemmaculatus* females in a stream affected by a mixture of urban-industrial activity and agriculture in the neotropical region. The masculinization was associated with reduced reproductive success, both in number and survival of progeny; consequently it could potentially affect population viability. Due to their wide distribution, tolerance to aquatic pollution and the potential use as bio-indicator we proposed the *C. decemmaculatus* as a sentinel species of EDCs exposure. The use of a sentinel species allows the early detection of sources of contaminants and prevents ecosystem impacts and human health risk. However, it is important to continue the research to unravel the causes and mechanisms of the masculinization process.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethicl approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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