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Effects of 17α-ethinylestradiol on sex ratio, gonadal histology and perianal hyperpigmentation of *Cnesterodon decemmaculatus* (Pisces, Poeciliidae) during a full-lifecycle exposure

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ABSTRACT

The effects of 17α-ethinylestradiol (EE_2) on sex ratio, gonopodium morphology, and gonadal histology of *C. decemmaculatus* were assessed by a full-lifecycle exposure experiment. Newborn fish were waterborne exposed to 30, 100, and 300 ng EE_2/L for 90 d, using 50 fish per treatment. Additionally, in December of 2016, a field survey was conducted on a *C. decemmaculatus* population inhabiting the Girado Creek downstream of the Chascomus city wastewater effluent discharge. After 90 d of exposure, EE_2 was able to histologically skew the sex ratio toward females and inhibit the full gonopodium development since the lowest tested concentration (LOEC = 30 ng/L). At higher concentrations, EE_2 was toxic, inducing mortality in a concentration-dependent fashion (90 d-LC_{50} = 109.9 ng/L) and altering the gonadal histoarchitecture, causing neither testes nor ovaries discernible histologically (LOEC = 100 ng/L). In addition, a novel response, perianal hyperpigmentation, was discovered been induced by the EE_2 exposure in a concentration-dependent fashion (90 d-EC_{50} = 39.3 ng/L). A higher proportion of females and perianal hyperpigmentation were observed in wild fish collected from the Girado Creek. The major reached conclusions are: i) EE_2 induce different effects on the sexual traits of *C. decemmaculatus* when exposed from early-life or adult stages. ii) The most sensitive effects observed in the laboratory occur in a creek receiving wastewater effluent. iii) The perianal hyperpigmentation comes-up as a promising biomarker of exposure to estrogenic compounds.

1. Introduction

The reproduction of vertebrates is controlled by the hypothalamic-pituitary-gonadal axis through a cascade of hormones whose concentrations are finely regulated by feedback systems and different control mechanisms (Rosenfeld et al., 2017). Some chemicals can alter the concentration of endogenous hormones, mimics or antagonizes their action, binding or interfering with the binding sites of hormones to their receptors and so are known as Endocrine Disrupting Chemicals (EDCs) (Kime, 2001). There is well-founded evidence on alterations induced at the level of reproduction in fish environmentally exposed to EDCs (Tyler et al., 1998; Kidd et al., 2007; Ankley et al., 2009; Söffker and Tyler, 2012). It is also known that early-life stages are more sensitive than others when exposed to EDCs since they are capable to affect sex determination and/or differentiation processes (Brion et al., 2004; González et al., 2015). For instance, the exposure of genotypic males of *Oryzias latipes* to 17β-estradiol (E_2) early in development can induce sex reversal with the formation of functional ovaries (Kobayashi and Iwamatsu, 2005). Also, the exposure of *Odontesthes bonariensis* larvae to 17α-ethinylestradiol (EE_2) during the sex differentiation period had the ability to feminize the gonad (Pérez et al., 2012). Moreover, several studies have found that EE_2 was able to change the sex ratio in fish, increasing the proportion of females (Andersen et al., 2003; Hill and Janz, 2003; Örn et al., 2006; Xu et al., 2008) and it was included in a

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recent review on the topic (Dang and Kienzler, 2019).

Particularly, secondary sexual characteristics of Poeciliids can be affected by EDCs during early-life stages. Several authors have reported effects linked to the reduction of gonopodium size in males exposed to estrogenic compounds (Drèze et al., 2000; Doyle and Lim, 2002; Angus et al., 2005; Rawson et al., 2006) and antiandrogens (Bayley et al., 2002). Masculinization of the anal fin in females exposed to androgens (Sone et al., 2005; Brockmeier et al., 2013) and progestogens (Frankel et al., 2016; Hou et al., 2017) were also reported. Effects of EDCs on gonads of Poeciliid fish were less studied, and only one study has reported the formation of testis-ova in juvenile females of *Gambusia affinis* exposed to 17β-trenbolone, an anabolic-androgenic steroid (Sone et al., 2005).

In a previous study, it was demonstrated the occurrence of testis-ova in adult males of the Neotropical Poeciliid, *Cnesterodon decemmaculatus*, exposed to EE_2 (Young et al., 2017). These males also presented liver alterations, but no phenotypical effects were seen at the level of the gonopodium (Young et al., 2017). Such results contrasted with observations done on other Poeciliids such as *Gambusia affinis* (Angus et al., 2005) and *Gambusia holbrooki* (Doyle and Lim, 2002; Rawson et al., 2006). In this context, the objective of the present study was to assess the effects of 17α-ethinylestradiol on sex ratio, gonadal sex, phenotypic sex, and phenotypic traits in *C. decemmaculatus* from newly born stages during a full life-cycle exposure.

2. Materials and methods

2.1. Fish

Newborn *C. decemmaculatus* (Jenyns, 1842) were obtained from the broodstock of the Laboratory of Ecotoxicology belonging to the *Instituto Nacional de Tecnología Agropecuaria* (INTA), Argentina.

2.2. Chemicals

17α-ethinylestradiol (Sygma-Aldrich, USA, purity 99%) was diluted in dimethylsulfoxide (Biopack, Argentina, purity 99–100%). ^{13}C-labeled EE_2 isotope (Cambridge Isotope Laboratories, Inc., Canada, purity 99%). The purities of these compounds were certified by their manufacturers. Used solvents for chemical analysis (i.e. methanol and acetonitrile) were HPLC grade from JT Baker.

2.3. Experimental design

A single-factor fixed-effect completely randomized model was established as the experimental design. Four experimental levels were set, exposing newborn *C. decemmaculatus* to 0, 30, 100, and 300 ng EE_2/L. Exposure duration was 90 d, period the species normally reaches sexual maturity. EE_2 concentrations were set according to those previously found in sewage effluents and receiving waters of the Pampas Region (Valdés et al., 2015; González et al., 2020). Stock solutions of EE_2 were prepared in DMSO and stored at −20 °C. In all treatments (including the control) the DMSO concentration was 0.025%. Hurlingham (Buenos Aires, Argentina) city's dechlorinated tap water (pH = 8.4 ± 0.2; electrical conductivity = 789 ± 15 μS/cm) was used for the experiment. The experiment was conducted in accordance with the principles and procedures approved by the Institutional Committee for the Care and Use of Experimental Animals of INTA-CNIA (46/2014). A total of 200 fish (average total length = 7.54 ± 0.64 mm) were randomly allocated to each treatment. To better simulate wild conditions (i.e., social behavior), the experimental populations (50 fish/treatment) and aquaria (40 L) were larger than the used in standardized tests for the species. Also, to avoid the stress associated to test media renewal, a given volume of the EE_2 work solution was added every 48 h to compensate for the steroid dissipated mass during that period. The 48 h dissipation rates were estimated by preliminary tests and adjusted every time EE_2 was analyzed in the water samples during the experiment. Exposure was conducted in a climatized room at 24 ± 2 °C and a photoperiod of 16 L:8D. Fish were daily fed *ad libitum* with commercial fish food (Shulet®, Argentina). Gentle aeration was provided to each aquarium throughout the experiment. Liquid wastes were collected and treated by a hazardous-waste specialized company.

2.4. Measurements of EE_2 concentrations in the test water

At the start of the experiment and every week, water samples (10 mL) were collected from the tanks of each treatment (including controls), immediately filtered through 0.45 nylon filters, and stored at −20 °C until processing. Samples were extracted using OASIS-HLB (Waters) SPE cartridges, eluted with methanol, dried under a gentle flow of N_2, and resuspended in 1 mL of mobile phase immediately before the injection. EE_2 concentrations were quantified by HPLC-MS using a Thermo Fisher Scientific Surveyor Plus HPLC equipped with an autosampler and coupled to an LTQ XL Ion Trap, according to the method described by Young et al. (2017). Recovery percentages were determined using an internal standard, spiking the water samples with 10 μg/L of ^{13}C-labeled EE_2 isotope. A recovery percentage of 75% was obtained with detection and quantification limits of 5 and 17 ng/L, respectively.

2.5. Tissue sampling and analysis

2.5.1. Phenotypical fish sexing

Fish were anesthetized by immersion in 80 mg/L of benzocaine solution, observed at a stereomicroscope (Leica GZ6) with a transilluminator device. Fish with a differentiated gonopodium were classified as male phenotype (MP), whereas fish without gonopodium were classified as female phenotype (FP).

2.5.2. Morphology

Fish wet weight (W) and standard body length (SL) were recorded and used to calculate Fulton's body condition factor (*K*) [(W (mg)/SL^3 (mm)) × 100]. Head length (HL) was measured and used to determine the cephalic index (CI) [HL (mm)/SL (mm) × 100]. The secondary sexual characteristics measured were the length of the fourth and the sixth rays of the gonopodium (R4 and R6, respectively). The gonopodium length (R4) was defined as the length from the anterior base of the anal fin to the gonopodial tip (Doyle and Lim, 2002). The elongation index (EI) was calculated as the R4/R6 ratio (Angus et al., 2001). The gonopodial index (GI) was calculated as the R4/SL ratio (Game et al., 2006). The morphological parameters SL, R4, and R6 were measured using an electronic digital caliper. Previously to euthanasia by decapitation, fish were photographed with a digital camera in a ventral position in order to observe the pigmentation in the perianal area. Fish were classified as hyperpigmented when the pigmented area was higher than 0.6 mm^2, 3-fold the maximum area in fish with normal pigmentation. The pigmented area was measured using an image analysis software (ImageJ 1.52, USA).

The classification used to describe the gonopodium development was based on the typical morphological characteristics of this species (Fig. 1): GP) "gonopodial primordium" (i.e., incipient R4 elongation), EG) "elongated gonopodium" (i.e., elongated R4 but without serraes, hooks, and cirrus), and FMG) "fully mature gonopodium" (i.e., elongated R4 and the presence of serraes, hooks, and cirrus).

2.5.3. Gonadal histology

Tissues were fixed in Bouin's fluid at 4 °C for 24 h, dehydrated, and embedded in paraffin for histological analysis. The trunk region containing the gonads was fully sectioned at transversal 6 μm-thick slices, that were serially mounted and stained with hematoxylin-eosin. The histological slides were observed and photographed with a light microscope (Nikon Eclipse E600) equipped with a digital camera (Nikon DS-Fi1). Pictures were processed using the software NIS-Elements F3.00.

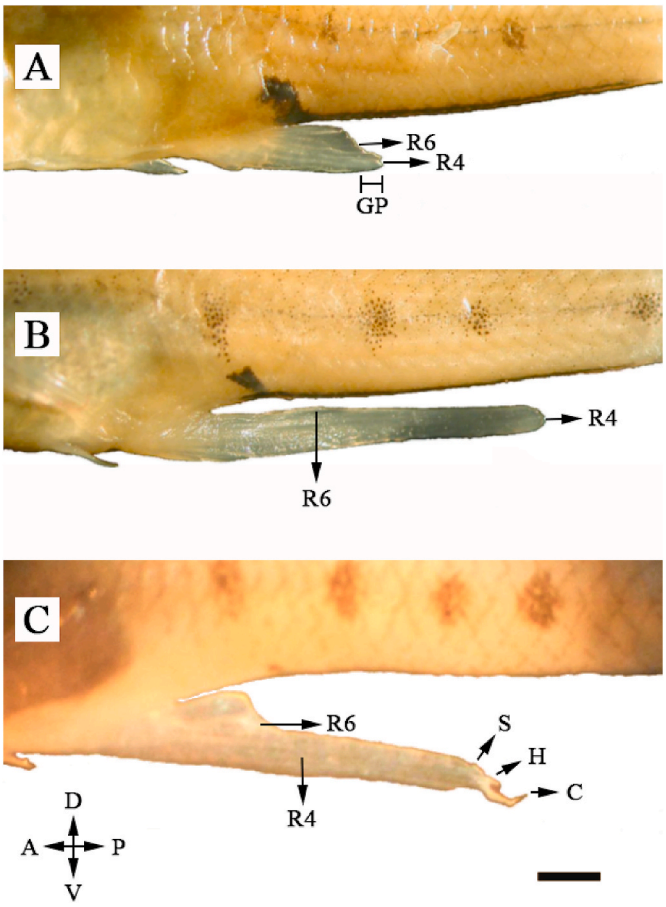

Fig. 1. Stages of gonopodium development of *C. decemmaculatus*. **A**: Gonopodial primordium (GP). **B**: Elongated gonopodium (EG) without serraes, hooks, and cirrus (SHC). **C**: Fully mature gonopodium (FMG) with the presence of SHC. R4: ray 4, R6: ray 6, A: anterior, P: posterior, V: ventral, D: dorsal, S: serraes, H: hooks, C: cirrus. Scale bar = 1 mm.

2.6. Sampling of fish in a freshwater stream

Complementary to the laboratory experiment, a field fish sampling was carried out at the "Girado Creek", close to the wastewater effluent outfall of Chascomús city, Argentina (35°37′57″ S, 58°00′26″ W). EE_2, along with other natural estrogens such as estrone (E_1), 17β-estradiol (E_2), and estriol (E_3), was detected in previous surveys performed in this place at concentrations up to 187 ng/L (Valdés et al., 2015). Sampling was carried out at the site located in the mixing zone between surface water and wastewater effluent. Body condition parameters, secondary sexual characteristics, gonadal histology, sex ratio, and perianal pigmentation were evaluated in the individuals of the *C. decemmaculatus* local population. Complementarily, the relative abundance of *C. decemmaculatus* in the small fish community associated with the aquatic vegetation was recorded.

2.7. Data analysis

The effect of the EE_2 exposure on body condition and secondary sexual characteristics was assessed by analysis of variance (ANOVA). If the *F* value of the ANOVA was significant ($p < 0.05$), Bonferroni's multiple comparisons test was used. The Chi-square test was used to compare proportions of male and female phenotypes, as well as the normal and altered gonadal histology percentages. Toxicity endpoints for mortality (LC_{50}) and hyperpigmentation (EC_{50}) were estimated from the concentration-response curve using a four-parameter non-linear model. Data were analyzed using Prism 5.1 for Windows (GraphPad Software, CA, USA).

3. Results

3.1. Nominal and actual concentrations

The average (±SD) measured concentrations in the tanks of each treatment during the experiment were 32.3 ± 0.2, 99.1 ± 3.2, and 296.2 ± 10.7 ng/L. In all cases, deviation percentages from the nominal values (30, 100, and 300 ng/L) were lower than 10% (7.7, 0.9, and 1.3%, respectively). In addition, EE_2 was not detected at any of the samples from the control tanks during the whole experiment.

3.2. Mortality, secondary sexual characteristics, and condition parameters

A concentration-dependent lethal effect was observed during the exposure period (Fig. 2). The 90 d-LC_{50} was estimated in 109.9 ng/L of EE_2, with a 95% confidence interval of 61.6 and 196.0 for the lower and upper limits, respectively. Mortality recorded in the control group was 30%. This is considered to be an acceptable value for a full-lifecycle experiment with this species.

Statistically significant differences ($p < 0.001$) were found for the assessed morphological features R4, EI, and GI between FP and MP of the control group (Fig. 3). On the other hand, no statistical differences ($p > 0.05$) were observed for these features in fish exposed to any of the EE_2 tested concentrations. All other assessed parameters and indexes (i.e., SL, HL, W, R6, CI, and *K*) were not significantly different ($p > 0.05$) between FP and MP, either in the controls or in the tested treatments (Supplementary Tables 1 and 2).

3.3. Phenotypic sex ratio and gonopodium development

Phenotypic sex proportions were significantly affected by EE_2 exposure ($p < 0.001$; Fig. 4A), inducing a reduction of MP (<22.2%) when compared to the control group (42.9%) from the lowest tested concentration (30 ng/L). In addition, gonopodium development was delayed in the few remaining MPs and the delay was increased with the EE_2 concentration (Fig. 4B). FMG males were only observed in the control group, EG males at the lowest EE_2 concentration, and only GP males were recorded from 100 ng/L.

3.4. Gonadal histology

The sex ratio determined by gonadal histology in the control group was 58.3% males and 41.7% females. This proportion was modified in fish exposed to 30 ng EE_2/L, showing an increase of the females (70%) respect to the males (30%) (Fig. 5). However, the main observed effects of the EE_2 were not only on the sex proportion, but also on the alteration

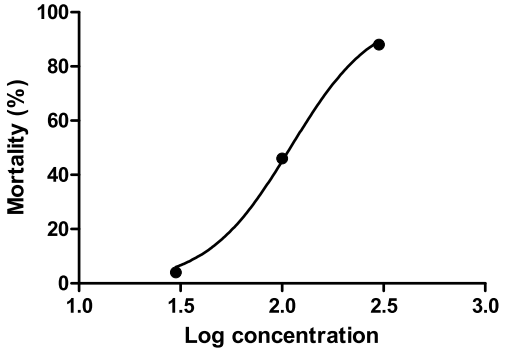

Fig. 2. Concentration-response relationship for the mortality obtained for *C. decemmaculatus* after 90-d exposure to EE_2 (ng/L).

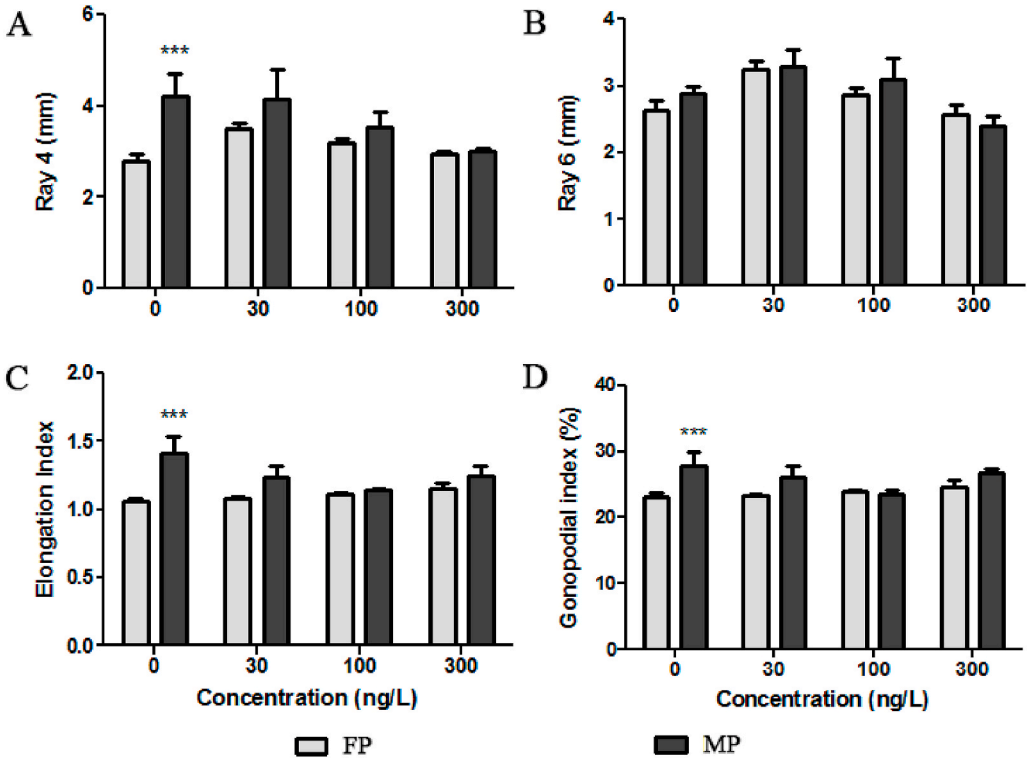

Fig. 3. Effect of EE_2 on secondary sexual characteristics and gonopodial indexes. Asterisks indicate statistically significant differences between phenotypic females (FP) and males (MP) ($p < 0.001$, N = 117).

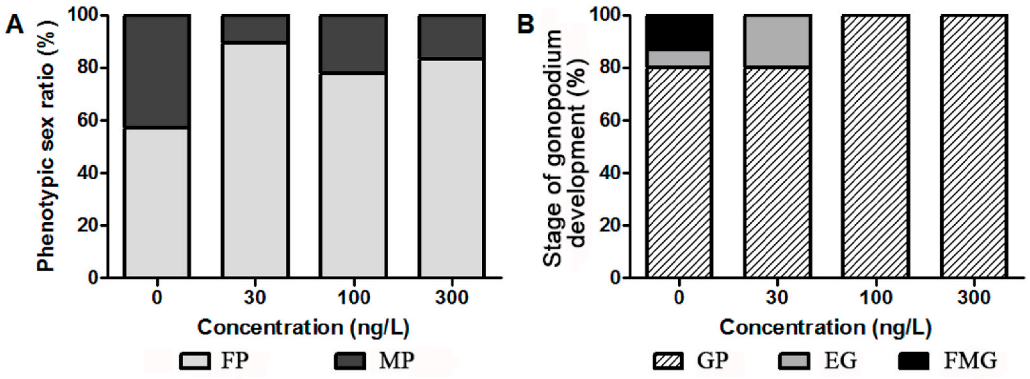

Fig. 4. Effect of EE_2 on the phenotypic sex ratio and gonopodial development. **A:** Phenotypic sex ratio. **B:** Gonopodium development stages in MP: gonopodial primordium (GP), elongated gonopodium (EG), and fully mature gonopodium (FMG). MP: male phenotype, FP: female phenotype.

of the normal gonadal histology affecting both male and female fish.

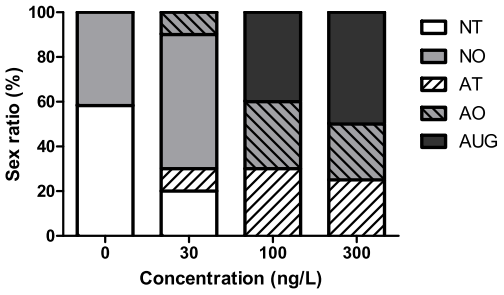

Fig. 5. Sex ratio based on gonadal histology of *C. decemmaculatus* after 90-d exposure to EE_2: normal testis (NT), normal ovary (NO), altered testis (AT), altered ovary (AO), and altered and undeveloped gonad (AUG; neither testes or ovary discernible histologically).

Gonad histological alterations were clearly increased showing a concentration-dependent relationship. Only 20% of the testis and ovaries of fish exposed to 30 ng EE_2/L were affected, but all of the gonads were abnormal in fish exposed to 100 and 300 ng EE_2/L. Moreover, a proportion of 40 and 60% of the fish in 100 and 300 ng EE_2/L, respectively, were found presenting a sexually non-differentiable gonad, that was not possible to histologically identify as an ovary or a testis. Fully feminized gonads in MP at the GP stage were observed since 100 ng EE_2/L. The percentage of fish presenting altered gonads were significantly different from controls at the 100 and 300 ng EE_2/L treatments ($p < 0.001$). According to these results, the estimated No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) for histological alterations of the gonads were 30 and 100 ng/L, respectively.

A normal testis (Fig. 6A) was described as a unique structure formed by the two testicular lobules merged and joined to the mesentery, presenting efferent ducts, cysts of spermatogonia, spermatocytes, and/or

spermatids in different proportions and stages depending on development degree. A normal ovary (Fig. 6B) was also described as a unique structure formed by the two ovarian lobules merged and joined to the mesentery, dorsally showing an ovarian cavity surrounded by oogonia, primary oocytes, and/or cortical alveoli in different proportions and stages depending on the development degree. The alterations of the testicular morphology were related to interstitial-cell hyperplasia, the consequent reduction of the number of germinal cells cysts, and the formation of increasingly bigger peripherical internal cavities (Fig. 6C). The alterations in the ovaries were related to cell necrosis and increased oocyte maturation in a first stage of disruption (Fig. 6D). Hyperplasia of stromal-cell, ovarian cavity-size increase, and oocyte number reduction were found in a more advanced stage (Fig. 6E). The sexually non-differentiable gonad was characterized by somatic-cell hyperplasia (whether stromal or interstitial cells), the formation of ducts or cavities (efferent ducts or ovarian cavity), and the presence of only immature germ cells (Fig. 6F).

3.5. Perianal hyperpigmentation

Fish with normal perianal pigmentation were observed in the control group (Fig. 7A). While the occurrence of hyperpigmentation in the perianal area was only observed in fish exposed to EE_2 (Fig. 7B). The average pigmented area in fish with normal pigmentation was 0.18 ± 0.07 mm^2 (min = 0.09; max = 0.27), whereas in fish with hyperpigmentation was 1.35 ± 0.44 mm^2 (min = 0.63; max = 2.35). The pigmented area was significantly different in comparison to the control group in fish exposed to 100 and 300 EE_2 ng/L ($p < 0.001$, Supplementary Table 3). Therefore, the estimated NOEC and LOEC for the perianal hyperpigmentation were 30 and 100 ng EE_2/L, respectively. In addition, a concentration-dependent increase was recorded in the percentage of fish with perianal hyperpigmentation. It was present in 37.5, 85.2, and 100% of the fish in the concentrations of 30, 100, and 300 ng/L, respectively (Fig. 7C). The overall estimated 90 d-EC_{50} was 39.3 ng EE_2/L, with a 95% confidence interval of 23.5 and 65.6 ng/L for the lower and upper limits, respectively.

When the occurrence of the perianal hyperpigmentation was assessed separately for MP and FP no differences in the sensitivity between sex were observed. It was present in 60 and 35% of the MP and FP exposed to 30 ng/L, in 75 and 91% for MP and FP exposed to 100 ng/L, and in all individuals exposed to 300 ng/L.

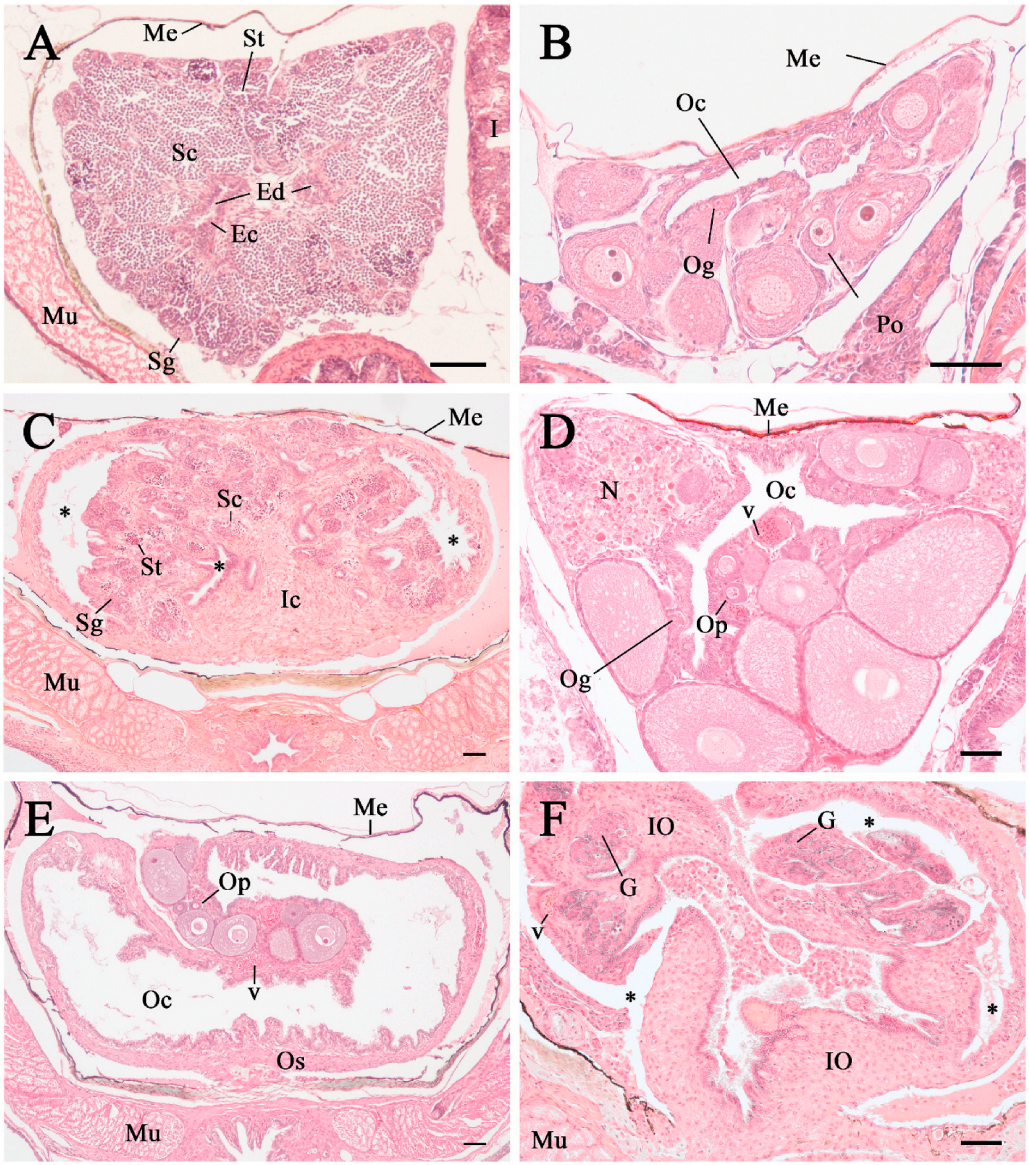

Fig. 6. Effects of EE_2 on *C. decemmaculatus* gonadal histology. A: Normal testicular histology (0 and 30 ng/L). B: Normal ovary histology (control and 30 ng EE_2/L). C: Altered testicular histology (30, 100, and 300 ng EE_2/L): interstitial-cell hyperplasia and ducts/cavities. D–E: Altered ovary histology (30, 100, and 300 ng EE_2/L): necrosis (D) and stromal-cell hyperplasia (E). F: Histological alterations in sexually non-differentiable gonads (100 and 300 ng EE_2/L): hyperplasia, ducts/cavities, and immature germ cells. Hematoxylin-eosin staining. Asterisks (*) indicate ducts or cavities in the gonad. Mu: muscle, I: intestine, Me: mesentery, v: vein, Ed: efferent duct, Ec: epithelial cells from the efferent duct, Sg: spermatogonia, Sc: spermatocytes, St: spermatids, Ic: interstitial cells, Oc: ovarian cavity, Og: oogonia, Po: primary oocyte, Os: ovarian stroma, G: immature germ cells, N: cell necrosis, IO: interstitial cells or ovarian stroma. Scale bar = 50 μm.

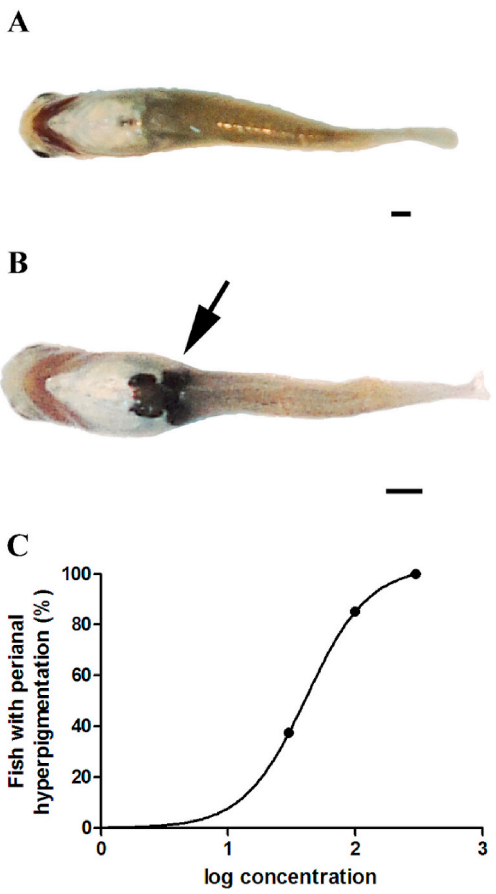

Fig. 7. Effect of EE_2 on perianal hyperpigmentation of *C. decemmaculatus*. **A**: normal pigmentation in fish from the control group. **B**: hyperpigmentation in the perianal area of fish exposed to EE_2. **C**: concentration-response curve of fish with perianal hyperpigmentation. Arrow indicates the location of the perianal hyperpigmentation. Scale bar = 1 mm.

3.6. Survey of a C. decemmaculatus wild population under wastewater effluent discharge

During the field sampling, *C. decemmaculatus* was the most abundant species of the aquatic vegetation-associated fish community of the Girado stream under the influence of the Chascomús city wastewater effluent (N = 69). In number, *C. decemmaculatus* was 86% of the fishing catch, followed by the following species *Jenynsia multidentata* (10%) and *Corydora paleatus* (4%). The phenotypic sex ratio of *C. decemmaculatus* in the area was skewed to the females (67%) over the males (33%), but no alterations of secondary sexual characteristics or gonadal histology were found (Supplementary Table 4). Among the male fish, 52.9% were categorized as FMG, 29.4% as EG, and17.7% as GP, according to the gonopodium development degree. In addition, the perianal hyperpigmentation was observed in 50% of the sampled females, but none of the caught males showed that feature.

4. Discussion

Several Nearctic and Neotropical Poeciliids have been used to evaluate effects induced by EDCs. As examples, *Gambusia affinis* (Angus et al., 2005), *Gambusia holbrooki* (Doyle and Lim, 2002), and *Poecilia reticulata* (Larsson et al., 2002) were used as model species because their sexually dimorphic anal fin has found to be sensitive to EDCs. Much less it is known about other South American Poeciliids not only on their use as sentinel species but also about the effects of endocrine disruptors on development and reproduction (Hued et al., 2013; Roggio et al., 2014; Young et al., 2017; Vidal et al., 2018; Zambrano et al., 2018). The biological characteristics of the Poeciliid *Cnesterodon decemmaculatus* (Cyprinodontiformes) have allowed to propose it as a sentinel species for ecotoxicological studies (Vidal et al., 2018; Zambrano et al., 2018). Furthermore, it is considered as a tolerant fish because it usually lives in polluted waters (Chalar et al., 2013; Hued et al., 2013; Vidal et al., 2018). In the present study, it was described for the first time in *C. decemmaculatus* the effect of EE_2, as a xenoestrogen model commonly found in wastewater effluents, on the survival and development of the gonopodium and other secondary sexual characteristics in parallel with the gonadal development during a full lifecycle exposure.

Regarding the effects of EE_2 on the *C. decemmaculatus* long-term survival, a significant reduction was induced by the xenoestrogen within the range of assayed concentrations, showing an LC_{50} around 100 ng/L. Although the precise mechanism of action associated with EE_2 long-term lethality on fish is still unknown, complex underlying processes would be expected considering the pleiotropic nature of the natural estrogen E_2. Long-term lethal effects induced by EE_2 have been also observed in *D. rerio* exposed to 100 and 2 ng/L for 60 d and 90 d, respectively (Hill and Janz, 2003; Xu et al., 2008). The observed spontaneous mortality rate in the control group was among the range that could be considered normal for a full lifecycle experiment with *C. decemmaculatus*. It was similar to that reported for *Danio rerio*, in the previously mentioned experiments, and for *G. affinis* in a 150-d test (Angus et al., 2005). The EE_2 lethal concentrations found for *C. decemmaculatus* were in the range of those commonly reported in wastewater effluents of the Pampas region but they were higher than the ones usually found in nearby receiving waters (Valdés et al., 2015). Therefore, shortening of the long-term life expectancy induced by EE_2 would be fairly improbable in *C. decemmaculatus* populations inhabiting these receiving waters, even at very close distance from the wastewater effluent discharge. In particular, considering that particulate and dissolved organic matter in surface waters tend to significantly reduce the bioavailability of this xenoestrogen (Yamamoto et al., 2003; Sun et al., 2006).

Additionally, to the long-term lethal effect, EE_2 was capable to alter, in a concentration-dependent manner, all the other assessed sexual features. It is currently known that sex steroids are not the initiators of sex differentiation, however, this process is dependent on the estrogens/androgens levels during a sensitive window early in life (Guiguen et al., 2010; Li et al., 2019). Moreover, EE_2 as an agonist of estrogen receptors is able to trigger molecular mechanisms that modify the gene expression profile in estrogen-sensitive tissues (Filby et al., 2007). In particular, it has been shown that estrogens are able to alter the normal testicular development, inhibiting the gene expression network involved in such process and inducing the expression of genes involved in ovarian development (Guiguen et al., 2010; Díaz and Piferrer, 2017).

In the present study, EE_2 was able to deeply alter the differentiation of the gonads of *C. decemmaculatus* when fish were exposed from early life stages. At the lowest tested concentration, the effects were associated with an increase of fish proportion developing an ovary. However, at higher concentrations, EE_2 caused the complete alteration of the gonad histoarchitecture and proliferation of the germ cells, to the point it was not possible to histologically differentiate if they were testes or ovaries. Unexpectedly, although testis-ova was induced by EE_2 on *C. decemmaculatus* exposed in the adult stage after gonad differentiation (Young et al., 2017), in the present study intersex gonads were not observed at any of the tested concentrations. Together these results would be showing different sensitive windows for the effects induced by EE_2 on *C. decemmaculatus* depending on the moment of the lifecycle that the fish are exposed.

The gonad alterations were characterized by a proliferation of somatic cells (interstitial and stromal cells hyperplasia), as well as cell necrosis induction and absence of advanced stages of the spermatogenesis or oogenesis. In particular, the mitogenic effects of estrogens have been well documented in mammals. For example, it has been

described that estradiol plays an important role in promoting ovarian stromal proliferation (Britt and Findlay, 2003; Britt et al., 2004; Pask, 2012). Moreover, it has been found that this estrogen was able to induce tumors in the ovary and the mammary gland (Hall and Korach, 2003). More specifically in fish, it is known that estradiol participates in ovarian morphogenesis (Nakamura, 2010; Kobayashi et al., 2013). This estrogen is particularly involved in the proliferation of somatic cells that allow closing the ovarian cavity (Ito et al., 2005), a fact that is also coincident with an increase in the expression of *cyp19a1a*, gonadal aromatase (Fernandino et al., 2008). Therefore, the sexually undifferentiated gonads driven by the somatic cell proliferation in response to EE_2 exposure was consistent with the previously mentioned studies. In addition, the induction of sexually undiscernible gonads has been also described for zebrafish exposed to 10 and 2 ng/L of this xenoestrogen for 60 and 90 dph, respectively (Hill and Janz, 2003; Xu et al., 2008).

In the Poeciliid fish *Gambusia holbrooki*, the release of androgens from gonads during the sexual differentiation period was found to play a central role in the development of the major male secondary sexual characteristic, the gonopodium (Ogino et al., 2004, 2018). Assuming the same mechanism for *C. decemmaculatus*, the feminization of the gonad induced by EE_2 should reduce the production of androgens and consequently impair the normal gonopodium development. Precisely a clear concentration-dependent inhibition of gonopodium development was observed in *C. decemmaculatus* exposed from early life stages to this xenoestrogen and males showing a fully mature gonopodium (FMG) were only observed in the control group. These results were coincident with those published by Angus et al. (2005), who demonstrated that EE_2 was able to inhibit gonopodium development in juvenile male *G. affinis*. However, it was contrasting to the response observed in a previous study conducted with adult male *C. decemmaculatus*, which showed no alteration of the gonopodium morphology when exposed to concentrations of EE_2 up to 176 ng/L (Young et al., 2017). These results together would support the findings reported by Ogino et al. (2004, 2018) and would be indicating that androgens are no longer necessaries for maintaining the gonopodium structure after it was completely developed. This was different from the response described for the gonads, which was altered by EE_2 exposure both during early life and adult stages. In this study some fish presenting a gonopodium primordium and showing ovaries inside were observed. Even though no information is available on the genetic sex determination of *C. decemmaculatus*, considering that androgens are needed for early gonopodium development, it would be right to suppose those fish initially had testis and therefore were *a priori* classified as phenotypic males. However, after long-term exposure to EE_2 from the early life stage, the gonad could be feminized, androgen production stopped and gonopodium development arrested. If so, to our knowledge, this would be the first report showing a synthetic estrogen has the ability to induce such effect on a Poeciliid fish.

In the present study, it was also found that EE_2 was able to increase the female to male ratio, both at phenotypic and histologic level, already from the lowest assayed concentration (30 ng/L). Similar effects were observed on other fish species exposed to EE_2 from early-life stages, such as medaka *O. latipes* (Örn et al., 2006) and zebrafish *D. rerio* (Hill and Janz, 2003; Xu et al., 2008). Moreover, complete feminization was reported by Andersen et al. (2003) in zebrafish exposed to 15 ng/L of EE_2 for 60 d from hatching. Interestingly in most of those studies the feminizing effect was related to an impairment of the reproduction functions on gamete development, fecundity, or egg and offspring viability.

An unexpected response to EE_2 exposure, the hyperpigmentation of the perianal area, was found both in female and male fish during the laboratory experiments. A small pigmented mark close to the anal fin insertion is usual for this species. However, the hyperpigmentation was only observed in fish exposed to EE_2. Moreover, the concentration-response relationship found for this feature was showing this effect was driven by the estrogenic stimulation. Even though it has not been previously described in fish, the melanin synthesis induced by estrogens has been observed in human melanocytes, being regulated through a nonclassical membrane-bound receptor: a G protein-coupled estrogen receptor (GPER) (Filardo et al., 2002; Natale et al., 2016). This finding, encourage future studies focused on the elucidation of the regulatory mechanisms of the EE_2, and probably E_2, on the pigmentation of the cells surrounding the perianal area of *C. decemmaculatus*. A better understanding of this response to estrogens could help to propose it as an easy and low-cost biomarker of exposure for this species, and probably other Poeciliid fish.

Concentrations of EE_2 ranging from 64.2 to 187 ng/L and 43.0 to 47.6 ng/L have been previously found, respectively, in wastewater effluents and receiving waters of the Pampas region (Valdés et al., 2015; González et al., 2020). Moreover, in those studies, EE_2 has not been found alone but accompanied by other natural estrogens (i.e., E_1, E_2, and E_3). Therefore, it was expected that some of the described effects in the laboratory could occur in *C. decemmaculatus* wild populations inhabiting surface waters of the Pampas receiving wastewater effluents. That hypothesis was confirmed by the field survey conducted in a sector of the Girado stream near the Chascomús city wastewater effluent outfall, finding a skewed ratio of females over males and perianal hyperpigmentation in half of the sampled female fish. However, no altered gonads or testis-ova were found. Neither a male phenotype (i.e., gonopodium primordium stage) with a female gonad (i.e., ovary) was observed at any sampled fish. Therefore, only the more sensitive responses were observed in the field. Considering that only those responses induced in the laboratory by 30 ng EE_2/L were found and that the reported concentrations in the surveyed area of the Girado stream were generally greater than that value (Valdés et al., 2015; González et al., 2020), it would seem that the bioavailable concentrations of estrogens in the field are significantly less than the ones measured. Such reduction of estrogen's bioavailability in surface water due to the adsorption on the dissolved and particulate organic matter has been well documented (Yamamoto et al., 2003; Sun et al., 2006). However, despite the abated impact due to the reduction of the bioavailability, the field survey showed for the first time that those sensitive and specifically estrogen-induced biological endpoints were affected in a *C. decemmaculatus* wild population inhabiting downstream a wastewater discharge.

5. Conclusions

According to the obtained experimental results, it is possible to conclude that induced effects by EE_2 on newborn *C. decemmaculatus* exposed during the full lifecycle are different from those induced in adult fish. No testis-ova was induced but instead, the whole gonad was feminized and the gonopodium development inhibited, causing an increase of the female to male ratio at the lowest assayed concentration (LOEC = 30 ng/L). At higher concentrations, EE_2 was toxic, inducing lethality (90 d-LC_{50} = 109.9 ng/L) and severe alterations of the gonads, characterized by somatic cell proliferation and necrosis, together with the inhibition of the germ cell development provoking neither testes or ovaries discernible histologically (LOEC = 100 ng/L). In addition, EE_2 induced, in a concentration-response manner, perianal hyperpigmentation that, to our knowledge was not previously described neither in *C. decemmaculatus* nor in other Poeciliid fish (90 d-EC_{50} = 39.3 ng/L). A skewed ratio of females over males and perianal hyperpigmentation were observed in a *C. decemmaculatus* wild population inhabiting a wastewater-impacted stream where EE_2 concentrations up to 47.6 ng/L were reported. Although only the most sensitive responses were observed in the field, the study found for the first time the laboratory-observed estrogen-specific effects on *C. decemmaculatus* in the wild. In addition, the perianal hyperpigmentation comes up as a potential novel biomarker of exposure to estrogenic compounds.

Credit author statement

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Investigation, Formal analysis, Writing- Original draft preparation, Writing- Reviewing and Editing. **Diego Sebastián Cristos**: Methodology, Investigation. **Diana Cristina Crespo**: Resources, Funding acquisition. **Gustavo Manuel Somoza**: Conceptualization, Writing- Reviewing and Editing, Visualization, Supervision, Funding acquisition. **Pedro Carriquiriborde**: Conceptualization, Writing- Reviewing and Editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111176.

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