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Review

Environmental pollution affects molecular and biochemical responses during gonadal maturation of *Astyanax fasciatus* (Teleostei: Characiformes: Characidae)

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ABSTRACT

Endocrine disrupting compounds (EDCs) have the potential to alter fish reproduction at various levels of organization. The aim of this study was to assess the impact of a natural environment with heavily anthropogenic influence on the physiological processes involved in reproduction in the freshwater fish lambari (*Astyanax fasciatus*) using different biomarkers. Adult males and females were collected in different seasons from two distinct sites in the same watershed: Ponte Nova Reservoir (PN) considered a pristine or small anthropogenic influence reference point; and Billings Reservoir (Bil), subjected to a large anthropogenic impact. Biological indices, such as hepatosomatic index and gonadosomatic index (GSI), gonadal histomorphology, fecundity, and biomarkers such as plasma levels of estradiol (E2) as well as hepatic gene expression of its alfa nuclear receptor (ERα), were analyzed. Hepatic vitellogenin (VTG) gene expression was evaluated in both sexes, as an indicator of xenoestrogen exposure. Females collected at PN presented a typical annual variation reflected in GSI, whereas for those sampled at Bil the index did not change through the seasons. The higher concentration of E2 in males collected at Bil during spring/2013, together with the detection of VTG gene expression, suggest the presence of EDCs in the water. These EDCs may have also influenced fecundity of females from Bil, which was higher during winter and spring/2013. Gene expression of ERα and ovarian morphology did not differ between fish from both sites. Water conditions from Bil reservoir impacted by anthropic activity clearly interfered mainly with biomarkers of biological effect such as plasma E2 levels and absolute and relative fecundity, but also altered biomarkers of exposure as VTG gene expression. These facts support the notion that waterborne EDCs are capable of causing estrogenic activity in *A. fasciatus*.

1. Introduction

The introduction of contaminants into the environment due to anthropogenic activities may cause adverse effects on human health as well as wildlife populations (Vázquez et al., 2009). Among the contaminants affecting physiological processes of the aquatic biota are endocrine disrupting compounds (EDCs), which are defined as compounds which can interfere with the synthesis, secretion, transport, binding, action or elimination of naturally secreted hormones. A large number of EDCs present in natural environments originate from improper elimination from sewage in wastewater treatment plants (WWTPs) (Tan et al., 2007; Hassell et al., 2016). EDCs can act on different pathways involving many physiological processes, such as reproduction, mimicking estrogen hormones (Vázquez et al., 2009),

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affecting sexual differentiation (Baroiller and Guiguen, 2001), gonadal development (Vested et al., 2014), and inducing vitellogenin synthesis in males (Moncaut et al., 2003; Vetillard and Bailhache, 2006). Several types of compounds are able to act as EDCs and affect reproductive processes in fish, including non-steroidal anti-inflammatory drugs (NSAIDs) (Fernandes et al., 2011; Ji et al., 2013) and metals (Driessnack et al., 2016), displaying estrogen-like activity and interference with estradiol receptors (Orn et al., 2006; Xu et al., 2008; Flick et al., 2014) and vitellogenin (VTG) synthesis (Scholz et al., 2004; Muncke and Eggen, 2006; Salierno and Kane, 2009). These studies have shown that different pollutants can deleteriously alter several physiological processes involved in reproduction at different organizational levels, potentially leading to population losses.

The presence of different EDCs in the aquatic environment has been well characterized in several studies around the world (Kinney et al., 2006; Kellar et al., 2014; Dias et al., 2015; Gorga et al., 2015; Minh et al., 2016). A wide range of water contaminants with EDC potential, such as NSAIDs (ibuprofen and diclofenac), that may bioaccumulate in organisms have been reported in Brazilian reservoirs, as is the case for the Billings reservoir (Bil) located in the metropolitan region of São Paulo, Brazil (Almeida and Weber, 2005). Bil reservoir is characterized by an intense anthropogenic activity, such as domestic sewage disposal and industrial effluents, deforestation and extensive land use (Mariani and Pompêo, 2008; Moschini-Carlos et al., 2010). Metals such as lead, chromium, mercury and zinc have also been detected in fish tissues since the 1980s at several points in this reservoir (Rocha et al., 1985), like the Taquacetuba and Bororé branches, where metal water concentrations are above those recommended by resolution of the Special Secretariat for the Environment. In fact, Oliveira (2012) detected high chromium concentrations in the liver of 60% of the sampled individuals of acará, *Geophagus brasiliensis*, 67% of trahira, *Hoplias malabaricus*, 98% of lambari, *Astyanax* sp. and 100% of tilapia, *Tilapia rendalli*, which are in disagreement with the limit established by ANVISA Decree 55.871/65 (National Health Surveillance Agency). In addition, in the Taquecetuba branch, the concentration of metals (lead, copper, mercury, nickel and zinc) in the water has also been reported above those allowed by CONAMA Resolution 357 (Gomes et al., 2015).

Water and sediment monitoring studies led by the State Government of São Paulo demonstrated the presence of several contaminants and 21 out of 27 points analyzed were considered eutrophic (CETESB, 1996). In a later study, the Taquacetuba branch was considered super-eutrophic (Gomes et al., 2016), evidencing that Bil contains a large diversity of pollutants. Only a few fish species such as tilapia (*Oreochromis niloticus*), lambari (*Astyanax* sp.) and catfish (*Rhamdia* sp.) can be found in this water body as representative species (Castro et al., 2009). However, when the ecosystem is degraded, as is the case of Bil, it is difficult to understand whether the effects observed in organisms that inhabit in this area, are due to the action of contaminants or due to other stressors, like hypoxia or changes in the food chain (Hook et al., 2014). Then, in order to evaluate the effect of environmental pollution on those species, biomarkers of biological effects and exposure have to be used (Broerg et al., 2005; Hook et al., 2014). According to Hook et al. (2014), biomarkers of biological effects are indicators of physiological or biochemical changes as a consequence of exposure, such as condition indices or hormonal circulating levels; while biomarkers of exposure are defined as single or multiple pollutants with similar modes of action than can show an early response to contaminants and are typically specific to a class of contaminants, e.g., the induction of the vitellogenin in males induced by estrogenic compounds.

Astyanax fasciatus, commonly known as red-tailed lambari, is one of the species frequently found in Bil. reservoir. It is a small omnivorous fish considered an important prey for carnivorous species (Vilella et al., 2002; Gurgel, 2004). This species presents different reproductive strategies, which may vary according to the environmental conditions, presenting both asynchronous or synchronous oocyte development (Silva et al., 2010). Its distribution is very wide, reaching all of Central and South America, being found in both clean and polluted areas. All these features contributed to choose *A. fasciatus* as a sentinel species for environmental and toxicological test studies (Schulz and Martins-Júnior, 2001; Alberto et al., 2005; Carrasco-Letelier et al., 2006; Prado et al., 2011). Therefore, the aim of this study was to assess the impact of a heavily anthropogenic impacted environment on the physiological processes involved in reproduction in the freshwater fish lambari (*Astyanax fasciatus*) using different biomarkers at different organizational levels.

2. Material and methods

2.1. Study area

The study area comprised two important reservoirs of the Tietê River Basin, located in the Metropolitan Region of São Paulo (MRSP) in the State of São Paulo, Southeastern Brazil (see map at Gomes et al., 2015). Ponte Nova reservoir (PN), located at the Upper Reaches of the Tietê River Basin, is part of an environmental protected area occupied by rainforests, preserved ciliary forests and flood plains along the water course. Based on these characteristics, the PN reservoir was considered in this study as a reference site with minimal anthropogenic impact. Samples were collected in the Rio Claro branch (PN, 23°34′36.5″S, 45°54′23.9″W), considered in a previous study as mesotrophic according to its trophic state index (TSI) (Table 1).

Bilings reservoir (Bil), located inside the MRSP, is a heavily impacted area due to its used for generation of electric power and as a public water supply. This reservoir is considered the second largest water reservoir of the MRSP, with a drainage area of 1560 km^2 and a water surface of 106.6 km^2 (Marceniuk and Hilsdorf, 2010). The Bil reservoir has seven branches with spatially differentiated biotic and pollutant characteristics, such as domestic sewage and industrial effluents, deforestation and uncontrolled soil occupation (Gomes et al., 2015). Samples were collected in the Taquacetuba branch (Bil

Table 1
Hydrogeomorphical, physical, chemical, and biological characterization of the Ponte Nova (PN) and Billings (Bil) reservoirs.

Variables	PN	Bil
Surface area (km^2)	25.7	106.6
Water retention time (days)	720	80
Elevation (m)	840	745
Temperature (°C)	16–23	17–25.5
pH	6.5	7.5–9.5
Chlorophyll-a (μg L^{-1})	0.7–4.1	33.3–867.0
Nitrate (μg L^{-1})	9.5–200	288.9–1045.9
Nitrite (μg L^{-1})	2.0–9.0	8.1–25.7
Total nitrogen (μg L^{-1})	500.00–900.0	430.0–473.6
Total phosphorus (μg L^{-1})	8.0–20.0	54.6–402.2
TSI	Mesotrophic	Super–hypereutrophic
Ibuprofen (ng L^{-1})	NF	10.0–78.2
Diclofenac (ng L^{-1})	NF	8.1–394.5
Total cadmium (mg/kg)	< 0.005	0.005
Total lead (mg/kg)	< 0.01	0.01–0.06
Total copper (mg/kg)	< 0.005	0.005–0.1
Total mercury (mg/kg)	< 0.0002	0.0002–0.27
Total nickel (mg/kg)	< 0.01	0.01–0.11
References	Gomes et al. (2015); Carvalho (2003)	Almeida and Weber (2005); Carvalho (2003); Gomes et al., (2015, 2016)

TSI Trophic State Index; NF not found.

23°48′16.5″S, 46°38′33.26″W), a region of large importance as a water supply for São Paulo city, considered hypereutrophic due to pollution by metals and frequent cyanobacterial blooms identified in previous studies (Table 1).

2.2. Animals and experimental design

The study was conducted from 2012 to 2014, sampling during each season, according to the following criteria: in 2012, the sampling period occurred between January and December, characterized by the capture of *A. fasciatus* females. During 2013–2014, the sampling period was held between September 2013 and March 2014, capturing both males and females. From those captures, the following groups were defined according to the seasons: Sum/12 (January to March 2012); Fal/12 (April to June 2012); Win/12 (August 2012); Spr/12 (October and December 2012); Win/13 (August 2013); Spr/13 (October and December 2013); Sum/14 (January to March 2014). A total of 162 females (body mass 15.2 ± 4.1 g, total length 10.3 ± 0.8 cm) and 72 males (body mass 12.3 ± 2.6 g, total length 10.0 ± 0.8 cm) were caught. Captures were authorized by the Federal Agency System ("Sistema de Autorização e Informação em Biodiversidade"; SISBIO 31935).

At the collection site, fish were anesthetized with 0.1% benzocaine. Total weight and length and gonad weight were measured. Blood samples were obtained by puncture of the caudal vein using heparinized syringes, and then centrifuged (655.1*g*) for 5 min to obtain plasma. Animals were then euthanized by decapitation, as was approved by the Animal Ethics Committee of the Institute of Biosciences, University of São Paulo (Protocol N° 154/2012). Liver and gonads were quickly removed and immediately weighed for calculation of the hepatosomatic and gonadosomatic indexes (HSI and GSI) (HSI = [liver mass/body mass]x100; GSI = [gonad mass/body mass]x100). A portion of the gonad was fixed in Bouin's solution for histological analysis. Another portion of known mass was placed in Gilson's solution for fecundity analysis (Vazzoler, 1981). The stages of oocyte maturation were classified according to Dias et al. (1998) and Honji et al. (2006). Plasma and liver tissue samples were stored on dry ice, then transported to the laboratory and stored at −80 °C for further analysis.

2.3. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Hepatic gene expression of vitellogenin A (VTG-A; Finn and Kristoffersen, 2007) and estrogen receptor alpha (ERα) were quantified by RT-qPCR. Total RNA was isolated by the RNeasy® mini total RNA isolation kit (Qiagen®) according to the manufacturer's protocol. RNA purity was assessed spectrophotometrically (NanoDrop1000; Thermo Fisher Scientific) and RNA integrity was evaluated by determining the ratio of 28 S and 18 S ribosomal RNA abundance with RNA 6000 Nanokit (Agilent Technologies®). Contaminating genomic DNA was removed with DNase using the DNA-free Turbo kit (Life Technologies®). One microgram of RNA was used for cDNA synthesis by reverse transcriptase using the Quanti Tect Reverse Transcription Kit (Qiagen®).

PCR was used to amplify partial cDNA sequences for VTG-A and ERα using primers designed against conserved regions of *Astyanax mexicanus* and some Cypriniformes. Each reaction consisted of 2.5 μL of Ecotaq Buffer (10X) with Mg (Lucigen®),18.3 μL of water,1 μL of 10 nM deoxynucleotide Mix Solution (dNTP) for each dNTP (BioLabs®), 1 μL of each primer and 0.2 μL of Ecotaq DNA Polymerase 100U to 5 U/μL (Lucigen). The amplification protocol with the thermalcycler was programmed a 90 °C (60 s), followed by a 40 °C cycle (10 s), 60 °C (0.30 s), 72 °C (60 s) and 72 °C (5 min). PCR products were separated through electrophoresis on 1% agarose gel and purified using the QIA quick PCR Clean up Kit (QIAGEN ltd.). Primers for the 18 s ribosomal RNA gene (housekeeping used as a normalizer) were designed using the published sequence for *A. fasciatus* (genbank KJ129623.1).

Table 2
Primers used for quantitative reverse-transcriptase PCR.

Genes	Primer	DNA sequence
VTG	Forward	GCCTCTGCGTTGTTGATCTT
VTG	Reverse	AAACTCTGACCCTGCTGGAA
ERα	Forward	CAGGACATGTACACTGCAGC
ERα	Reverse	GGTTGGTTGCTGGACACA
18 S rRNA	Forward	CCCTATCAGCTGTCGATGGT
18 S rRNA	Reverse	TTACAGGGCCTCGAAAGAGA

2.3.1. Real-time quantitative assays (RT-qPCR)

The primers designed and used for RT-qPCR are shown in Table 2. Genes were quantified using the Stratagene MX3000P real time PCR system using delta-delta Ct (normalized and relative quantification). Each reaction contained 2 μL of sample (dilution 1:10), 12.5 μL of Power SYBR Green PCR Master Mix, 8.5 μL of water and 1 μL of primers. The thermos cycling schedule started at 95 °C (15 min) followed by 40 cycles of 95 °C (10 s), 60 °C (0.30 s), 72 °C (60 s), and a melt curve cycle at 95 °C (60 s), 55 °C (0.30 s), 95 °C (0.30 s). The standard curve, constructed by the PCR products of interest, showed a linear relationship for the Ct values (cycle threshold). Based on the slope of the curve, the efficiency of the PCR was calculated as 111.9%, 99.8% and 95.5% for VTG-A, ERα and 18 S, respectively.

2.4. 17β-estradiol levels

Plasma concentration analysis of 17β-estradiol (E2) was performed using ELISA immunoassay kits (Cayman®), following the manufacturer's guidelines. The microplate reader (Spectra Max250, Molecular Devices) was at a wave length of 450 nm. Samples were diluted 5 times with buffer solution from the kit. The lower limit of detection was 15 pg/mL. The minimum and maximum intervals of intraspecific and interspecific coefficients of variation (CV) were 2.2–19.2% and 0.67–0.57%, respectively.

2.5. Histological analysis

Gonad samples were dehydrated through an ascending series of ethanol solutions, cleared in dimethylbenzene and embedded in Paraplast® (Erv-Plast, Erviegas) following routine for histological procedures. Samples were serially sectioned at 5 μm on a microtome (Leica RM2255), mounted on Poly-L-lysine covered slides (Sigma, USA) and stained with haematoxylin-eosin or Periodic-Acid-Schiff (PAS)/Weigert's Haematoxylin/Metanil Yellow (Quintero-Hunter et al., 1991). Sections were analyzed and photographed using a computerized image capture system (microscope Leica DM1000, photographic camera Leica DFC295, and image capture Leica Application Suite Professional software, LAS V3.6). Stages of oogenesis were classified according Prado et al. (2011) and based on different stages of oocyte development, maturation stages of the ovary were adapted and established in accordance with Dias et al. (1998) and Honji et al. (2006).

2.6. Fecundity and diameter of oocytes

In order to determine absolute fecundity (AF; the number of oocytes released by a female in each spawning cycle) (Vazzoler, 1996), one-third of the gonad was weighed and placed in Gilson's solution for 30 days, allowing oocyte dissociation. Later, oocytes were transferred to 70°GL and the volume was adjusted to 1 L. A sample of 2.5 mL of oocyte solution was collected using a *Stempel* pipette, and the number of oocytes were then counted. For each individual, AF was calculated using three sub-samples of oocyte solution divided by total ovarian mass (RF; relative fecundity). Oocyte diameter was measured using the same image capture program mentioned in the histological section. Percentage of residual oocytes (RO) and vitellogenic oocytes (VO) were

Table 3
Gonadosomatic Index (GSI, %), Hepatosomatic Index (HSI, %), Absolute and Relative Fecundity (AF and RF), Residual and Vitellogenic Oocytes (RO and VO) of *A. fasciatus* females and 17β-estradiol (E2, pg mL^{-1}) and expression of vitellogenin (VTG) and estrogen receptor (ERα) genes of females and males of *A. fasciatus* (Mean ± SEM).

	Sum/12	Fal/12	Win/12	Spr/12	Win/13	Spr/13	Sum/14
Females							
GSI - PN	11.0 ± 0.89^{ab}	6.9 ± 0.53^{c}	6.1 ± 0.63^{c}	13.3 ± 0.82^{a}	6.4 ± 0.63^{c}	11.3 ± 0.70^{ab}	7.6 ± 0.93^{b}
GSI - Bil	11.1 ± 0.85	8.8 ± 0.66	10.3 ± 0.21*	10.7 ± 0.91	9.5 ± 0.96*	9.4 ± 1.19	11.7 ± 2.69*
HSI - PN	1.2 ± 0.24	1.1 ± 0.05	1.7 ± 0.58	1.0 ± 0.14	1.3 ± 0.22	0.9 ± 0.11	0.9 ± 0.14
HSI - Bil	0.7 ± 0.04	1.1 ± 0.14	10.7 ± 0.85	0.7 ± 0.05	1.0 ± 0.10	0.9 ± 0.16	0.8 ± 0.07
AF - PN	8056.5 ± 1113.74	8289.4 ± 1395.6	1443.6 ± 375.30	7542.0 ± 1391.11	2823.5 ± 1731.59	6630.9 ± 1731.59	5347.2 ± 966.86
AF - Bil	12988.9 ± 2189.30^{b}	11153.6 ± 1890.45^{b}	3097.2 ± 780.47^{b}	4867.8 ± 1073.99^{b}	25385.7 ± 8226.28^{ab*}	61322.3 ± 16873.90^{a*}	1972 ± 787.48^{b}
RF - PN	4272.9 ± 294.84	4601.2 ± 456.30	2330.2 ± 473.07	3798.2 ± 505.03	3678.6 ± 910.86	3762.2 ± 287.11	3847.8 ± 910.86
RF - Bil	6098.1 ± 893.91^{b}	9094.8 ± 3472.03^{b}	2269.7 ± 402.04^{b}	3104.0 ± 431.78^{b}	18043.3 ± 7818.92^{ab*}	43322.8 ± 10176.49^{a*}	1166.9 ± 393.81^{b}
RO - PN	79.2 ± 2.41	83.1 ± 7.28	89.4 ± 6.09	77.1 ± 9.30	84.6 ± 3.77	81.2 ± 2.43	85.6 ± 1.13
RO - Bil	79.2. ± 5.11	83.1 ± 13.23	89.4 ± 1.20	77.1 ± 3.23	83.7 ± 2.66	77.4 ± 4.41	83.8 ± 8.10
VO - PN	20.7 ± 2.85	12.3 ± 1.80	9.0 ± 0.41	23.0 ± 5.75	17.2 ± 4.81	19.5 ± 2.26	14.9 ± 1.01
VO - Bil	22.1 ± 5.74	12.0 ± 3.23	8.8 ± 2.00	11.0 ± 3.23	16.0 ± 2.25	22.7 ± 4.47	16.2 ± 8.10
E2 - PN	200,2 ± 33,13	104,4 ± 10,37	134,2 ± 18,62	50,1 ± 10,58	256,2 ± 109,46	101,2 ± 20,30	299,3 ± 123,72
E2 - Bil	78,3 ± 9,74	218,6 ± 47,43	101,2 ± 24,87	168,6 ± 53,89	146,1 ± 20,65	886,7 ± 543,17	241,2 ± 45,32
VTG (mRNA) - PN			1.4 ± 0.45 (*n = 4*)	1 ± 0.10 (*n = 2*)	1.3 ± 0.36 (*n = 6*)	3.0 ± 1.98 (*n = 5*)	1.2 ± 0.75 (*n = 5*)
VTG (mRNA) - Bil			0.6 ± 0.36^{b} (*n = 4*)	0.7 ± 0.17^{b} (*n = 6*)	1.0 ± 0.35^{b} (*n = 4*)	0.5 ± 0.30^{b} (*n = 6*)	255.9 ± 96.48^{a*} (*n = 6*)
ERα (mRNA) - PN			1.1 ± 0.29 (*n = 4*)	1.0 ± 0.17 (*n = 2*)	1.5 ± 0.54 (*n = 6*)	1.9 ± 0.88 (*n = 5*)	1.2 ± 0.33 (*n = 5*)
ERα (mRNA) - Bil			0.8 ± 0.32 (*n = 4*)	0.2 ± 0.05 (*n = 6*)	1.3 ± 0.28 (*n = 4*)	0.04 ± 0.01 (*n = 6*)	1.4 ± 0.32 (*n = 6*)
Males							
E2 - PN					172,2 ± 92,023	218,8 ± 31,81	351,0 ± 68,16b
E2 - Bil					268,1 ± 61,65^{b}	1675,8 ± 224,31^{a*}	497,3 ± 118,38^{b}
VTG (mRNA) - PN					6.0 ± 5.64 (*n = 4*)	6.2 ± 5.30 (*n = 5*)	1.5 ± 1.41 (*n = 6*)
VTG (mRNA) - Bil					911.6 ± 706.2 (*n = 4*)	5.4 ± 2.61 (*n = 4*)	0.7 ± 0.30 (*n = 3*)
ERα (mRNA) - PN					1.1 ± 0.24	1.2 ± 0.30	1.4 ± 0.50
ERα (mRNA) - Bil					1.4 ± 0.37 (*n = 4*)	0.7 ± 0.40 (*n = 4*)	3.2 ± 0.60 (*n = 3*)

n = 6 of total number of individuals analyzed/species/reservoir exceped for expression genes data (shown in table). ^{abc} Means statistically different between females and males in the same reservoir.* Significant differences at 95% confidence limit between the reservoirs in the same season (ANOVA, $P < 0.05$). PN – Ponte Nova; Bil, Billings; VTG (mRNA) – Vitellogenin expression; ERα (mRNA) – Estradiol Receptor expression, Sum/2012- Summer of 2012; Fal/12 – Fall of 2012; Win/12 – Winter of 2012; Spr/2012 – Spring of 2012; Win/13 – Winter of 2013; Spr/2013 – Spring of 2013; Sum/14 – Relative genic expression Summer of 2014. Summer of 2012 and Fall of 2012 were not analyzed due absence of sample.

estimated.

2.7. Statistical analysis

Data were expressed as mean ± SEM (standard error of the mean). Comparisons of animals between both environmental sites sampled throughout the year were performed by two-way analysis of variance (ANOVA), followed by the Holm-Sidak multiple comparison test. The significance level adopted was 95% ($P \leq 0.05$). Statistical analyses were performed using the software SigmaStat for Windows, version 3.5 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Gene expression of VTG-A and ERα

Gene expression of VTG-A showed a significant increase during Sum/14 in females from Bil reservoir compared to those sampled during the other seasons from PN reservoir ($P < 0.001$; Table 3). This difference was not observed in case of ERα gene expression. Males from Bil showed a very large inter individual variability of gene expression of ERα ($P = 0.933$) and VTG-A ($P = 0.258$) although no statistically differences were observed (Table 3).

3.2. E2 concentration

Concentration of plasma E2 did not vary between females throughout the different seasons sampled ($P = 0.173$ and $P = 0.357$, respectively; Table 3) nor between the PN and Bil reservoirs ($P = 0.134$). In contrast, males in the Bil reservoir presented a higher plasma E2 concentration during Spr/13 ($P < 0.001$) compared to males from PN ($P < 0.001$; Table 3).

3.3. Gonadal histology

Histological analysis showed that ovaries, from both PN and Bil reservoirs, were in an advanced stage of maturity. In relation to the stage of oocytes development, there was a higher frequency of vitellogenic oocytes, followed by perinucleolar oocytes and cortical alveoli oocytes in females from both reservoirs throughout the year (Table 4). Atretic oocytes were only observed in Spr/12, Spr/13 and Sum/14 in females from the PN reservoir and in Sum/12 in animals from the Bil reservoir (Table 4). Histological analysis of the ovaries of females from both reservoirs allowed the identification of oogonia (Fig. 1A), cortical alveoli oocytes (Fig. 1B), perinucleolar oocytes (Fig. 1C), vitellogenic oocytes with a central nucleus (Fig. 1D), vitellogenic oocytes with nuclear migration (Fig. 1E) and atretic oocytes (Fig. 1F).

3.4. GSI and HSI

The GSI of females collected in the PN reservoir were higher in Spr/12 and Sum/12 than in Fal/12, Fal/13, Win/12 and Win/13 ($P < 0.001$, Table 3). There was no statistical difference for females collected in the Bil reservoir throughout the seasons (Table 3). Related to the sampling sites, higher values of GSI were observed for females from Bil reservoir during Win/12, Win/13 and Sum/14 ($P < 0.001$, Table 3). No significant statistical differences were observed for HSI values between sampling sites and seasons (Table 3).

3.5. Fecundity and oocyte diameter

Females from the Bil reservoir during Spr/13 showed the highest AF, except for Win/13 ($P = 0.030$; Table 3). No differences were found between seasons for females collected at the PN reservoir (Table 3). In relation to the reservoirs, females from Bil exhibited higher AF and RF values than those collected at PN during Win/13 and Spr/13 ($P = 0.05$ and $P = 0.010$, respectively) (Table 3). The percentage of RO and OV did not show any statistical differences between females collected in either site, neither throughout the year (Table 3). Finally, females in both sampling sites and throughout the year showed a higher diameter frequency of OR and lower of OV (Fig. 2).

4. Discussion

In the present study, the reproductive physiology of *A. fasciatus* was evaluated using biomarkers related to the gonadal maturation process to determine the effects of diminished water quality by anthropogenic impact of a major urban reservoir in Brazil. Molecular biomarkers, such as ERα and VTG-A gene expression, biochemical parameters, such as plasma concentration of E2, and morphological characteristics, such as gonadal morphology and oocyte recruitment dynamics (AF, RF and diameter) generated differing responses, evidence that fish possess diverse regulatory abilities, even in adverse environmental conditions. In addition, the physiological responses of males and females of *A. fasciatus* occurred at specific moments, which may be a consequence of the seasonal variation in the presence of contaminants in the Bil reservoir and/or their mode of action (MoA), defined as the series of key processes that begin with the interaction of a contaminant with a target site (e.g. receptor) and proceeds through operational and anatomical changes in an organism that result in sub lethal or lethal effects (USEPA, 2000; Beyer et al., 2014).

Gene expression of VTG-A, biomarker of estrogen exposure, showed alterations in females at distinct seasons. In females, during Sum/14 the mean value of gene expression was well above that of others seasons sampled. This suggests environmental interference and the presence of estrogenic compounds in Bil at that time. A similar outcome with this biomarker was found by Cocci et al. (2017), who studied Gilthead seabream (*Sparus aurata*) hepatocytes exposed to seawater extracts,

Table 4
Classification of ovarian maturation stage based on oocyte development of *A. fasciatus* females.

	PN					Bil					Maturation Stage
	PNO	COA	VO	AO	POF	PNO	COA	VO	AO	POF	
Females											
Sum/12	+ +	+	+ + +	—	—	+ +	+	+ + +	—	+	Advanced
Fal/12	+ +	+	+ + +	–	–	+ +	+	+ + +	–	–	Advanced
Win/12	+ +	+	+ + +	–	–	+ +	+	+ + +	–	–	Advanced
Spr/12	+ +	+	+ + +	+	–	+ +	+	+ + +	–	–	Advanced
Win/13	+ +	+	+ + +	–	–	+ +	+	+ + +	–	–	Advanced
Spr/13	+ +	+	+ + +	+	–	+ +	+	+ + +	–	–	Advanced
Sum/14	+ +	+	+ + +	+	–	+ +	+	+ + +	–	–	Advanced

PNO: perinucleolar oocyte; COA: cortical alveoli oocyte; VO: vitellogenic oocyte; AO: atretic oocyte; POF: postovulatory follicles; +: current; + +: frequent; + + +: predominant;: absent.

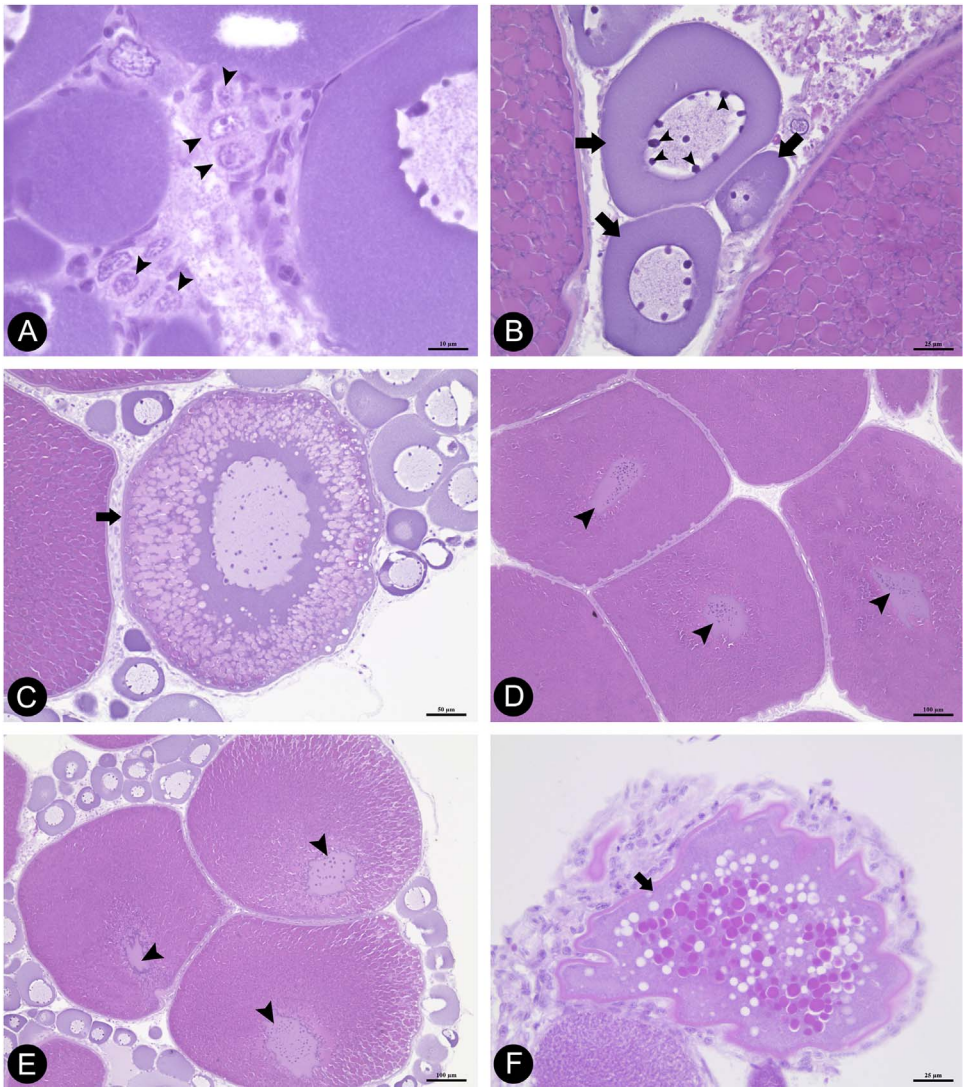

Fig. 1. Stages of oocyte maturation of *Astyanax fasciatus*. A) oogonia (arrowhead), small cells that develop in nests (delimited by arrows' heads); B) Perinuclear oocytes (arrows) with several nucleoli (arrowhead); C) Cortical alveoli oocytes (arrows); D) Vitellogenic oocytes with a central nucleus (arrowhead); E) Vitellogenic oocytes with peripheral nucleus (arrowhead); F) Atretic oocyte exhibiting cellular disorganization (delimited by arrows). Stain: Periodic-Acid-Schiff (PAS)/Weigert's Haematoxylin/Metanil Yellow. Bar: 10 μm (A), 25 μm (B, F), 50 μm (C); 100 μm (D, E).

showing a correlation between presence of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) in seawater and VTG gene expression. Although females seem to adjust their cycle to different environments, the fact that males exhibit evidences of VTG gene expression and high levels of E2 (Win/12–13), clearly reflect the presence of xenobiotics, acting as estrogens, in Bil reservoir, reinforcing the notion that the presence of EDCs in the Bil reservoir can be interfering with reproductive processes of *A. fasciatus*.

These EDCs may also explain the results of AF and RF in females from this reservoir, which were higher in Win/13 and Spr/13 in relation to the other seasons of the year and to females from the PN reservoir. The diverse alterations for different parameters at different sampling seasons (E2 concentration, AF, RF and VTG-A mRNA in females) may be the result of a complex mix of contaminants in the environment. A study with female zebrafish (*Danio rerio*) evaluated the interaction of contaminants in a complex mixture, and showed several alterations with exposure to a single dose of 17a-ethinylestradiol (EE2), with changes of steroid acute regulatory protein (StAR), 17b-hydroxysteroid dehydrogenase type 1 (17b-HSD1) and aromatase (cyp19a1), whereas exposure to a mixture of eleven estrogenic compounds previously quantified at the Douro River estuary (Portugal), only caused differences at cyp19a1 (Urbatzka et al., 2012). Chemical contaminants, whether with similar or different MoA, may influence the toxicity of another compound, resulting in interactive effects, such as additive, synergistic or antagonistic (Beyer et al., 2014). An efficient way of assessing the effects of mixtures is the weight-of-evidence approach (Sanchez and Porcher, 2009), which proposes the need for multiple studies, both in the field and in the laboratory, in order to understand more precisely how these compounds are capable of affecting the biota.

The annual variation pattern of GSI from females sampled in the PN reservoir was different from that observed in the Bill reservoir animals. Prado et al. (2011) studied *A. fasciatus* in five distinct reservoirs in the South eastern region of Brazil (one of them considered uncontaminated and the others contaminated) and showed that females at all reservoirs presented lower GSI values in July (winter) and higher in October (spring). The results of the present study for the PN reservoir are in agreement with these data and as discussed by these authors, variables other than the presence of contaminants can influence the GSI, such as food availability, genetic profile, abundance of predators and environmental status. Furthermore, the pattern of GSI seasonal change may be related to the life history of the population in a particular reservoir, taking into account the reproductive plasticity already reported for this species (Silva et al., 2010). Both GSI and ovarian histology are widely used as biomarkers in studies investigating the role of environmental variables on the oocyte maturation process, together with the synthesis of vitellogenin and the presence of intersex individuals, both in the natural environment and in the laboratory (Bjerregaard et al., 2006; Jobling et al., 2006; Harris et al., 2011; Lange et al., 2011; Tetreault et al., 2011; Bahamonde et al., 2014). In this study, no change was observed in gonadal histomorphology, even when alterations were

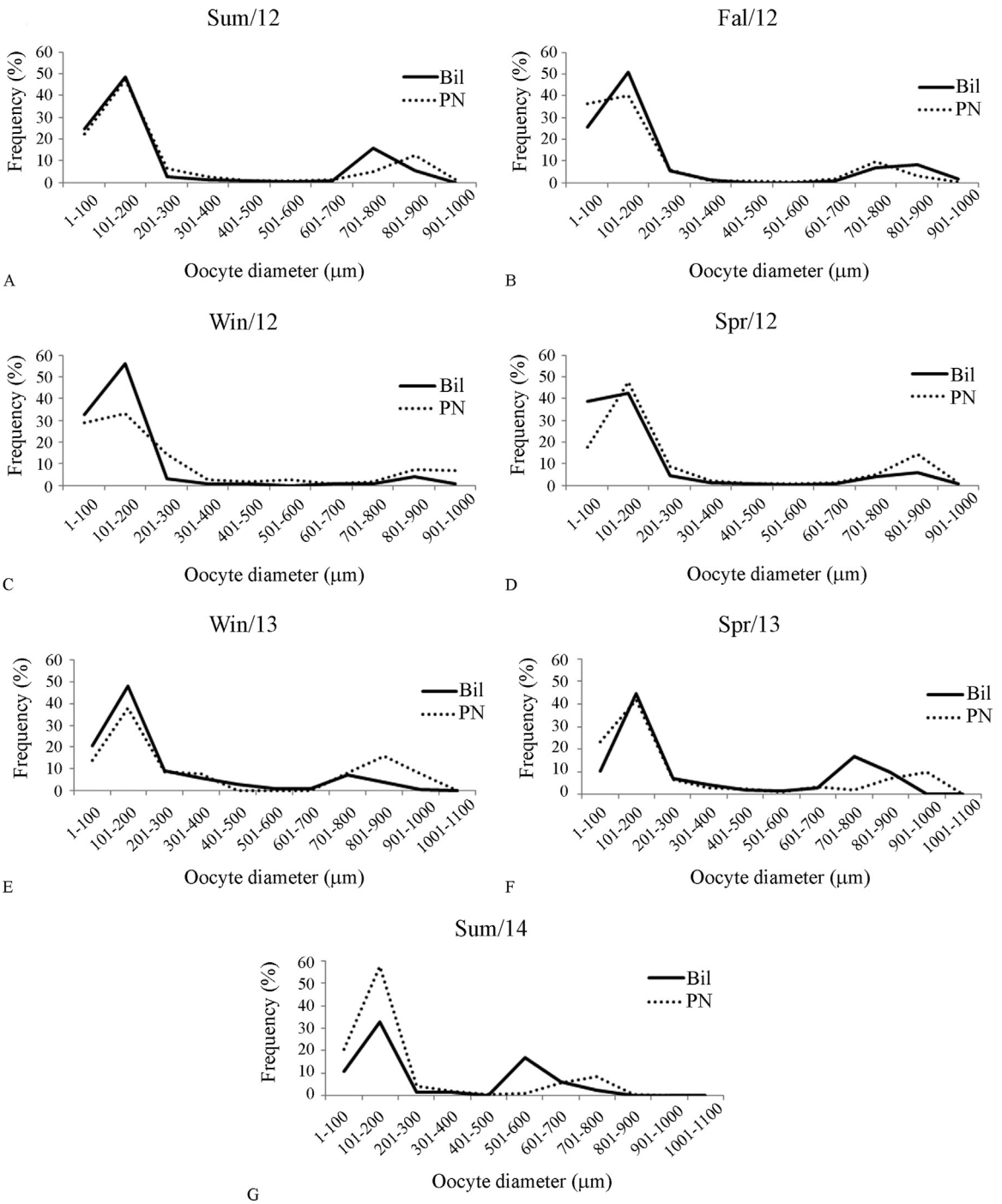

Fig. 2. Percentage of oocyte diameter (frequency) of females of *Astyanax fasciatus* collected in 2012 in the Ponte Nova (dotted line) and Billings (solid line) reservoir. A) Females collected in the summer of 2012. B) Females collected in the fall of 2012. C) Females collected during the winter of 2012. D) Females collected in spring 2012. E) Females collected in the winter 2013. F) Females collected in the spring of 2013. G) Females collected in the summer of 2014. PN: Ponte Nova reservoir; Bil: Billings reservoir; Sum/12 - Summer 2012; Fal/12 - Fall 2012; Win/12 - Winter 2012; Spr/12 - Spring 2012, Win/13 - Winter 2013; Spr/2013 - Spring 2013; Sum/14 - Summer 2014.

found at other biomarkers of exposure at different levels of organization, associated with physiological, genetic and/or biochemical mechanisms. These results point to regulatory mechanisms that minimize the effect of the observed changes in these biomarkers at the histomorphological level.

Taken together, the results found in this study suggest that EDCs present in the Bil reservoir mainly influenced those biomarkers of biological effects, mainly regarding the results found in males. Dang (2016), reviewing several studies with EDCs on biomarkers of exposure and effect, such as vitellogenin concentration, secondary sex characteristics and sex ratio in *P. promelas*, *Oryzias latipes* and *D. rerio*, mentions that changes in biomarkers result of a multifactorial sequence of complex processes that may be influenced by chemical, non-chemical, isolated biological factors and other interactions. This assertion can be corroborated in this study, because after data collection during a long period, the animals exhibited alterations at specific moments in biomarkers of exposure, possibly caused by different contaminants and/or MoA. As stated by Cortes et al. (2016), this study reinforces the importance of including different biomarkers for assessing risk analysis. In addition, we believe that the continued reassessment of these risk

analyses should be considered, especially in areas susceptible to varying environmental or anthropogenic contamination, such as locations close to urban centers.

5. Conclusion

The use of different biomarkers allowed not only to describe the alterations on the physiological processes involved in reproduction in *A. fasciatus* using different biomarkers at various levels of organization, but also to confirm the presence and effects of EDC in a natural environment with heavily anthropogenic influence. The conditions found in the water from the Bil reservoir interfered on biomarkers of biological effects (plasma concentration of E2, AF and RF) in *A. fasciatus*. Additionally, presence of biomarkers of exposure (VTG-A gene expression in males) supports the hypothesis that EDCs present in this water body are interfering with estrogenic activity in *A. fasciatus*. On the other hand, the histomorphological data and frequency of ovarian maturation stages indicate that there was not damage to the process of oocyte maturation process and how it may reflect in the population dynamics. The next step is to study deeply the origin, nature and mechanisms of the alterations produced by pollutants of emerging concern and, secondly, to provide solid evidence for incorporation into national regulatory regulations.

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