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Environmental exposure to oestrogenic endocrine disruptors mixtures reflecting on gonadal sex steroids and gametogenesis of the neotropical fish *Astyanax rivularis*

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ABSTRACT

Discharge of municipal wastewater promotes the entry of diverse oestrogenic compounds into the water bodies. This complex mixture of substances interferes in the steroidogenic pathway, being able to promote severe reproductive impairment in freshwater fish populations. The purpose of the present study was to evaluate the effects of oestrogenic endocrine disruptors (EDCs) mixture on gonadal sex steroids (testosterone, T; 11-ketotestosterone, 11-KT; 17β-oestradiol, E2; 17-hydroxyprogesterone, 17-OHP) in the peak of the reproductive season of *Astyanax rivularis*, correlating the results obtained with the proportion of germ cells and gonadal histopathology. Three sampling sites were chosen to conduct the study, one reference site (S1), without contamination by municipal wastewater and two sites (S2 and S3) receiving discharge of municipal wastewater. Males of *A. rivularis* presented higher concentrations of E2, lower androgens (T and 11-KT) in gonads when compared to males from site S1. Concentrations of 17-OHP did not present significant difference among sites. In sites S2 and S3, the proportion of early spermatocytes, spermatids and Leydig cells increased while spermatozoa decreased compared to fish from S1. The following gonadal histopathologies were detected in the male fishes: intersex gonads (28% in S3) and testicular degeneration with germinal epithelium exhibiting agglutinated germ cells masses and empty cysts (57% in S2 and 71% in S3). In females, concentrations of T, E2 and 17-OHP did not present significant difference among the sites, however higher 11-KT concentrations were detected in females from sites S2 and S3. A lower proportion of perinucleolar follicles and a higher incidence of vitellogenic follicles, besides, aged oocytes and the presence of eosinophilic proteinaceous fluid in the interstitial compartment were also found in females from impacted sites. These results indicate that the urbanization and consequent release of municipal wastewater containing oestrogenic compounds in the headwater creeks are altering the levels of sex hormones and gametogenesis of *A. rivularis*. Further studies should be performed to determine whether oestrogenic endocrine disrupters are disrupting the reproduction of *A. rivularis*.

1. Introduction

Worldwide, natural and synthetic hormones, pharmaceutical and personal care products (PPCPs), plasticizers are found in inland waters due to contamination by domestic and municipal sewage, without an adequate treatment (Adeel et al., 2016; Bahamonde et al., 2015; Barber et al., 2012). Exposure to natural oestrogens and xenoestrogens causes increase in hepatic levels of vitellogenin (Vtg) and zona radiata proteins (Zrp) in male fishes, and these changes are related to alterations in the concentration of 17 β-oestradiol (Bahamonde et al., 2014; Ibor et al., 2016; Prado et al., 2014). In fish, sex steroids play important roles in sexual differentiation and gonadal development, and are involved in the embryonic development, metabolism, immune responses and reproduction (Lubzens et al., 2010; Schulz et al., 2010). Exposure to environmental oestrogens can cause gonadal histopathologies, like proteinaceous fluid and intersex, and these alterations can be transitory or permanently (Luzio et al., 2016).

In vertebrates, the hypothalamus-pituitary-gonad (HPG) axis controls the biosynthesis of sex steroids through gonadotrophins, follicle stimulating (FSH) and luteinizing hormones (LH) (Lubzens et al., 2010; Schulz et al., 2010). In females, folliculogenesis is mainly dominated by 17 β-oestradiol (E2) during oocyte growth and by maturation inducing

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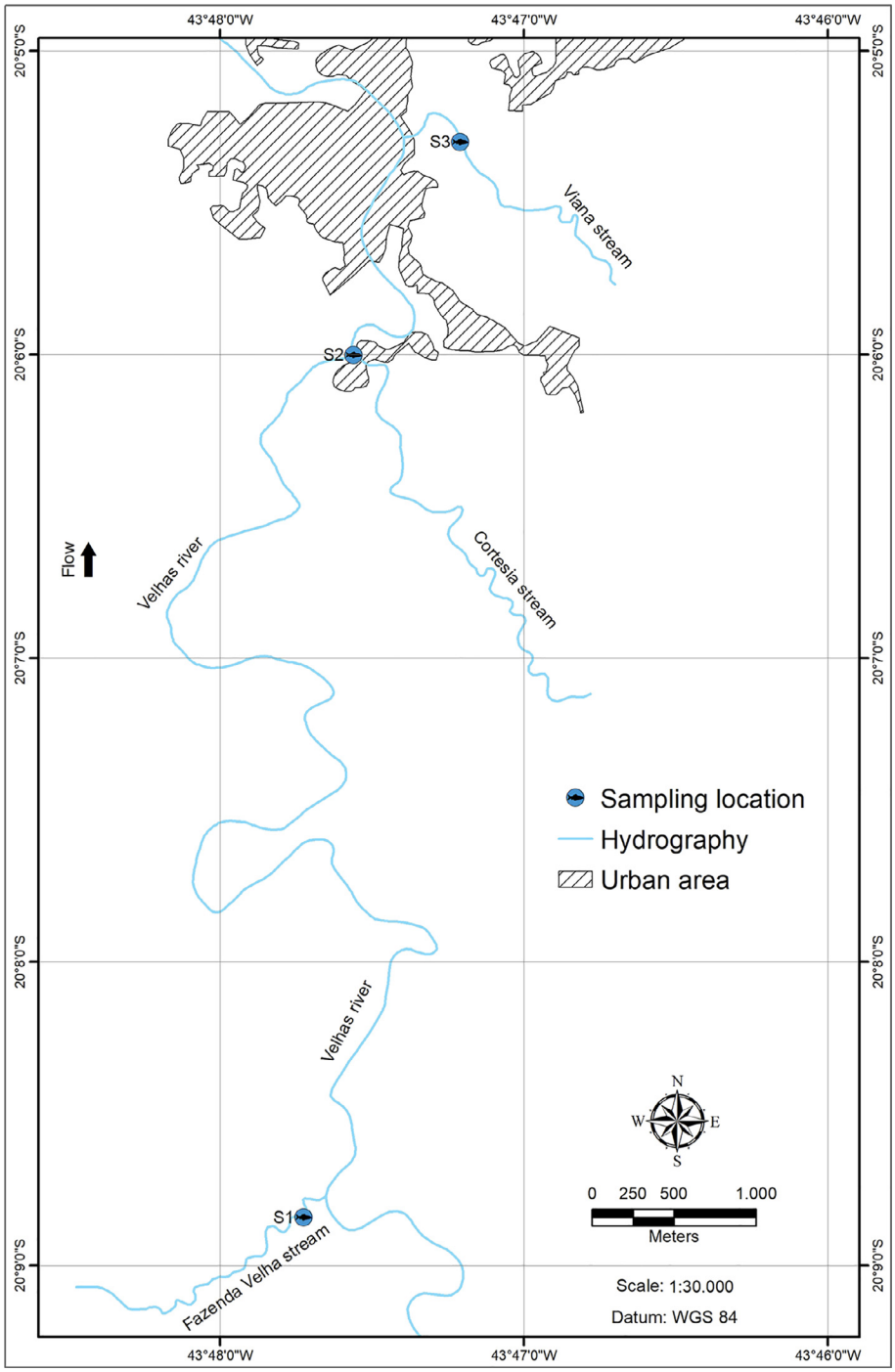

Fig. 1. Location of sampling sites, reference site (S1) and impacted sites (S2 and S3) in the Velhas River, São Francisco River basin.

steroid (MIS) during oocyte final maturation (Moore et al., 2000). Under influence of FSH, testosterone is converted to E2 by P450 aromatase in the follicular cells, regulating the oocyte growth. Then, E2 is released into circulation, and promotes the synthesis of vitellogenin and zona radiata proteins in the liver, which will be key proteins in the vitellogenic oocyte (Arukwe and Goksøyr, 2003; Lubzens et al., 2010). In *Gadus morhua*, both testosterone (T) and 11-ketotestosterone (11-KT) induce the growth and development of previtellogenic follicles, in vitro (Kortner et al., 2008). After oocyte growth, a steroidogenic shift lead to a decrease in E2 levels due to low ovarian aromatase (*cyp19a1a*) activation (Kime, 1993), while MIS levels increase through LH stimulation. MIS binds to membrane progestin receptors (mPRs) and stimulates processes associated with oocyte maturation (Kohli et al., 2005; Nagahama and Yamashita, 2008; Thomas, 2012).

Spermatogenesis is a finely regulated process in which diploid stem cells proliferate and differentiated in highly specialised haploid cells, spermatozoa. The early stages of spermatogenesis are mainly regulated by FSH, while sperm maturation is mostly under LH control (Schulz et al., 2010). Both FSH and LH act on Leydig cells by stimulating the synthesis and secretion of androgens, T and 11-KT. In addition, FSH regulates the function of Sertoli cells to support many aspects of sperm cell maturation (Huhtaniemi and Themmen, 2005; Themmen and Huhtaniemi, 2000; Xie et al., 2017). At the beginning of spermatogenesis, 11-KT acts directly by inhibiting the anti-Mullerian hormone (AMH) and stimulating the expression of activin B, thus promoting spermatogonial proliferation (Miura et al., 2002, 1995). In addition, 11-

KT also acts at the final maturation process inducing spermiation (Milla et al., 2008). Otherwise, E2 participates in the spermatogonial self-renewal under control of Sertoli cell (Nader et al., 1999; Schulz et al., 2010) and at final maturation of spermatozoa (Delalande et al., 2015; Rolland et al., 2009). In teleosts, progestins are essential for spermatogenesis playing crucial roles in final sperm maturation, spermiation, sperm motility and in early stages of spermatogenesis (Tubbs and Thomas, 2008; Ueda et al., 1985). Progestins are also important in the conversion of 11β-hydroxytestosterone to 11-KT by stimulating 11β-hydroxysteroid dehydrogenase (Chen et al., 2010).

Besides that, sex steroids, gonadotropins and growth factors regulate the fish gametogenesis (Tokarz et al., 2015). Spermatogenesis is frequently divided into spermatogonial phase with successive generation of spermatogonia, spermatocytary phase with meiotic division of the spermatocytes and spermiogenic phase when spermatids become spermatozoa (Leal et al., 2009; Schulz et al., 2010). In females, oogonia proliferation and differentiation is followed by primary follicular growth including the perinucleolar and cortical alveoli follicles, secondary follicular growth, when vitellogenesis occurs, and final oocyte maturation which results in the formation of the mature oocyte apt to fertilisation (Forsgren and Young, 2012; Lubzens et al., 2010). Endocrine disrupting chemicals (EDCs) bind to cell membrane or intracellular receptors and can alter cellular homeostasis and some gene transcription (Denslow and Sepúlveda, 2007). During gametogenesis, the proportion of germ cells is altered by exposure to oestrogenic EDCs (Bahamonde et al., 2015; Luzio et al., 2015; Prado et al., 2011). Moreover, fishes exposed to nonylphenol (Lin and Janz, 2006), natural oestrogens (Panter et al., 1998) and bisphenol A (Mihaich et al., 2012) indicate that these EDCs can cause gametogenesis inhibition as well as degeneration of seminiferous tubules and gonadal histopathology. In severe cases, these alterations promote a feminization of the population and in a long-term a total collapse of populations (Kidd et al., 2007).

In South America, lambaris of the genus *Astyanax* are found in water bodies with different levels of water contamination, so they are suitable sentinel models for ecotoxicological studies (Prado et al., 2014, 2011; Weber et al., 2017). *Astyanax rivularis* (Lutken, 1875) is an endemic fish of streams and creeks in the upper Velhas River, São Francisco River basin, Brazil. Considering the lack of studies that address the effects of EDCs on sex steroids and how these effects can affect the proportion of germ cells, the present study aims to evaluate the effects of estrogenic EDCs on sex steroids and its consequences on gametogenesis of the wild fish *Astyanax rivularis*.

2. Material and methods

2.1. Sampling sites and oestrogenic compounds in water

Mature females and males of *A. rivularis* were captured using 3 gillnets of 10 m with a 0.8 cm stretched mesh size deployed for about 4 h in pools of the streams, at a distance of 100 m from each other. Sampling ocurred in the peak of the reproductive season (June) in three streams of the Upper Velhas River: S1, reference site, with a good conservation status, S2 and S3, contaminated sites that receive municipal domestic sewage of the neighbouring towns (Fig. 1). In this study, only mature females and males were used, with ovaries filled with vitellogenic oocytes and lumen seminiferous tubules filled with spermatozoa. Males of *A. rivularis* presents anal fin spicules when they are in advanced maturation (Vieira et al., 2015). Thus, this pattern was utilized to identify males and females.

In each collection site, the main physicochemical parameters were obtained from the water and oestrogenic compounds were assessed using three 500 ml water samples: oestrone (E1), oestradiol (E2) and oestriol (E3), bisphenol A (BPA) and nonylphenol (NP). The samples collected at each site had a distance of approximately 50 m from one another. Detection of the compounds was performed by liquid chromatography (HPLC) coupled to mass spectrometry (MS), according to protocol previously described (Weber et al., 2017). Oestrogenic potential of the samples was evaluated in terms of the oestradiol equivalent concentrations ($EEQs = C_i.EEF_i$), where C_i is the concentration of compound i in the sample, and EEF_i is the oestradiol equivalency factor of compound i. The oestradiol equivalency factor (EEF) is defined by the following expression: $EEF_i = EC50_{E2}/EC50_i$, where $EC50_{E2}$ is the concentration that half of the maximum response for the oestradiol, and $EC50_i$ is the concentration that half of the maximum response for compound i (Wagner and Oehlmann, 2009). To determine the estrogenic potential (EEQ_t) of each site was calculated the sum of the concentrations for each EDC after normalising with their oestradiol equivalency factors (EEFs) by the formula: $EEQ_t = \Sigma\ (EEF_{E1}) + (EEF_{E2}) + (EEF_{E3}) + (EEF_{NP}) + (EEF_{BPA})$ (Welshons et al., 2003).

2.2. Fish sampling

A total of 42 specimens (n = 7 fish/sex/site) of *A. rivularis* in the mature stage were used in this study. Alive fish were euthanized by immersion in eugenol 85 mg.L^{-1}. Total length (TL; 0.01 cm), body weight (BW; 0.01 g), gonad weight (GW; 0.001 g) were measured, and gonadosomatic index (GSI = 100.GW/BW) and Fulton condition factor ($K = 100.BW/TL^3$) were calculated for each fish. Fish collection procedures followed the ethical principles established by the Brazilian College of Animal Experimentation (COBEA), and the study was approved by the Ethics Committee on Animal Use (CEUA, protocol N°189, 2016) of the Federal University of Minas Gerais, Brazil. Gonad samples were obtained of each fish for morphological and hormonal analyses.

2.3. Histology and morphometry

For histological analyses, the whole left testis and ovaries of each fish were fixed in Bouin's fluid for 24 h and then kept in 70% ethanol. Then, the samples were gradually dehydrated in ethanol, embedded in paraffin, sectioned at 5 μm thickness taking the whole midline plan of the organ, and stained with haematoxylin-eosin (HE). For each fish were analysed five histological sections.

To assess gametogenesis, morphometric analyses (n = 7 sex/site) were carried out on histological sections. For males, the proportion (%) of the germ cells (undifferentiated spermatogonia A, differentiated spermatogonia A, spermatogonia B, early spermatocytes, late spermatocytes, spermatids, spermatozoa), myoid cells, connective tissue, Leydig cells, Sertoli cells, acidophilic granulocytes and blood vessels was determined. For females, the proportion (%) of perinucleolar, cortical alveoli and vitellogenic follicles, and the connective tissue was determined. From each histological slide, six images at 400× magnification were obtained. A grid with 540 intersections between lines was overlaid on each image, and the counts were made using the Image J software, totalling 15,120 points analysed per site for both males and females.

The following gonadal histopathologies were assessed: intersex, testicular degeneration, eosinophilic proteinaceous fluid and oocyte ageing (Kwak et al., 2001; Luzio et al., 2016; OECD, 2009). The histopathological alterations were measured as percentage of total number of individuals. Testicular degeneration and eosinophilic proteinaceous fluid were also assessed by the ratio of affected area and total area of the tissue × 100 (% damage tissue). Oocyte ageing was evaluated as a percentage of the total number of vitellogenic follicles (Prado et al., 2014).

2.4. ELISA for sex steroids

During fish collection, whole right gonad samples from the same fishes used at morphometric analyses were stored in liquid nitrogen and kept in a freezer at −80 °C. The frozen samples were homogenised in extraction buffer (50 mM Tris-HCl pH 8.0 with 0.002% aprotinin and 1 mM phenylmethylsulfonyl) at a 1:2 ratio of tissue weight: buffer

volume using a Potter S (Braun, Melsungen, Germany) homogeniser. Subsequently, the extracts were sonicated using a GEX 600 EC ultrasonic processor and then centrifuged at 15000*g* for 60 min at 4 °C. After centrifugation, the supernatants were stored in aliquots at −80 °C until analysis. The dosage of gonadal sex steroids was performed in duplicate samples of testis and ovaries by using commercial ELISA kits following the manufacturer's instructions: 17β-oestradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT) (Cayman Chemicals, Ann Arbor) and 17-hydroxyprogesterone (17-OHP) (DRG Diagnostics, GmbH, Germany). The sensitivity of the assays was 6.6 pg/ml (oestradiol), 3.9 pg/ml (testosterone), 0.8 pg/ml (11-ketotestosterone) and 34.0 pg/ml (17-hydroxyprogesterone).

2.5. Statistical analyses

Prism Graph Pad 5.0 (Graph Pad software, La Jolla, USA) was used in the statistical analyses. One-way analysis of variance followed by Tukey's test was used to compare biometry, GSI, K and germ cells proportion (%). For gonadal sex steroids, ageing oocytes index, proteinaceous fluid and testicular degeneration area (%) and water oestrogenic compounds among sites was used Kruskal-Wallis followed by Dunn's. Data were expressed as mean ± standard error and considered significant at $p < 0.05$.

3. Results

3.1. Water quality

In the upper Velhas River, the water temperature and dissolved oxygen showed similar values among sites (Table 1). In S1, reference site, all oestrogenic substances presented mean values of < 90 ng/L. Mean values of E1 were higher in S2 (153 ng/L), while NP and BPA were higher in S3 (926 and 230 ng/L, respectively). High levels of E2 (260 ng/L) and E3 (368 ng/L) were observed in S3 site (Table 1). The oestrogenic potential (EEQ_t) of S1 site was 161.7 ng/L, while in site S2 and S3 were 667 ng/L and 1300 ng/L, respectively.

3.2. Biometry and biological indexes

Mature females of *A. rivularis* had 11.09 ± 0.51 cm of total length and 19.48 ± 3.16 g of body weight in site S1, with no significant difference among sites, $p > 0.05$ (Table 2). Males from S2 and S3 showed significantly lower values for total length ($p = 0.03$) and body weight ($p = 0.01$) than males from reference site (S1). No significant variations among sites were found for GSI in both females and males, $p > 0.05$. The Fulton condition (K) in females showed no statistical differences between sites ($p > 0.05$). Males from S1 site showed no statistically differences in K value among sites, but S2 males had statistically lower values of K when compared with S3 males ($p = 0.03$) (Table 2).

Table 2
Biometric data and biological indices of *Astyanax rivularis* in three sites from the upper Velhas River, São Francisco River basin.

	S1	S2	S3
Males			
Total length (TL, cm)	9.04 ± 0.75[a]	8.20 ± 0.39[b]	8.15 ± 0.42[b]
Body weight (BW, g)	9.78 ± 2.62[a]	6.45 ± 0.37[b]	7.16 ± 1.09[b]
Gonadosomatic index (GSI)	10.59 ± 1.89[a]	11.17 ± 2.00[a]	9.59 ± 5.13[a]
Fulton condition factor (K)	1.30 ± 0.09[ab]	1.17 ± 0.11[a]	1.32 ± 0.05[b]
Females			
Total length (TL, cm)	11.09 ± 0.51[a]	10.56 ± 0.66[a]	10.50 ± 0.42[a]
Body weight (BW, g)	19.48 ± 3.16[a]	16.30 ± 3.61[a]	16.96 ± 1.72[a]
Gonadosomatic index (GSI)	14.02 ± 3.74[a]	15.42 ± 2.14[a]	13.35 ± 3.38[a]
Fulton condition factor (K)	1.42 ± 0.09[a]	1.37 ± 0.08[a]	1.47 ± 0.18[a]

Values are expressed as mean ± standard error of 7 fish per site. In each line, different letters indicate significant differences among sites, $p < 0.05$. (S1) reference site with a good conservation status, (S2 and S3) contaminated sites that receive municipal wastewater.

3.3. Gametogenesis

In males, no significant difference was observed in the proportion of the different types of spermatogonia among the sampling sites ($p > 0.05$) (Fig. 2A, G). However, the proportion of early spermatocytes was significantly higher at S3 site (Fig. 2A, G) ($p = 0.0016$) and spermatids (Fig. 2A, G) was significantly higher in both impacted sites ($p = 0.0110$). The proportion of spermatozoa (Fig. 2A, G) was statistically lower in S3 site ($p = 0.0017$). Sertoli cells (Fig. 2I, G), myoid cells (Fig. 2K, G), and acidophilic granulocytes (Fig. 2B, G) did not differ significantly among sites ($p > 0.05$). However, Leydig cells ($p = 0.047$) (Fig. 2G) and blood vessels ($p = 0.04$) (Fig. 2G) had higher proportion in the fish from site S3, and connective tissue was lower at S3 site ($p = 0.018$) (Fig. 2G).

The females of S2 site presented a lower proportion of perinucleolar follicles when compared to site S1 ($p = 0.04$) (Fig. 3A, F). The proportion of follicles with cortical alveoli (Fig. 3B, F) and connective tissue (Fig. 3F) did not have statistical differences between sites ($p = 0.76$ and $p = 0.10$, respectively). The females of site S3 had a higher proportion of vitellogenic follicles compared to S2 ($p = 0.02$) (Fig. 3C, F).

3.4. Gonadal histopathology

Degeneration of the testicular germinal epithelium exhibiting agglutinated germ cells masses (Fig. 2C-E) was also found in males from S2 (57%) and S3 (71%) occupying 1.19 ± 0.34% and 2.09 ± 0.58% of the tissue area, respectively. On S1 site, this alteration was less frequent and occupied 1.15 ± 0.34% of the tissue. In males, perinucleolar follicles associated with spermatogenic cells cysts into the seminiferous tubules (i.e. intersex gonad) were observed in 28% of the males from site S3 (Fig. 2F). No intersex gonads were observed at sites S1 and S2.

In females, the histopathology commonly found was a diffuse eosinophilic proteinaceous fluid, spread within the interstitial tissue

Table 1
Water physicochemical parameters and concentration oestrogenic pollutants at different collection sites from the upper Velhas River, São Francisco River basin.

	S1	S2	S3	*p* value
Temperature (°C)	17.4 ± 2.1[a]	17.2 ± 1.8[a]	17.2 ± 1.5[a]	0.69
Dissolved oxygen (mg/L)	10.9 ± 2.3[a]	11.1 ± 2.3[a]	11.2 ± 1.7[a]	0.99
Oestradiol (E2) (ng/L)	32.3 ± 10.2[a]	133.3 ± 57.7[ab]	260.0 ± 196.9[b]	0.03
Oestrone (E1) (ng/L)	13.6 ± 11.8[a]	153.3 ± 124.4[a]	66.67 ± 57.7[a]	0.24
Oestriol (E3) (ng/L)	43.0 ± 19.9[a]	295.0 ± 208.6[a]	368.7 ± 165.2[a]	0.32
Nonylphenol (NP) (ng/L)	85.6 ± 64.2[a]	664.7 ± 386.8[ab]	926.8 ± 592.1[b]	0.04
Bisphenol A (BPA) (ng/L)	69.2 ± 2.69[a]	114.2 ± 78.86[ab]	230.6 ± 175.9[b]	0.01

Values are expressed as mean ± standard error of 3 measures per site. In each line, different letters indicate significant differences among sites, $p < 0.05$. (S1) reference site with a good conservation status, (S2 and S3) contaminated sites that receive municipal wastewater.

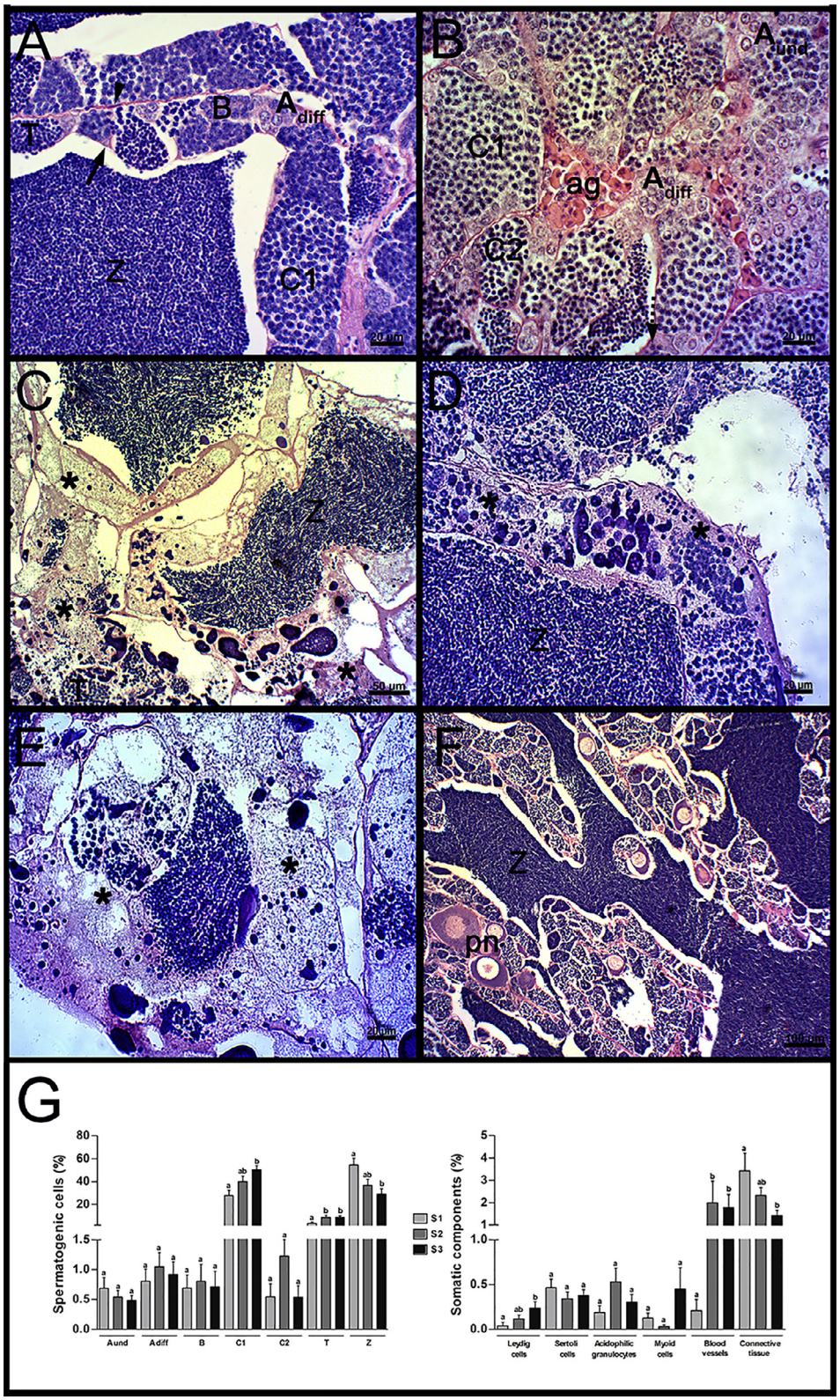

Fig. 2. Histological sections of testis and spermatogenic cells proportion in *Astyanax rivularis* from the Velhas River, São Francisco River basin. (A) Testis with normal architecture of seminiferous tubules, (B) Infiltration of acidophilic granulocytes (ag), (C-E) Testicular degeneration of the germinal epithelium with agglutination of germ cells (asterisks), (F) Intersex gonad with presence of perinucleolar follicles (pn) within seminiferous tubules, (G) Proportion (%) of spermatogenic cells in *Astyanax rivularis* (S1) reference site with a good conservation status, (S2 and S3) contaminated sites that receive municipal wastewater. Sertoli cells (arrow); myoid cells (arrowhead); spermatogonia A undifferentiated (A_{und}), spermatogonia A differentiated (A_{diff}), spermatogonia B (B), early spermatocytes (C1), late spermatocytes (C2), spermatids (T), spermatozoa (Z), acidophilic granulocytes (I), perinucleolar follicle (pn). Scale bars: (A, B, C, E and F) 20 µm, (D) 50 µm, (E) 100 µm.

(Fig. 3D, E). This gonadal alteration was found in S2 (57%) and S3 (85%) of the females. It occupied 2.12 ± 0.65% and 2.92 ± 1.47% of the tissue area, with significant difference regarding the reference site S1 (0.27 ± 0.13%) ($p = 0.35$). Oocyte ageing was recognized by aggregation of the cortical alveoli and yolk liquefaction (Fig. 3E). The proportion of vitellogenic follicles exhibiting this alteration was more elevated in females from S2 (17.5%) than S1 (12.5%) and S3 (8%), with significant difference between S2 and S3 ($p = 0.01$).

3.5. Gonadal steroids level

In males, concentrations of E2 were higher in sites S2 (3061 ± 1116 pg/ml) and S3 (3065 ± 1389 pg/ml) when compared to reference site, S1 (423 ± 107 pg/ml) ($p = 0.024$) (Fig. 4A). Male

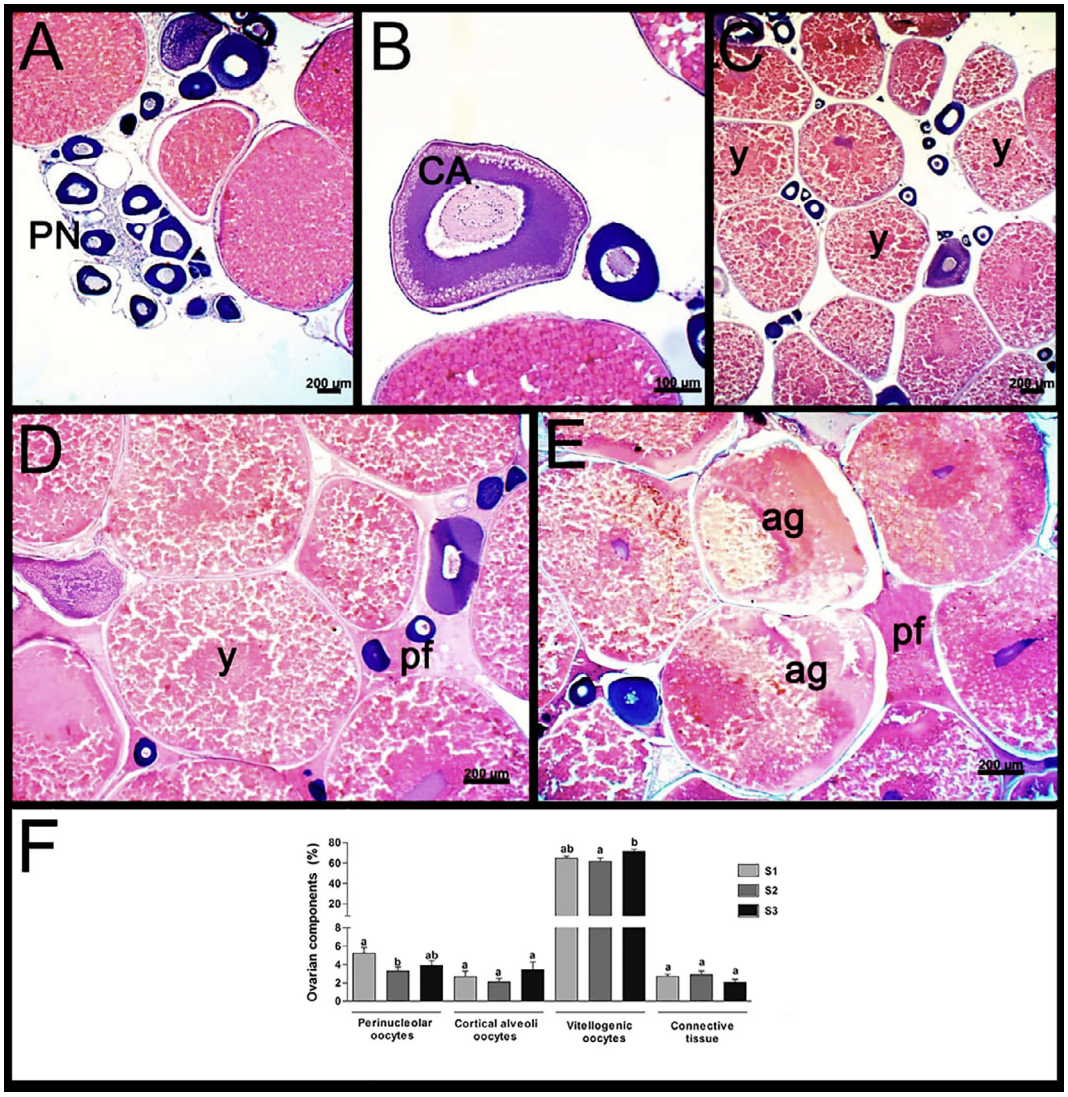

Fig. 3. Histological sections of ovary and follicles proportion (%) in *Astyanax rivularis* from the Velhas River, São Francisco River basin. (A) Perinucleolar follicle (PN), (B) cortical alveoli follicle (CA), (C) vitellogenic follicle (y), (D) Proteinaceous fluid (pf) in the interstitial compartment, (E) Ageing oocytes (ag) with aggregation of cortical alveoli and yolk liquefaction, associated to proteinaceous fluid (pf), (F) Proportion (%) of perinucleolar, cortical alveoli and vitellogenic follicles, and connective tissue in three sites: (S1) reference site with a good conservation status, (S2 and S3) contaminated sites that receive municipal wastewater. Scale bars: (A, C, D and E) 200 µm, (B) 100 µm.

fish from site S2 had lower values of 11-KT (91.5 ± 33.3 pg/ml) and T (78.5 ± 25.0 pg/ml) when compared to reference site ($p = 0.034$, $p = 0.011$, respectively) (Fig. 4B, C). In females, E2 and T showed similar values in the three sites, $p > 0.05$ (Fig. 4E, G). On the other hand, the 11-KT values were significantly higher in fish from site S3 (5.21 ± 0.13 pg/ml), $p = 0.03$ (Fig. 4F). No significant difference was observed in the concentrations of 17-OH in males and females of the different sites, $p > 0.05$ (Fig. 4D, H).

4. Discussion

Studies on endocrine disruption with native fish species have been conducted to assess the level of conservation of aquatic environments (Bahamonde et al., 2015; Ibor et al., 2016; Noaksson et al., 2005, 2003; Prado et al., 2014; Randak et al., 2009; Tetreault et al., 2014; Tolussi et al., 2018). These studies are often arduous to be performed due to the large number of different contaminants released in the aquatic environment and the performance of these compounds in synergy can cause different changes from those observed in laboratory studies that use only one contaminant (Silva et al., 2012). To our knowledge, this is the first study that associates the concentration of sex steroids in the gonad to proportion of germ cells and gonadal histopathologies in males and females in *Astyanax rivularus*.

The high variability in the concentration of oestrogenic compounds in the analyzed samples is related to the type of environment that the study was carried out. Riverine environments such as rivers and streams have a greater water renewal, preventing a smaller variation in the concentrations of these contaminants (Campanha et al., 2015). This same pattern of variation is observed in other studies in which these compounds were analyzed in these types of environment (Moreira et al., 2011; Schultz et al., 2013).

Lower values of total length and body weight were observed in males of *A. rivularis* at sites contaminated in the present study. This pattern is common when fishes are exposed to oestrogenic endocrine chemicals in both field and laboratory (Bahamonde et al., 2015; Ibor et al., 2016; Prado et al., 2014; Randak et al., 2009; Silva et al., 2012). Some oestrogenic compounds, as BPA, promote the reduction of thyroid hormones, T3 and T4, affecting the growth of the fishes (Naderi et al., 2014).

Astyanax rivularis is a partial spawning species that reaches the peak

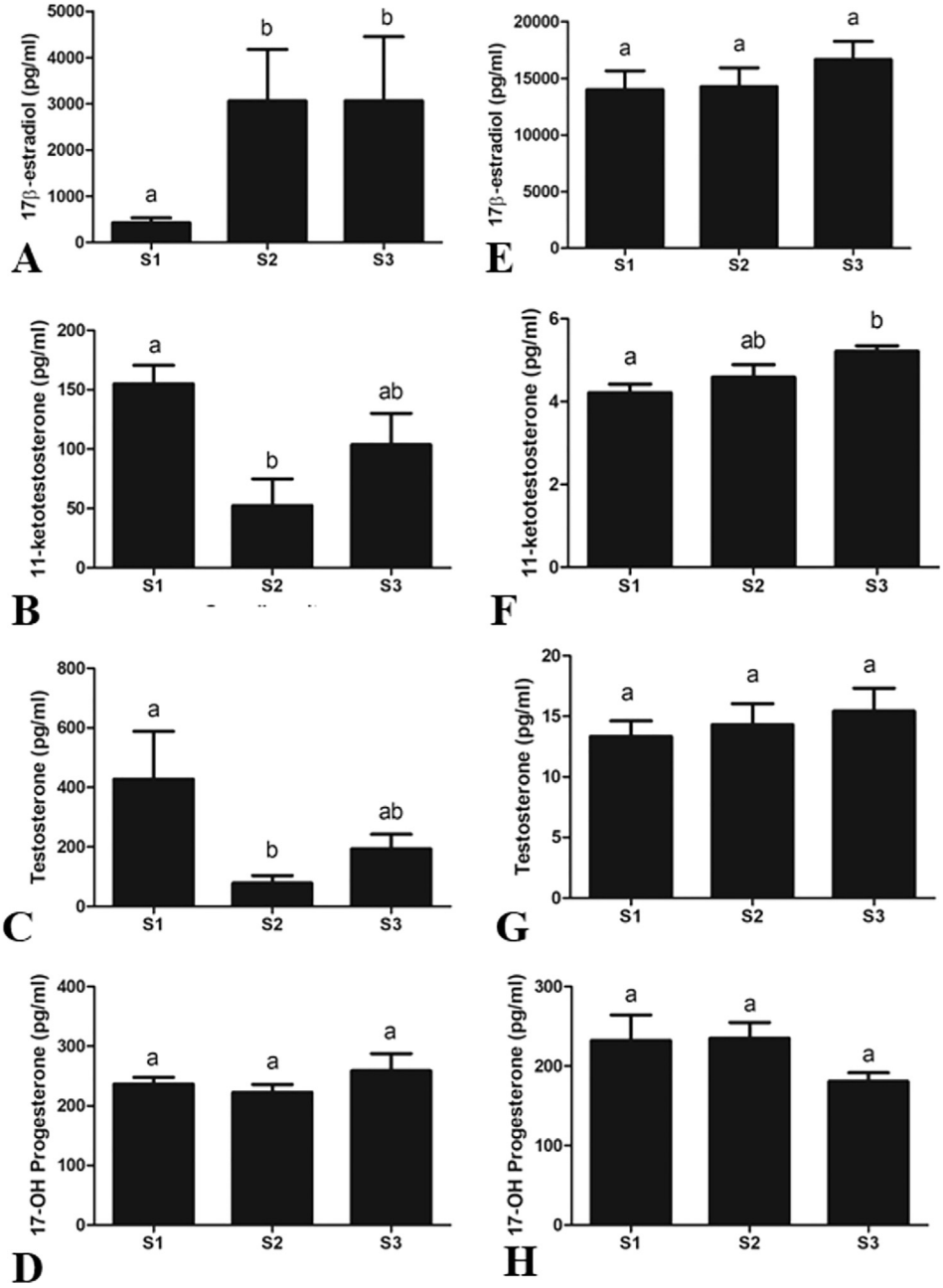

Fig. 4. Concentrations of gonadal sex steroids in gonads of *Astyanax rivularis* sampled at reference site (S1) and impacted sites (S2 e S3). (A, B, C, D) males and (E, F, G, H) females. Values are expressed as means ± SEM. Different letters indicate statistical differences between sites ($p < 0.05$).

of gonad maturation in June at upper Velhas River (Weber et al., 2017). GSI is a biomarker widely used in studies that evaluate endocrine dysregulation (Bahamonde et al., 2015; Ibor et al., 2016; Prado et al., 2014). Most studies have shown that these compounds cause a reduction of this index in females and males contaminated with oestrogenic EDCs (Bahamonde et al., 2015; Prado et al., 2011; Randak et al., 2009; Tetreault et al., 2011). However, it is important that studies using partial spawning species ensure that the individuals are in the same gonadal maturation stage, since the individuals of these species may be in different stages of gonad maturation in the same season of the year (Carvalho et al., 2009; Martins et al., 2012). Thus, in the present study only males and females in advanced maturation were used to make this comparison. Therefore, this index should not be used isolated to evaluate effects of EDCs.

In fish, 17β-oestradiol (E2) plays a role in the spermatogonial self-renewal (Schulz et al., 2010). In this sense, several studies have shown an increase in the proportion of spermatogonia, when males are exposed to estrogenic EDCs (Ibor et al., 2016; Kwak et al., 2001; Luzio et al., 2016; Silva et al., 2012). However, in the present study no significant increase in proportion of spermatogonia was observed in contaminated sites, but early spermatocytes increased significantly, probably reflecting on the spermatogonial differentiation. Similarly, increase in the proportion of spermatocytes was also observed in the spermatogenesis of the *Oreochromis niloticus*, exposed to the herbicide, tebuthiuron (Almeida et al., 2018). Probably, this compound decreased rate of meiosis and spermatid differentiation, promoting an accumulation of spermatocytes in exposed animals. In support to these findings, inhibition of the aromatase activity in *Halichoeres trimaculatus* reduced the proportion of early spermatocytes, expose to E2 (Kobayashi et al., 2011). Therefore, these studies demonstrate that oestrogens are associated with the proliferation and differentiation of spermatogonia to spermatocytes.

The spermiogenic phase occurs when the spermatids form spermatozoa, which are released into the lumen of seminiferous tubules. The increase in the proportion of spermatids and a decrease of spermatozoa in males exposed to oestrogenic compounds has also been found in the present study and in some other studies (Almeida et al., 2018; Bahamonde et al., 2015; Prado et al., 2011). Some oestrogenic compounds such as tebuthiuron may decrease the rate of differentiation of spermatids and thus, reduce the proportion of spermatozoa (Almeida et al., 2018). These alterations can be related to the low concentrations of androgens, especially 11-KT, as detected in males of *A. rivularis*. In addition, constant stimulation of oestrogenic compounds promotes an up-regulation of aromatase activity (*cyp19A*) increasing E2 levels and reducing T in testis (Almeida et al., 2018; Martins-Santos et al., 2017). Besides that, oestrogenic mimics, like EE2, down-regulate 11β-hydroxysteroid dehydrogenase type 2 (*hsd11b2)* and 17β-hydroxysteroid dehydrogenases type 3 (*hsd17b3*) in males, enzymes responsible for the production of androgens (Filby et al., 2007).

Acidophilic granulocytes are frequently found in fish testis as detected in *A. rivularis* most frequently in contaminated sites. In *Sparus aurata,* administration of 17α-EE2 caused an inflammatory process in the testis, with a consequent infiltration of acidophilic granulocytes, self-regulating the gene expression of cytokine, chemokines and adhesion molecules (Cabas et al., 2011; Liarte et al., 2011; Szwejser et al., 2017). This result confirms that oestrogenic EDCs stimulate the infiltration of these cells into the testis.

The concentrations of E2 and T in females of *A. rivularis* did not present significant variations at the different sampling sites. This demonstrates that folliculogenesis is less affected by oestrogenic compounds than spermatogenesis (Bahamonde et al., 2015). Exposure to oestrogenic compounds up-regulates 17β-hydroxysteroid dehydrogenases type 3 (*hsd17b3*) expression in fish females. This enzyme is responsible for the conversion of 11-ketoandrosterone to 11-KT (Filby et al., 2007). In field studies, an increase in 11-KT levels was observed in tilapia species contaminated with polychlorinated biphenyl (PCB) (Adeogun et al., 2016). Previous studies, *Oncorhynchus kisutch*, have associated the influence of 11-KT on folliculogenesis only by stimulating the development of primary follicles and the transition to secondary growth (Monson et al., 2017). In the present study, a slight increase in the proportion of vitellogenic follicles was also observed in the S3 site, which may be associated with an increase in 11-KT ovarian levels.

In the present study, a higher proportion of over ripening, which indicates the presence of oocytes ageing within the ovary, was found in females from the site S2. This alteration could be associated to delayed steroidogenic exchange of E2 to progestins due to contamination by oestrogenic compounds as shown in *Perca fluviatilis* (Noaksson et al., 2005) and in *Astyanax fasciatus* (Prado et al., 2014). Concentrations of 17-OHP, which is a precursor of the major fish progestin, 17, 20-dihydroxy-4-pregnen-3-one (17a,20b-DP), did not present statistically lower values at site S2. Studies carried out with zebrafish exposed to EE2 did not show a reduction in the enzyme 20b-hydroxysteroid dehydrogenase (*hsd20b*), that is responsible for the conversion of 17-OHP to 17a,20b-DP (Urbatzka et al., 2012). Thus, spawning delay can be associated with the acting of the EDCs directly in progestins.

The exposure of female fishes to oestrogenic compounds may lead to several gonadal histopathologies (Luzio et al., 2016; OECD, 2009). In the present study, the majority of females in impacted sites presented the formation of eosinophilic proteinaceous fluid in the interstitial tissue. This histopathology is associated with the exposure of females to oestrogenic endocrine disruptors and can be due to vitellogenin degradation in the interstitial compartment as suggested for *Danio rerio* exposed to 17α-ethinylestradiol (Luzio et al., 2016; Silva et al., 2012).

The intersex condition is that in which the presence of oocytes occurs along the testicular tissue as detected in males from site S3 of the present study. Hinck et al. (2009) demonstrated that E2 levels are higher in intersex fish than in animals not exposed to oestrogenic compounds. It is noteworthy that androgen levels (T and 11-KT) are also lower in intersex males than non-intersex males exposed to oestrogenic contaminants (Bahamonde et al., 2015). Exposure of males to oestrogenic compounds increases the expression of the aromatase enzyme, and hence promotes an increase in oestrogen production and reduction in T concentrations and consequently 11-KT (Almeida et al., 2018; Martins-Santos et al., 2017). These changes in hormone levels promote the feminization of males in environments contaminated by oestrogenic compounds (Bahamonde et al., 2013).

Another histopathology commonly found in S2 and S3 males was the degeneration of the germinal epithelium with the presence of germ cell agglutination. Laboratory studies with *Oryzias latipes* (Kang et al., 2002) and *Pimephales promelas* (Leino et al., 2005) exposed to E2 and flutamide, respectively, have shown increased germinal epithelium degeneration of the seminiferous tubules with clusters of apoptotic germ cells, multinucleated cells and degeneration of the epithelial architecture. A study carried out with *Xiphophorus helleri*, exposed to BPA and NP and to the mixture of these two oestrogenic compounds (Kwak et al., 2001) showed similar histopathologies to those found in the present study. However, additional studies should be performed to determine the molecular mechanisms involved in the performance of endocrine disruptors on the degeneration of the seminiferous tubules.

In summary, the present study reveals that the environmental contamination by oestrogenic endocrine disruptors in the Velhas River headwaters promotes changes on the sex steroids levels and can alter the proportion of germ cells and the gonadal morphology, especially in male fishes. Thus, we can conclude that sex steroids and gonadal histopathology are important biomarkers to use in studies of oestrogenic endocrine dysregulation. Further studies should be performed to determine whether oestrogenic endocrine disrupters are disrupting the reproduction of *A. rivularis*.

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