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# Reproductive biomarkers responses induced by xenoestrogens in the characid fish *Astyanax fasciatus* inhabiting a South American reservoir: An integrated field and laboratory approach

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ABSTRACT

Field studies evaluating the effects of endocrine disruption chemicals (EDCs) on the fish reproduction are scarce worldwide. The goal of this study was to assess hepatic levels of vitellogenin (Vtg), *zona radiata* proteins (*Zr*p) and insulin-like growth factors (IGF-I and IGF-II), and relating them to reproductive endpoints in a wild fish population habiting a reservoir that receive domestic sewage, agricultural and industrial residues. Adult fish *Astyanax fasciatus* were sampled during the reproductive season in five sites from the Furnas Reservoir, Grande River, and Paraguay–Paraná basin. As a control to field data, fish were experimentally exposed via dietary intake, to oestradiol benzoate (OB) for 7 days. Fish from site with little anthropogenic interference showed hepatic levels of Vtg, *Zr*p and IGF-I and IGF-II similar to those from the non-treated experimental group. In sites located immediately downstream from the municipal wastewater discharges, the water total oestrogen was $>120$ ng/l, and male fish displayed increased Vtg and *Zr*p and decreased IGF-I levels similar to OB treated fish. In females, levels of Vtg, *Zr*p, IGF-I and IGF-II suggest an impairment of final oocyte maturation and spawning, as also detected by frequency of over-ripening, follicular atresia and fecundity. At the sites that receive agricultural and industrial residues, the water total oestrogen was $<50$ ng/l and females showed decreased *Zr*p and increased IGF-II levels associated to reduced diameter of vitellogenic follicles, indicating an inhibition of oocyte growth. Overall, the current study reports oestrogenic contamination impairing the reproduction of a wild fish from a hydroeletric reservoir and, the data contribute to improving the current knowledge on relationship between hepatic Vtg, *Zr*p and IGF-I and IGF-II, and reproductive endpoints in a teleost fish. In addition, our data point out novel reproductive biomarkers (IGF-I, IGF-II and over-ripening) to assessing xenoestrogenic contamination in freshwater ecosystems.



## 1. Introduction

Over recent decades, there is growing interest in evaluating the adverse effects of endocrine disrupting chemicals (EDCs) in aquatic environments. EDCs are exogenous compounds able to disrupt the activity of the endocrine system responsible for homoeostasis, reproduction, and development of organisms (Diamanti-Kandarakis et al., 2009). Among EDCs, xenoestrogens receive more attention due to their ability to mimic natural oestrogens, binding to oestrogen receptors, which have relatively low specificity (WHO/IPCS, 2002). Municipal wastewater effluents are considered the main source of oestrogens in aquatic ecosystems since the consumption of oestrogens and progestrogens in human medicine and animal farming has been progressively increasing (Ying et al., 2002). Oestrogen concentrations as low as 10–100 ng/l in water can adversely disrupt the endocrine system, affecting the reproductive biology and the long-term sustainability of fish populations (Hanselman et al., 2003; Kidd et al., 2007).

Understanding the effects of endocrine disrupting chemicals on aquatic organisms is a challenge since they are continuously exposed to increasing amounts of a mixture of contaminants in their natural habitat (Allen and Moore, 2004). A range of reproductive parameters has been reported as endpoints of xenoestrogen exposure in fish gonads, i.e. intersex, gonadal growth, oocyte production, fertility and follicular atresia (Jobling et al., 2002; Arukwe and Goksøyr, 2003; Robinson et al., 2003, Mills and Chichester, 2005; Hutchinson et al., 2006; Tetreault et al., 2011).

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In addition, cellular and molecular biomarkers are essential to study the basic mechanisms of the action of EDCs (Denslow and Sepúlveda, 2007, Arukwe and Røe 2008). Vitellogenin (Vtg) and zona radiata proteins (*Zr*p) are typically synthesised in the liver of females under the regulation of endogenous 17β-oestradiol (E2), transported via the bloodstream to the ovary and taken up into maturing oocytes (Arukwe and Goksøyr, 2003). Usually, Vtg and *Zr*p concentrations are very low or absent in male and juvenile fish, but can be induced upon xenoestrogen exposure, so they are sensitive biomarkers of oestrogenic EDCs contamination in the aquatic environment (Arukwe and Goksøyr, 2003; Genovese et al., 2011, Truman and van den Hurk, 2012).

Recent studies have demonstrated that growth hormone (GH) – insulin-like growth factor (IGF) – gonad interaction may be an alternative axis in fish reproduction that acts in concert with the hypothalamus – pituitary – gonad pathway (Huang and Weng, 2010). In several teleosts, IGF-I as well as IGF-II may induce oocyte maturation, each possibly having a species-specific role (Mukherjee et al., 2006; Lokman et al., 2007; Weber et al., 2007; Reinecke, 2010b). In addition, IGF-I can stimulate steroid biosynthesis during previtellogenic oocyte growth (Campbell et al., 2006; Lokman et al., 2007). Both in vivo and in vitro studies provide early evidences of an interaction between EDCs and IGF-I (Reinecke, 2010a). In particular, E2 appears to be able to down-regulate hepatic IGF-I expression at environmentally relevant concentrations, but environmental oestrogen effects on the IGF-II system still remains to be clarified (Shved et al., 2008; Reinecke, 2010b).

In South America, some native fish have been utilised as bioindicator species for evaluating the environmental pollution in field or experimental assays (Cazenave et al., 2009; Leonardi et al., 2011; Fernandes et al., 2013). Among them, we highlight the lambari *Astyanax fasciatus* from the Characidae family, a species widely distributed in the La Plata River basin, abundant in several Brazilian ecosystems, and that supports the fishing at the Furnas Reservoir, Grande River, and Paraguay–Paraná basin. This small characid species displays a non-migratory behaviour, omnivorus feeding habit, prolonged reproductive season, multiple spawnings through the year and first gonadal maturity at about 6.5 cm standard length (Carvalho et al., 2009). Although some reports have indicated this lambari as an appropriate species for ecotoxicological studies in South America (Schulz and Martins-Júnior, 2001; Alberto et al., 2005; Carrasco-Letelier et al., 2006; Prado et al., 2011), studies focused on the reproductive molecular responses are missing.

Although the IGF system is involved in the fish reproductive physiology, reports on IGF-I and IGF-II levels in environments contaminated by EDC's are scarce. The goal of this study was to assess the effects of xenoestrogenic contamination on hepatic levels of Vtg, *Zr*p and IGF-I and IGF-II, and relate them to the reproductive endpoints in *A. fasciatus* from the Furnas Reservoir.

## 2. Material and methods

### 2.1. Study area

The Furnas Reservoir (Fig. 1) is a complex ecosystem with $22 \times 10^9$ m$^3$ of water and 1440 km$^2$ of flooded total area, distributed in two major regions related to the Grande River and Sapucaí River, each measuring about 250 km. This reservoir situated on the Upper Paraná River, south-eastern Brazil, supplies water to a local population of over 1 million people and receives more than 2000 t of waste solids daily from the surrounding cities. Untreated municipal and industrial sewage and pollution by pesticides has gradually reduced the water quality, requiring special attention from Brazilian public institutions for conservation of fish fauna, which is dominated by small and medium-sized species (Heleno, 2004; IGAM, 2008; Sadauskas-Henrique et al., 2011).

### 2.2. Field collection

All procedures performed during fish collection followed the ethical principles established by the Brazilian College of Animal Experimentation (COBEA) and the study was approved by the Ethics Committee for Animal Experimentation (CETEA) of the Federal University of Minas Gerais, Brazil. A total of 278 adult specimens of *A. fasciatus* were caught during the reproductive season peak (November 2010 to January 2011) at five sites: Barranco Alto, BA (21°11′ S; 45°58′ W); Fama, F (21°24′ S; 45°50′ W); Boa Esperança, BE (21°04′ S; 45°33′ W); Guapé, G (20°45′ S; 45°55′ W) and Turvo, T (20°40′ S; 46°13′ W). The main environmental pollution sources in each site are indicated in Fig. 1. Fish were sampled using 100 m of gillnets with a 3–4 cm stretched mesh size deployed for about 14 h on the surface of the water and placed 30 m from the shoreline of the reservoir. The fish collection was supported by a team of professional fishermen from the Furnas Hydrobiology and Hatchery Station.

### 2.3. Physic-chemical parameters and total oestrogen in the water

During the fish collections, temperature, pH, dissolved oxygen and conductivity at 25 °C (specific conductance) were measured in each sampling site at 1 m depth from the water's surface using an YSI 650 MDS multi-parameter instrument (Table 1). Total phosphorus was determined in 1 l water samples processed at the laboratory of Furnas Hydrobiology and Hatchery Station by the colorimetric method (APHA, 1999).

Water samples (0.5 l from each site) were stored in plastic bottles and transported in ice for the analysis of total oestrogen (oestrone, E1; 17β-oestradiol, E2 and oestriol, E3) (Table 1) by applying the colorimetric test (ELISA kit Ecologiena, JapanEnviroChemicals, Ltd.). In the laboratory, the samples were filtered by glass fibre filters with 1 μm pore size and, then 5 ml of methanol (MeOH) was added to elute the organic part of the pellet. The test was performed on duplicate samples following the manufacturer's recommendations. The competitive-ELISA coefficient of variation was up to 10% and the quantitative analysis ranged from 0.05 to 3.0 μg/l. For all samples, duplicate measurements were made at 450 nm using Versa Max Microplate Reader (Molecular Devices, USA).

### 2.4. Biological indices and fecundity

Live specimens were kept in plastic containers with water from the reservoir until dissection and samples collection. Total length (TL), body weight (BW), gonads weight (GW) and liver weight (LW) were obtained for determining the gonadosomatic index (GSI = 100 GW/BW), hepatosomatic index (HSI = 100 LW/BW) and Fulton condition factor ($K^1 = 100$ BW/TL$^3$). To eliminate the influence of the gonads and liver, $K^2$ was also calculated ($K^2 = 100$ BW − (GW + LW)/TL$^3$).

For histology, a transversal section of the middle region of the left gonad was obtained from each fish, then fixed in Bouin's fluid for 8–12 h and kept in 70% ethanol for posterior processing in the laboratory. The right ovary from 20 fish/site was fixed in 10% formalin for determining the fecundity in the laboratory. Liver samples ($n = 10$ fish/sex) from each site were frozen in liquid nitrogen and preserved at −80 °C for subsequent ELISA assays.

The batch fecundity was determined according to methodology established previously (Otobo, 1978). Ovaries fixed in 10% formalin were washed with water and dried on paper towel. Three samples of ~100 mg were obtained from the cranial, middle and tail ovarian regions. The vitellogenic oocytes were counted manually under a stereomicroscopy for determining the batch fecundity (BF = NO × GW/SW, where NO = total number of vitellogenic oocytes in the sample, GW = gonads weight and SW = sample weight). The batch fecundity relative to TL was also calculated in order to eliminate the interference of fish size (Arantes et al., 2010).

### 2.5. Experimental exposure to oestradiol benzoate (OB)

As control of the oestrogenic contamination in the Furnas Reservoir, a laboratory study was conducted in December 2010. For this, adult specimens of *A. fasciatus* were captured from the site Turvo ($n = 75$), then maintained for three months in clean water of the fish culture tanks at the Furnas Hydrobiology and Hatchery Station, as a recovery time. Following, they were transported to the laboratory, separated at random into OB treated and non-treated groups at a density of 1.2 g of live fish/l of water, and were acclimatised over three days in aquaria with oxygenator, thermostat, mechanical, biological filters and a controlled photoperiod (12 h light:12 h dark) and temperature (27–28 °C). During the experiment, the treated group (positive control) was fed twice a day ad libitum with commercial diet (Tetra Tropical granules, Tetra GmbH, Herrenteich, Germany) containing Oestradiol Benzoate (OB, Tecnopec Brazil) dissolved in anhydrous alcohol, at a final concentration of 20 μg/g OB. Fish from the non-treated group (negative control) were fed with a commercial diet with ethanol only. After 7 days of the experiment, the fish were killed by trasnversal section of spinal cord. For each specimen, the following data were obtained: TL, BW, GW and LW for determining the GSI, HSI, $K^1$ and $K^2$. A transversal section of the middle region of the left gonad was obtained for histology. Liver samples ($n = 10$/sex/group) were also frozen in liquid nitrogen and