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The influence of seasonality, fish size and reproductive status on EROD activity in *Plagioscion squamosissimus*: Implications for biomonitoring of tropical/subtropical reservoirs

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ABSTRACT

Ethoxyresorufin-*O*-deethylase (EROD) activity is considered an important biomarker for aquatic environmental contamination. Although EROD activity has been widely used as a biomarker of exposure to polycyclic aromatic hydrocarbons in fish, this activity can be influenced in the field by spatial-, seasonal- or individual-related factors. We therefore performed a comparative study of hepatic EROD activity levels in the croaker *Plagioscion squamosissimus* to determine whether variations existed in enzyme activity levels, especially in relationship to reproductive status, fish size, age, and seasonality. For this purpose, we collected fish from three reservoirs with different pollution levels during the early-rainy (November 2012), rainy (March 2013), and dry (July–August 2013) seasons from the Tietê River, Brazil. We tested whether size, age and sex affected EROD activity among the localities and seasons. We found a marked effect of pollution during the dry season on variation among the localities in EROD activity in *P. squamosissimus*. An analysis of covariance indicated that sex had a significant negative effect on the seasonal variability of the EROD activity levels at the most polluted locality (near São Paulo). A possible explanation for the statistical association between EROD activity and sex is that the reproductive status of the females influenced the EROD activity levels. A possible explanation for the statistical association between EROD activity and sex is that the reproductive status of the females influenced the EROD activity levels, also largely reported in the literature. Our results suggest that reproductive status can be a significant confounding factor for determining EROD activity in female *P. squamosissimus* in freshwater ecosystems when compared to males. Moreover, our results suggest that the presence of phenanthrene during the dry season at Barra Bonita reservoir might explain the highest EROD activity responses in this period of study.

1. Introduction

Pollution biomarkers in fish have been widely used to assess chemical contamination in the aquatic environment (Whyte et al., 2000; van der Oost et al., 2003; Amiard-Triquet et al., 2013). Over the past few years, a significant increase in the number of studies using this type of biochemical endpoint has been observed (Trídico et al., 2010; Nahrgang et al., 2013; Danion et al., 2014), and ethoxyresorufin-*O*-deethylase (EROD) activity is one of the most widely used biomarkers of exposure to aquatic contaminants (van der Oost et al., 2003; Roméo and Giambérini, 2013; Santos et al., 2014). EROD activity is a useful tool for detecting the early signs of contamination by organic compounds, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Goksøyr and Förlin, 1992; Roméo and Giambérini, 2013; Ratia and Oikari, 2014), and has been standardized as a core

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biomarker in several countries of North America, Europe, and Asia (Pampanin et al., 2006; Collier et al., 2013). Although EROD activity has been frequently used as a biomarker of exposure in fish, this activity can be influenced in the field by spatial- (Barhoumi et al., 2012), seasonal- (Gorbi et al., 2005; Nahrgang et al., 2010) and individual-related factors (e.g., gender, size, age, and reproductive status) (Whyte et al., 2000; van der Oost et al., 2003; Gorbi et al., 2005; Koenig and Solé, 2012).

Previous studies have demonstrated that the differences between males and females are among the most important individual-related factors affecting fish EROD activity (Whyte et al., 2000), with downregulation occurring in females during the period of reproductive activity (Koenig and Solé, 2012; Chiang et al., 2012). Briefly, this downregulation has been observed in spawning females from different species (Whyte et al., 2000), in which EROD activity levels gradually decline toward the onset of ovulation and rise again during the postspawning period (van der Oost et al., 2003; Elskus, 2004).

Seasonality can also affect the EROD activity responses due to environmental variability (e.g., pH, dissolved oxygen (DO), temperature), influencing the enzymatic results obtained using this biomarker (Stegeman et al., 1992; Chiang et al., 2012; Nahrgang et al., 2013). These confounding factors (i.e., seasonality and individual-related factors) can sometimes mask the effects of contaminant-induced stress signals and prevent the correct interpretation of biomarker results (Sheehan and Power, 1999; Gagnon and Hodson, 2012).

Hence, when using biomarkers, it is important to distinguish between normal fluctuations of biological responses and fluctuations attributable to chemical stress induced by pollutants (Amiard-Triquet et al., 2013). Few studies have considered these confounding factors, analyzing EROD activity in males and females separately (Koenig and Solé, 2012) or using only sexually immature fish (Barhoumi et al., 2012). Thus, to avoid data misinterpretation and the underestimation of environmental pollutants, the relative contributions of seasonality and individual-related factors (e.g., size and reproductive status) must be determined, as all of these factors can potentially confound the EROD activity responses of the fish (Whyte et al., 2000; Gagnon and Hodson, 2012).

Over the past few decades, the Tietê River has been seriously altered by anthropogenic contamination caused by chemical industries and a population of nearly 20 million people in the metropolitan region of São Paulo (Mortatti et al., 2011; CETESB, 2013). Although the water quality has progressively decreased due to runoff from the metropolitan region of São Paulo, the non-native fish species are still highly abundant and possesses a certain tolerance to environmental stressors, both natural and human induced, compared with that of native species (Fedorenkova et al., 2013). The South American silver croaker, *Plagioscion squamosissimus* (Heckel, 1840) is a sedentary fish and native to the Amazonian region (Reis et al., 2003), that was introduced in the Paraná River basin in the 1960s by the Hydroelectric Company (Nomura, 1984 in Carnélos and Benedito, 2002). Currently, this species is widespread in the Tietê River basin (Moretto et al., 2008), and is considered a valuable resource for human consumption and recreational fishing (Barros et al., 2012). Because of its wide geographical distribution, abundance and tolerance to environmental stress in dammed river systems, the croaker *P. squamosissimus* is a good candidate as a freshwater sentinel organism, making it very useful in the design of studies involving aquatic biomarkers in the tropical/subtropical reservoirs.

Therefore, the aim of this study was to determine whether there are differences in the EROD activity levels in the croaker *P. squamosissimus* in the Tietê River, Brazil, with respect to seasonality, contaminant levels, size, sex, age, and reproductive status.

2. Materials and methods

2.1. Study area

The study site, the Tietê River Basin in southeast Brazil, has a total surface area of 71,988 km^2, 70% of which is affected by industrial and agricultural activities, primarily involving chemical industries and sugar cane culture (Barrella and Petrere, 2003; Mortatti et al., 2011; CETESB, 2012). Eight reservoirs have been built along the 1150-km length of the river. Sampling sites in the Tietê River Basin were selected based on their pollution levels (Fig. 1). We selected three reservoirs for this study, including two close to the metropolitan region of São Paulo (Barra Bonita and Bariri), with intense and moderate industrial activity, respectively, and one far from São Paulo (Promissão), which was used as the reference site. These reservoirs were chosen based on the "Sediment Quality Guidelines for the Protection of Aquatic Life" established by the Canadian Council of Ministers of the Environment and the Environmental Agency of the State of São Paulo (CETESB, 2012, 2013). These guidelines establish a criterion to classify water bodies based on the concentrations of chemical contaminants. This criterion is based on two threshold levels, one below which aquatic life is seldom affected (TEL = threshold effect level), and one above which effects on aquatic life are likely (PEL = probable effect level) (Burton, 2002). The Barra Bonita reservoir (20°31′S and 48°32′W) is close to the metropolitan area of São Paulo city (~200 km) and is the site of the region's most intense industrial activity. Barra Bonita is contaminated with polycyclic aromatic hydrocarbon (PAHs), polychlorinated biphenyls (PCBs), heavy metals, and pesticides (CETESB, 2012, 2013). The Bariri reservoir (22°09′S and 48°44′W) is located close to the Barra Bonita reservoir (~50 km), is the site of moderate industrial and agricultural activities (e.g., sugar cane culture) and is contaminated primarily by organochlorine pesticide residues (CETESB, 2012). The Promissão reservoir (21°19′S and 49°44′W) is located approximately 400 km west of São Paulo city and was chosen as the reference sampling site because recent studies have reported that this reservoir is still in a healthy state and is not contaminated (Rodgher et al., 2005; CETESB, 2012). Data on the physical-chemical characteristics, heavy metals, PAHs, PCBs and pesticides were extracted from a river monitoring database provided by the Environmental Agency of the State of São Paulo (CETESB, 2012, 2013) (see Tables 1 and 2).

2.2. Fish sampling

P. squamosissimus was collected from three sites at each reservoir (Fig. 1) in the early-rainy (November 2012), rainy (March 2013), and dry seasons (July and August 2013). The fish were caught using gillnets (3-cm to 14-cm mesh between opposite knots), hand nets (mesh sizes of 3, 5, and 8 mm), and hook-and-line using living prawns or small fishes as bait to minimize the potential bias caused by size selectivity. The sample size according to the sex/season/locality was in accordance with Gagnon and Hodson (2012), who estimated a minimum number of 4–12 fish/sex/season/site to detect a 3-fold induction of EROD activity at $\alpha = 0.05$. The fish were maintained live in tanks with aeration for less than 2 h until their arrival at the field laboratory, where they were sacrificed (by a sharp blow to the head), weighed (g), measured (standard length in cm) and dissected to obtain the livers for the determination of ethoxyresorufin-*O*-deethylase (EROD) activity. The livers were weighed (total wet weight in milligrams), snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis. Water physico-chemical parameters, including temperature (°C), pH, dissolved oxygen (mg/ml), conductivity (mS/cm), and total dissolved solids (g/L) were measured during fish collection

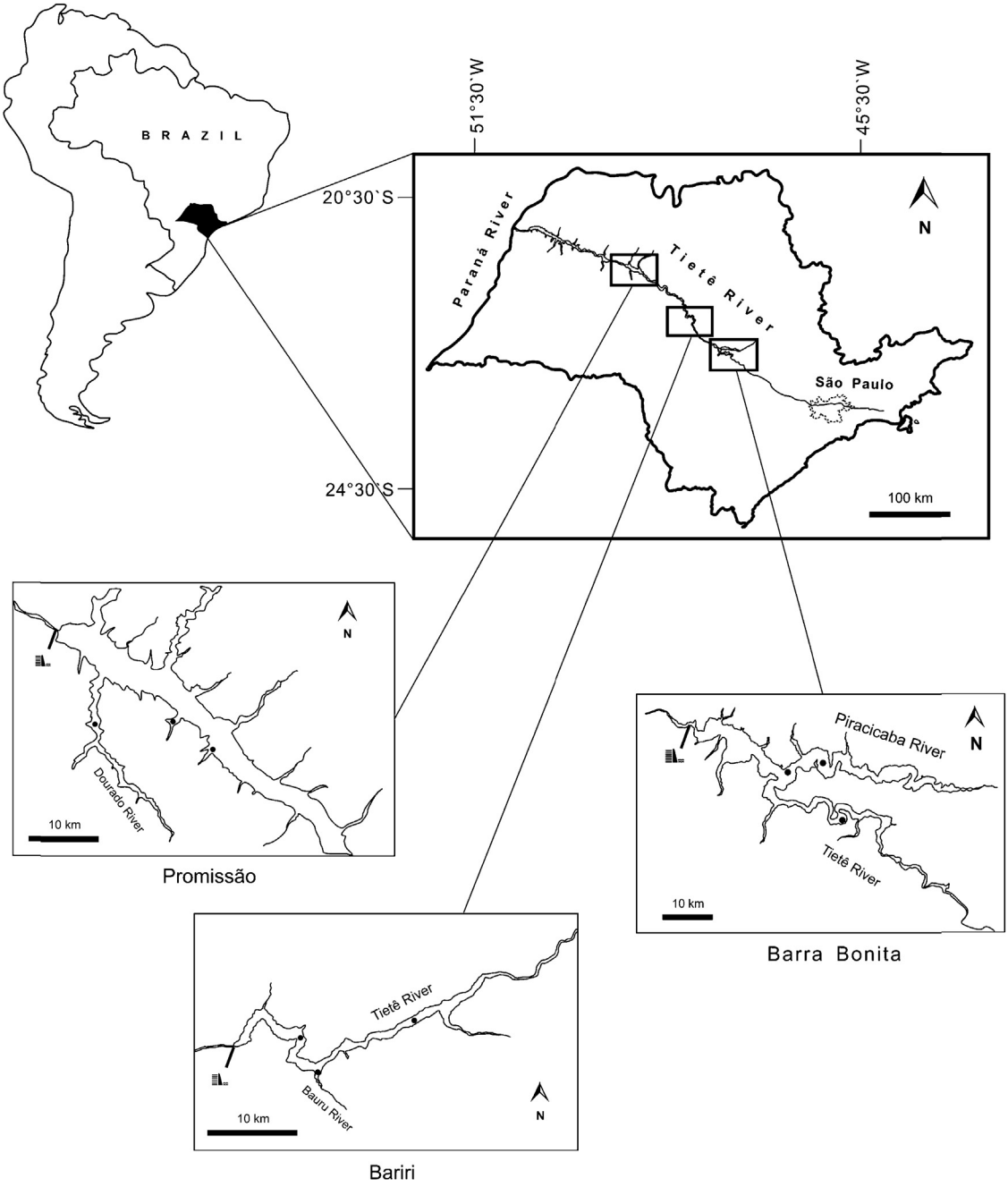

Fig. 1. Distribution of the sampling sites in the Tietê River, São Paulo, Brazil. Each square is a reservoir and black dots indicate the sampling sites in each reservoir.

using a multiparameter water quality meter (Horiba model U-50, Horiba Ltd., Kyoto, Japan).

2.3. Determination of maturity stages and hepatosomatic index

The stages of female maturity were determined macroscopically according to three stages of ovarian development and classified as immature (IMM), in maturation (IMA) and mature (MA) according to Vazzoler (1996) and Barbosa et al. (2012). These stages were defined visually by the characteristics of the gonads (i.e., color, transparency and vascularization) and by the presence and diameter of oocytes at the different maturity stages (for more detail, see Barbosa et al., 2012). The hepatosomatic index (HSI) was calculated according to the equation HSI = (LW/TW) × 100, where LW (g) is the liver weight and TW (g) is the total fish weight. We were not able to collect otoliths. As an alternative, we used fish size (=standard length) as a proxy of fish age based on the method proposed by Braga (1998) for *P. squamosissimus* from Barra Bonita reservoir. Age classes 1, 2, 3, and 4 years corresponded to average SL of 23, 33, 39 and 45 cm for females and 21, 27, 31 and 37 cm for males respectively.

2.4. EROD activity analysis

EROD activity was assessed in the post-mitochondrial fraction (S9) of the liver samples. To obtain the S9 fraction, the frozen liver samples were homogenized using a Polytron homogenizer (Ultra-Turrax, IKA, USA) at 4 °C to prevent a loss of enzymatic activity prior to the measurement. Homogenization was performed in a buffer at pH 7.8 containing 50 mM TRIS, 1 mM EDTA, 250 mM sucrose, and

Table 1
Seasonal water physical-chemical parameters in the three sites evaluated in the Tietê River. Mean ± standard deviation.

Parameter	CONAMA (WQI)	Barra Bonita			Bariri			Promissão		
		Early-rainy	Rainy	Dry	Early-rainy	Rainy	Dry	Early-rainy	Rainy	Dry
Temperature (°C)		26.9 ± 1.2	26.3 ± 1.1	21.5 ± 1.3	27.1 ± 1.1	29.5 ± 1.2	21.2 ± 1.4	28.7 ± 1.4	31.1 ± 0.8	24.1 ± 1.2
pH	6–9	6.9 ± 0.7	7.3 ± 1.1	7.6 ± 1.4	7.4 ± 0.8	6.7 ± 0.9	6.9 ± 0.5	8.2 ± 1.1	7.2 ± 0.9	7.9 ± 1.2
Dissolved oxygen (mg/L)	>5	6.2 ± 1.2	7.2 ± 0.7	7.6 ± 1.7	5.6 ± 0.9	4.1 ± 1.3*	6.1 ± 1.2	7.9 ± 1.3	7.8 ± 1.4	7.7 ± 0.7
Conductivity (mS/cm)		324 ± 18.5	315 ± 13.3	244 ± 9.8	304 ± 11.1	280 ± 8.7	252 ± 8.9	115 ± 7.1	220 ± 8.6	148 ± 6.7
Total dissolved solid (g/L)	<500	187 ± 6.8	168 ± 10.1	158 ± 9.1	179 ± 7.1	143 ± 8.1	166 ± 9.1	75 ± 6.3	111 ± 5.3	134 ± 7.8
DBO (mg/L)	<5	3	2	4	6*	10*	13*	4	3	<2
Turbidity (NTU)	<100	9	4.76	5.43	19	54	35	6.1	5.6	1.4
Al (mg/L)	<0.1	<0.1	n.a	n.a	0.19*	0.079	0.076	0.087	n.a	<0.05
Ba (mg/L)	<0.7	0.5	n.a	n.a	0.057	0.086	0.054	0.036	n.a	0.037
Cd (mg/L)	<0.001	<0.001	n.a	n.a	<0.002	<0.002	<0.002	<0.002	n.a	<0.002
Pb (mg/L)	<0.01	<0.01	n.a	n.a	<0.009	<0.009	<0.009	<0.009	n.a	<0.009
Cu (mg/L)	<0.009	<0.005	n.a	n.a	<0.005	<0.005	<0.005	<0.005	n.a	<0.005
Cr (mg/L)	<0.05	<0.05	n.a	n.a	<0.005	<0.005	<0.005	<0.005	n.a	<0.005
Fe (mg/L)	<0.3	<0.3	n.a	n.a	0.694*	0.311*	0.498*	0.169	n.a	0.074
P (mg/L)	<0.03	0.282*	0.01	0.1*	0.712*	0.315*	0.32*	<0.007	0.019	<0.02
Mn (mg/L)	<0.1	0.2*	n.a	n.a	0.087	0.1	0.103*	<0.03	n.a	<0.03
Hg (mg/L)	<0.0002	<0.0001	n.a	n.a	0.0002	<0.0002	<0.0002	<0.0002	n.a	<0.0002
Ni (mg/L)	<0.025	<0.02	n.a	n.a	<0.01	<0.01	<0.01	<0.01	n.a	<0.01
K (mg/L)		5	n.a	n.a	3.64	3.38	3.2	3.88	n.a	4.46
Na (mg/L)		24	n.a	n.a	23.2	20.1	17.6	17.9	n.a	14
Zn (mg/L)	<0.18	<0.1	n.a	n.a	0.006	0.016	0.014	<0.003	n.a	<0.003

WQI, water quality index based on Resolution nº 357/2005 of the Environment National Council (CONAMA) (Brasil, 2005); n.a, not available.
* Concentrations above the reference values.

20% glycerol. The homogenates were subsequently centrifuged at 9000 *g* for 30 min at 4 °C. Aliquots (1 ml) of the supernatant were transferred to two cryotubes and stored in liquid nitrogen prior to further biochemical analysis. EROD activity was assayed fluorometrically according to Burke et al. (1985), with modifications for 96-well plates using a microplate reader (Spectramax Gemini XS®, Molecular Devices Co., USA). Briefly, the samples of the S9 fraction were diluted to ~1.25 mg/ml in buffer (1 mM ethoxyresorufin diluted in 100 mM K_2HPO_4) and pipetted (10 μl) in triplicate into a 96-well microplate. To initiate the reaction, a regenerating system, which consisted of 0.25 mM b-NADP, 2.5 mM $MgCl_2$, 5 mM glycose-6-phosphate, and 0.5 units of glucose-6-phosphate dehydrogenase per milliliter of incubation mixture, was added. The transformation of ethoxyresorufin to resorufin was monitored at an excitation of 535 nm, and fluorescence emission was measured at 595 nm. The EROD activity levels were expressed as pmol of resorufin produced per minute per mg total protein in the S9 fraction. The protein assay was conducted according to Bradford (1976), using bovine serum albumin (BSA) as the standard, and the measurements were performed using a microplate reader at an absorbance of 595 nm.

2.5. Data analysis

The data were assessed for normality (Shapiro–Wilk test) and homogeneity of variance (Bartlett test). If the requirements of normality and homogeneity of variance were not met, nonparametric tests were used. An analysis of covariance (ANCOVA) was used to evaluate the effects of size and sex (covariates) on the EROD activity response among the localities and seasons. Prior to performing the ANCOVA test, the slopes of the interaction factors (size and sex) were assessed with a homogeneity test. A significant interaction effect indicated that the difference between the groups depended on the value of the covariate (Engqvist, 2005). This procedure is important for separating the effects of the differences among treatments from the differences due to covariates, such as size and sex, on the biomarker responses (Underwood, 1997; Engqvist, 2005; Koenig and Solé, 2012). Differences between males and females in size (=standard length), body weight, hepatosomatic index and EROD activity levels among the seasons were examined with a non-parametric Kruskal–Wallis test followed by a Dunn's post hoc test. We also used Mann–Whitney test to examine a possible influence of estimated age between males and females on EROD activity levels. The relationship between reproductive status and EROD activity level at each location/reservoir was determined using a Spearman's rank correlation (r_s) analysis. The statistical analyses were performed using Statistica software, version 7.1 (Statsoft, USA), and the graphs were plotted with R software (R Development Team; http://www.R-project.org). Differences were considered significant at $p < 0.05$, and the data were expressed as the mean ± standard deviation.

3. Results

3.1. Water quality assessment

The water physico-chemical parameters recorded at the three reservoirs are summarized in Table 1. The highest temperatures (above 26 ± 1.2 °C) were recorded during the early-rainy season and the rainy season at the three localities (Barra Bonita, Bariri and Promissão) together with the lowest dissolved oxygen values (below 7 mg/L), except for Promissão (DO above 7.7 mg/L). The water at Barra Bonita and Bariri also showed higher conductivity, total dissolved solid and DBO (biochemical oxygen demand) values than the water at Promissão reservoir (named reference site). Concentrations of metals such as Al, Fe, P and Mn were found at the two localities (Barra Bonita and Bariri) during the three seasons, compared to those found in Promissão (Table 1).

The contaminant data from sediments at the three reservoirs are summarized in Table 2. These data were obtained from the Environmental Agency of the State of São Paulo (CETESB, 2012, 2013). The sediment at Barra Bonita showed higher concentrations of metals (Cd, Cu, Cr, Ni and Zn), PAHs (phenanthrene and fluoranthene) and pesticides (DDT, Heptachlor, Heptachlor epoxide and Lindane) above to TEL (threshold effects level) in relation to the other reservoirs (Table 2). The water at Bariri and Promissão reservoirs showed some contaminants, but all with concentration below TEL index, indicating good conditions for these localities (Table 2). In September at Barra Bonita, all metals concentration increased in comparison to the other localities and seasons (Table 2). However, statistical analyses were not run for PAHs, PCBs and pesticides from

Table 2
Contaminant concentrations obtained from sediment in the three localities in the Tietê river in August 2012 and September 2013. This contaminant database was provided by the Environmental Protection Agency in São Paulo (CETESB, 2012, 2013).

Parameter	Sediment Quality Guidelines (SQGs)		Barra Bonita (BB)		Bariri (BA)		Promissão (PR)	
	TEL	PEL	Aug/2012	Sep/2013	Aug/2012	Sep/2013	Aug/2012	Sep/2013
Metals (mg/kg)								
Aluminum (Al)	–	–	56,948	66,079	6860	n.a	n.a	n.a
Arsenic (As)	5.9	17	4	<1	1	n.a	n.a	n.a
Cadmium (Cd)	0.6	3.5	<0.6	0.76	<0.6	n.a	n.a	n.a
Lead (Pb)	35	91.3	20	23.7	<10	n.a	n.a	n.a
Copper (Cu)	35.7	197	56.6	106	32	n.a	n.a	n.a
Chromium (Cr)	37.3	90	46.9	68.4	10.3	n.a	n.a	n.a
Iron (Fe)	–	–	37,094	52,490	15,570	n.a	n.a	n.a
Manganese (Mn)	–	–	426	609	246	n.a	n.a	n.a
Mercury (Hg)	0.17	0.486	0.06	0.12	<0.02	n.a	n.a	n.a
Nickel (Ni)	18	35.9	16.9	22.1	6.12	n.a	n.a	n.a
Zinc (Zn)	123	315	119	201	27	n.a	n.a	n.a
Polychlorinated biphenyls (μg/kg)	34.1	277			n.a	n.a	n.a	n.a
Congener 101			<2.5	<2.5	n.a	n.a	n.a	n.a
Congener 118			<2.5	<2.5	n.a	n.a	n.a	n.a
Congener 138			<2.5	<2.5	n.a	n.a	n.a	n.a
Congener 153			<2.5	<2.5	n.a	n.a	n.a	n.a
Congener 180			<2.5	<2.5	n.a	n.a	n.a	n.a
Congener 28			<2.5	<2.5	n.a	n.a	n.a	n.a
Congener 52			<2.5	<2.5	n.a	n.a	n.a	n.a
Polycyclic aromatic hydrocarbons (μg/kg)					n.a	n.a	n.a	n.a
Acenaphthene	6.71	88.9	n.a	<20	n.a	n.a	n.a	n.a
Anthracene	46.9	245	n.a	<20	n.a	n.a	n.a	n.a
Benzo[a]anthracene	31.7	385	n.a	<20	n.a	n.a	n.a	n.a
Benzo[a]pyrene	31.9	782	n.a	29	n.a	n.a	n.a	n.a
Benzo[b]fluoranthene	–	–	n.a	<20	n.a	n.a	n.a	n.a
Benzo(g,h,i)perylene	–	–	n.a	<80	n.a	n.a	n.a	n.a
Dibenz(a,h)anthracene	6.22	135	n.a	<30	n.a	n.a	n.a	n.a
Phenanthrene	41.9	515	n.a	44.1*	n.a	n.a	n.a	n.a
Fluoranthene	111	2355	n.a	119*	n.a	n.a	n.a	n.a
Fluorene	21.2	144	n.a	<20	n.a	n.a	n.a	n.a
Indeno[1,2,3-cd]pyrene	–	–	n.a	<80	n.a	n.a	n.a	n.a
Naphthalene	34.6	391	n.a	<30	n.a	n.a	n.a	n.a
Pyrene	53	875	n.a	<20	n.a	n.a	n.a	n.a
Pesticides (μg/kg)					n.a	n.a	n.a	n.a
Aldrin	–	–	<0.5	<0.5	n.a	n.a	n.a	n.a
DDT	1.19	4.77	<1.5	<1.5	n.a	n.a	n.a	n.a
Heptachlor	0.30	10	<1.25	<1.25	n.a	n.a	n.a	n.a
Heptachlor epoxide	0.60	2.74	<1.25	<1.25	n.a	n.a	n.a	n.a
Lindane	0.94	1.38	<1.25	<1.25	n.a	n.a	n.a	n.a

TEL, threshold effects level represents the concentration below which adverse effects on aquatic life are expected to occur only rarely; PEL, probable effects level represents the concentration above which adverse effects on aquatic life are frequently expected; n.a, not available.

* Concentrations above the reference values.

sediments due to the lack of data available necessary for investigating the relationship between the EROD activity levels and the contaminant levels.

3.2. Fish morphological parameters

The sample sizes, standard lengths, body weights, and estimated age for croakers of both sexes are given in Table 3. Among the seasons, males and females differed significantly in standard length (Kruskal–Wallis test, $H = 147.9$; df = 17; $p < 0.001$) and body weight (Kruskal–Wallis test, $H = 145.5$; df = 17; $p < 0.001$). The females were larger than the males (t-test, $t = 6.76$; $p < 0.001$; $n = 219$). The males differed in length and weight among the three seasons (Table 3), while the females differed in length ($F_{2,6} = 4.59$; $p = 0.029$) and weight ($H = 6.76$; df = 2; $p = 0.009$) among the seasons only at Bariri. The mean estimated age of fish among the seasons and localities varied from 1 to 3 for males and 1 to 4 for females (Table 3). Most of the fish were sexually mature during the early-rainy and rainy seasons rather than during the dry season. The highest numbers of mature females were found during the early-rainy season at all three localities (Table 4). The hepatosomatic index for both males and females differed significantly among the seasons (Kruskal–Wallis test, $H = 108.09$; df = 17; $p = 0.001$), with higher values observed during the dry season (Table 4).

3.3. Liver EROD activity

Comparisons were conducted for the males and females separately because the EROD activity levels varied between the sexes among the localities and seasons. The EROD activity levels among the seasons differed significantly, especially at Barra Bonita, for both sexes (Table 4; Fig. 2). The highest EROD activity levels for the males were detected during the early-rainy season (Barra Bonita and Bariri) and the dry season (Barra Bonita) (Table 4; Fig. 2A). The females showed the highest EROD activity level only during the dry season at Barra Bonita and showed the lowest activity level during the early-rainy season (Table 4; Fig. 2B). No relationship was found between age of males (Mann–Whitney U test = 1466; $n = 143$; $p = 0.91$) and females (Mann–Whitney U test = 756; $n = 76$; $p = 0.27$) of the croaker and EROD activity levels. There was a negative relationship between reproductive status and EROD activity only during the early-rainy season at Barra Bonita reservoir ($r_s = -0.916$; $p < 0.001$). We also observed a strong effect of seasonality on EROD activity at the most contaminated area (Barra Bonita) for the

Table 3
Morphometric measurements in males and females croaker *Plagioscion squamosissimus* from three sites in the Tietê River, Brazil. Values are shown as mean ± standard deviation. Different letters denote significant differences between seasons based on Dunn's test ($p < 0.05$).

Locality	Parameter	Early-rainy (Nov)		Rainy (Mar)		Dry (July–August)	
		Males	Females	Males	Females	Males	Females
Barra Bonita (BB)	Sample size	17	12	24	6	18	9
	Standard length (cm)	19.42 ± 1.85^{a}	19.77 ± 1.84^{a}	17.69 ± 1.10^{b}	20.87 ± 2.29^{a}	20.99 ± 2.36^{ac}	20.30 ± 2.05^{a}
	Range	16.2–23.5	17.1–22.5	14.2–19.9	18.5–23.4	16.4–24.5	16.6–23.2
	Body weight (g)	152.9 ± 43.4^{a}	164.3 ± 47.3^{a}	114.4 ± 22.2^{b}	183.2 ± 58.7^{a}	186.7 ± 57.2^{a}	170.6 ± 49.2^{a}
	Range	84.9–250.9	101.2–236.5	55.8–159.7	127.2–266.2	83.7–281.1	89.7–246.5
	Age (years)	2	2	2	2	2	2
	Range	1–2	1–3	1–2	1–3	1–3	1–3
Bariri (BA)	Sample size	16	5	13	5	16	7
	Standard length (cm)	15.65 ± 1.57^{a}	19.04 ± 2.78^{a}	15.65 ± 1.23^{a}	19.32 ± 2.27^{ab}	21.35 ± 3.28^{b}	22.56 ± 1.85^{b}
	Range	13.6–20.5	15.2–22.5	13.4–18.1	16.6–22.5	18.1–28.5	20.1–25.1
	Body weight (g)	74.1 ± 21.5^{a}	145.7 ± 67.6^{a}	74.5 ± 17.9^{a}	147.2 ± 52.1^{a}	195.5 ± 113.5^{b}	258.4 ± 63.6^{b}
	Range	47.1–128.9	63.6–233.1	46.9–110.1	87.5–220.6	13.4–440.9	180.3–340.4
	Age (years)	1	2	1	2	2	2
	Range	1–2	1–3	1–2	1–3	1–3	1–3
Promissão (PR)	Sample size	20	6	10	17	9	9
	Standard length (cm)	19.54 ± 1.62^{a}	24.11 ± 7.02^{a}	23.44 ± 2.16^{b}	25.88 ± 1.83^{a}	22.3 ± 2.22^{b}	25.44 ± 3.02^{a}
	Range	17.5–22.2	19.9–38.1	18.2–25.5	23.5–29.2	19.5–26.3	21.5–32.2
	Body weight (g)	148.8 ± 36.6^{a}	339.3 ± 377.6^{a}	253.7 ± 66.3^{b}	335.9 ± 67.1^{a}	226.1 ± 65.1^{b}	332.6 ± 115.7^{a}
	Range	104.3–218.3	150.4–1106	112.5–357.5	253.6–485.4	154.2–340.1	204.7–602.1
	Age (years)	2	2	2	3	2	3
	Range	1–3	1–4	1–3	1–3	1–3	1–3

Table 4
Measurement of reproductive status, hepatosomatic index and ethoxyresorufin-*O*-deethylase (EROD) activity in the croaker *Plagioscion squamosissimus* in Tietê River, Brazil. Values are shown as mean ± standard deviation. Different letters denote significant differences between seasons based on Dunn's test ($p < 0.05$).

Locality	Parameter	Early-rainy (Nov)		Rainy (Mar)		Dry (July–August)	
		Males	Females	Males	Females	Males	Females
Barra Bonita (BB)	Reproductive status*	88.2	91.7	91.7	83.3	50	33.3
	Hepatosomatic index	0.35 ± 0.14^{a}	0.33 ± 0.13^{a}	0.45 ± 0.08^{a}	0.42 ± 0.07^{a}	0.73 ± 0.15^{b}	0.73 ± 0.14^{b}
	EROD activity	70.6 ± 46.27^{a}	18.86 ± 12.89^{ac}	46.56 ± 21.39^{ab}	65.31 ± 19.84^{b}	170.5 ± 153.83^{d}	157.33 ± 191.7^{d}
Bariri (BA)	Reproductive status*	75	100	69.2	80	62.5	57.1
	Hepatosomatic index	0.63 ± 0.29^{a}	0.50 ± 0.24^{a}	0.51 ± 0.12^{a}	0.46 ± 0.12^{a}	1.02 ± 0.86^{ab}	0.81 ± 0.34^{a}
	EROD activity	62.25 ± 41.54^{a}	38.24 ± 23.52^{ab}	38.65 ± 25.10^{ab}	43.59 ± 39.81^{a}	42.43 ± 19.65^{ab}	49.86 ± 20.09^{ab}
Promissão (PR)	Reproductive status*	95	100	80	82.4	66.7	77.8
	Hepatosomatic index	0.51 ± 0.17^{a}	0.43 ± 0.21^{a}	0.46 ± 0.09^{a}	0.47 ± 0.13^{a}	0.74 ± 0.16^{b}	0.71 ± 0.14^{b}
	EROD activity	35.65 ± 23.04^{bc}	34.92 ± 29.01^{abc}	33.19 ± 10.06^{c}	29.34 ± 17.56^{ac}	23.89 ± 11.23^{c}	24.56 ± 13.24^{c}

* Percentage of individuals considered mature, as those with active gonads. The unit for hepatosomatic index is percentage and EROD activity is pmol min^{-1} mg $prot^{-1}$.

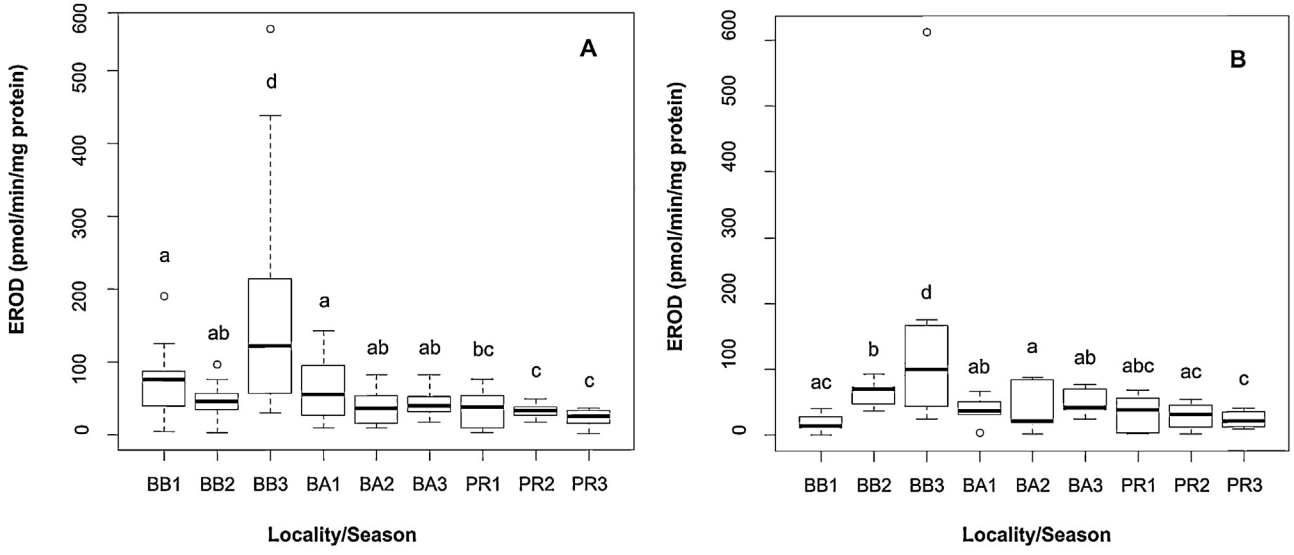

Fig. 2. Box-plots of ethoxyresorufin-*O*-deethylase activity of *Plagioscion squamosissimus* among the localities (BB = Barra Bonita; BA = Bariri; PR = Promissão), and among season (November 2012 = Early-rainy: 1; March 2013 = Rainy: 2; July and August = Dry: 3). Mean ethoxyresorufin-*O*-deethylase activity in (A) males and (B) females among sampling sites. Box-plots denoted by different letters biomarker values differed significantly among seasons and localities. Line = mean; box = mean ± standard error; whiskers = mean ± standard deviation.

Table 5
Results of the analysis of covariance (ANCOVA) using sex and size (standard length) as covariates on the EROD activity levels among localities and seasons. The significant values (* = <0.05; ** = <0.01) indicate that the differences found among the localities and seasons vary as a function of the covariates used in this model.

Model factors	*N*	*F*-statistic	*p*-values
Size	213	4.44	0.036*
Sex	213	4.16	0.042*
Size × Sex	213	4.43	0.036*
Locality	213	15.03	0.001**
Season	213	6.35	0.002**
Locality × Season	213	10.57	0.001**

males (Kruskal–Wallis test, $H = 34.68$; df = 8; $p < 0.001$) (Fig. 2A) and females (Kruskal–Wallis test, $H = 26.64$; df = 8; $p < 0.001$) (Fig. 2B).

The ANCOVA analysis indicated that sex ($F_{1, 178} = 4.44$; $p = 0.036$) and size ($F_{1, 179} = 4.16$; $p = 0.042$) had significant negative effects on the seasonal variability of the EROD activity levels (Table 5). After controlling for individual-level covariates, the interactions between size and sex and locality and season were statistically significant for the EROD activity levels among the localities and seasons in the Tietê river (Table 5).

4. Discussion

The differences in EROD activity levels found among the seasons at Barra Bonita suggest that reproductive status, sex and size had a significant negative effect on the variability of this biomarker at this highly polluted locality (near São Paulo). Our study indicated that this difference in Barra Bonita and Bariri was related to the reproductive status of the females, with a decrease of EROD activity levels in the females during the early-rainy season in comparison with the males. In addition, in Promissão at the dry season (the reference site), where the largest females examined in the study were found, the EROD activity levels were lower compared with those in Barra Bonita during at the same season, suggesting that Promissão reservoir was less polluted. This decrease in activity was even more evident during the early-rainy season at Barra Bonita (Fig. 2B), suggesting that the reproductive status of the females (more than 90% mature females; Table 4) may potentially explain these differences ($r_s = -0.916$; $p < 0.001$). In contrast, the males presented an elevated activity of this biomarker upon beginning of the early-rainy season in all the three reservoirs, with a highest EROD activity during the dry season at Barra Bonita (Fig. 2A). Webb and Gagnon (2002) and Webb et al. (2005), found a similar pattern in the Swan-Canning river, Western Australia, in which males at all sites, had a higher EROD activity than female fish. In the present study, male croaker had significantly higher EROD activity level than the females, most likely due to reproductive activity, supporting the finding by Hodson et al. (1991) that the reproductive status can significantly affect EROD activity in fish.

It is also important to stress that this elevated EROD activity in males occurred during the early-rainy season, when the highest temperatures (above $26 \pm 1.2\,^{\circ}C$) were recorded at the three localities (Barra Bonita, Bariri and Promissão). Clearly, for aquatic cold-blood animals, the increase in water temperature could also partially explain the elevated EROD activity in males due to increase in metabolic rates. Lyons et al. (2011) found a similar dependence of EROD activity with respect to temperature in cod (*Gadus morhua*).

Although mature *P. squamosissimus* females were found during all of the seasons, the peak in the number of mature females occurred during the early-rainy season (see Table 4), when spawning in this species is most intense (Petesse et al., 2007). In fact, the end of the spawning (March to April) period coincided with an increase in EROD activity in the females at Barra Bonita during the rainy and dry seasons (Fig. 2B), which reached activity levels similar to those of the males (see Fig. 2A). The decreased EROD activity observed in the females during the early-rainy season at Barra Bonita may have been due to the antagonist effects of a steroid hormone on the activity of this biomarker in the females (Whyte et al., 2000; Koenig and Solé, 2012). The mechanism for EROD activity downregulation in spawning females has been related to the increase in 17β-estradiol that occurs during the reproductive cycle (Shapiro, 1982; Snowberger et al., 1991; Buhler et al., 2000; Whyte et al., 2000; Elskus, 2004; Iwanowicz et al., 2009). This steroid hormone controls vitellogenin (egg yolk protein) production during gonadal recrudescence (Shapiro, 1982). Whyte et al. (2000) have suggested that the females contain more lipids and have a high depuration potential of hydrophobic contaminants during spawning than males through the release of their lipid rich eggs.

Although there is strong evidence that the reproductive status of the females decrease the EROD activity levels during the spawning period, we could not determine whether the increase in 17β-estradiol caused the suppression of the EROD activity levels at Barra Bonita based on our data. Our results are in accordance with those reported by Gorbi et al. (2005), Kopecka and Pempkowiak (2008), Chiang et al. (2012), and Koenig and Solé (2012), who have also shown that EROD activity levels in fish species differ markedly among the seasons, especially during the spawning period. Bucheli and Fent (1995) have found that in fish, EROD activity decreases shortly before and during spawning. According to Whyte et al. (2000), the EROD activity levels in the female fish that they studied gradually declined toward the onset of ovulation until spawning and then returned to basal levels during the postspawning period.

In the present study, the standard length of males was statistically different among the seasons in the three reservoirs and the females only during the seasons at Bariri reservoir (Table 2). For males the standard length did not influenced the EROD activity response among localities and seasons (Table 2). Although the standard length used as covariates had influenced the EROD activity as showed in the model (Table 3), when this effect was controlled, we still found a significant difference in the EROD activity responses among the seasons and localities ($p = 0.001$). In general, a larger fish has more exposure time, during which it can accumulate more hydrophobic contaminants. This effect has been noted by Hugla et al. (1995) and Sleiderink et al. (1995) in barbel and flatfish (dab), where mature fish collected from a PCB-contaminated site had higher levels of EROD activity than juveniles from the same site. However, in the present study, the difference between juveniles and adults cannot be investigated because the most of the individuals were adults. Moreover, the age has also been shown to influence EROD enzyme activities in fish (Whyte et al., 2000; van der Oost et al., 2003; Kashiwada et al., 2007). The influence of the age on biomarker response to toxicants is at least partly the consequence of differences in uptake of contaminants through the life history stage such as larvae, embryos, juveniles and adults (Amiard-Triquet et al., 2013). Although, in the present study, the most of the croaker collected were adults and with estimated age between 1 and 4 years, we did not find an influence of age between males ($p = 0.91$) and females ($p = 0.27$) on EROD activity levels among reservoirs and seasons in the Tietê river. An important study by Khan and Payne (2002) reported that adult males and juveniles fish had higher levels of EROD activity that prespawning females, providing evidence in support of the present study that the lower of EROD activity in pre-spawning females than in mature males is probably associated with sex steroids.

Furthermore, the highest EROD activity levels were found at Barra Bonita during the dry season, in contrast with the other localities, strongly reflecting the presence of xenobiotic chemicals in this part of the Tietê River (near São Paulo), particularly PAHs, which have been recognized as inducers of EROD activity in fish

(Whyte et al., 2000). An increased in phenanthrene and fluoranthene concentrations were observed in September at Barra Bonita reservoir (see Table 2). The presence of these PAHs in the sediment may explain the higher EROD activity levels found during the dry season at Barra Bonita. In contrast, at Promissão, EROD activity was low during all seasons (Fig. 2). Phenanthrene (Phe) is one of the most abundant PAHs in aquatic ecosystems as a result of petrogenic and pyrogenic sources (Sun et al., 2011). Previous studies (Stegeman et al., 1998; Shailaja and D'Silva, 2003; Correia et al., 2007; Oliveira et al., 2007; Cheikyula et al., 2008) have demonstrated that phenanthrene showed strong induction effects on EROD activity in fish. According to Oliveira et al. (2007), a limited number of studies are available on Phe CYP1A modulation showing divergent results (Goksøyr et al., 1986; Bols et al., 1999; Billard et al., 2004; Pathiratne and Hemachandra, 2010). However, a significant EROD activity induction was observed in an experiment in golden grey mullet (*Liza aurata*) exposure to phenanthrene when compared to control group (Oliveira et al., 2007). Another experimental study, also demonstrated that Phe induced the EROD activity in gilthead seabream (*Sparus aurata*), at which fish were exposed for 4 days to phenanthrene (0.11–0.56 μM) (Correia et al., 2007). Moreover, this last study showed that the concentration-dependent increase of Phe-type metabolites in bile followed by the increase of EROD activity indicates that Phe is metabolized in the liver of seabream.

Although there were studies (e.g., Goksøyr et al., 1986) that have shown an absence of induction of Phe on EROD activity levels, the studies of Correia et al. (2007) and Oliveira et al. (2007), demonstrated in an elegant and well replicated experiment that Phe can increase EROD activity at low exposures (e.g., 0.11 μm = 72.25 pmol min^{-1} $prot^{-1}$), but an inhibition at high concentrations (e.g., 0.56 μm = 42.60 pmol min^{-1} $prot^{-1}$). The experiment realized by Goksøyr et al. (1986) used only a concentration of 100 mg/ml, which could explain the lack of the induction of CYP1A. According to Correia et al. (2007), this provides evidences that EROD activity can handle only with toxicant stressors above a certain level, but for more severe exposures, other subcellular processes in liver can be induced.

Furthermore, the increase of hepatosomatic index is a clear indication of an abnormal high-metabolic activity in those exposed livers (Correia et al., 2007). We found a significant increase of hepatosomatic index ($H = 55.85$; df = 5; $p < 0.001$) during the dry season at Barra Bonita (Table 4), indicating a possible reaction of PAHs (e.g., phenanthrene) in this fish species.

Many studies have demonstrated that PAHs and PCBs are responsible for the induction of EROD activity in fish (Orrego et al., 2005; Parente et al., 2008; Trídico et al., 2010) and other aquatic organisms (Ren et al., 2014; Vranković and Slavić, 2015); however, few studies have included confounding factors (e.g., individual-related) in their analyses (Chiang et al., 2012; Koenig and Solé, 2012). Gagnon and Hodson (2012) have stressed the importance of considering fish size and sex in biomarker studies to avoid a gender bias in reported EROD activity levels. Burger et al. (2007) have also highlighted that many studies have ignored sex (i.e., did not distinguish between the sexes), choosing to analyze only one gender or to report data according to sex without commenting on any differences present. Moreover, assays of EROD activity in sexually maturing female fish approaching the spawning period produce inflated variances of EROD activities in mixed samples of male and female fish (Förlin and Haux, 1990; Gagnon and Hodson, 2012). Accordingly, it is important to assume that the variance may not always be equal between the sexes (Gorbi et al., 2005; Gagnon and Hodson, 2012). Another important point is that biomonitoring studies of EROD activity have generally used mixed populations of male and female fish (Fuentes-Rios et al., 2005; Traven et al., 2013), making it increasingly difficult to relate the causes and effects.

We suggest that future studies of EROD activity in fish should assess males and females separately. Thus, the natural variabilities (e.g., individual-related factors) of biomarkers in field conditions should be carefully considered to improve the use of these ecotoxicological responses in biomonitoring of tropical/subtropical reservoirs in freshwater rivers.

5. Conclusion

In conclusion, our study demonstrates the importance of considering reproductive status and size for females and males separately in studies of EROD activity. The direct comparison of contamination-induced EROD activities should not be conducted without controlling for the influence of these individual-related factors (e.g., reproductive status) in each fish species. Additionally, measurements of EROD activity levels at different times of the year would be valuable for determining whether this enzymatic process shows seasonal variation. This approach would help to avoid data misinterpretation and the underestimation of environmental contamination inducers.

We suggest that further studies should include the evaluation of 17β-estradiol and other stressors, including varying concentrations of aromatic hydrocarbons, bile metabolites (PAHs and PCBs) and parasites, in individual fish to assess the factors promoting variability in EROD activity levels.

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