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Research Paper

Endocrine disruption of phenanthrene in the protogynous dusky grouper *Epinephelus marginatus* (Serranidae: Perciformes)

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ABSTRACT

The dusky grouper *Epinephelus marginatus* is a protogynous hermaphrodite fish, that maintains high levels of plasma steroids as juveniles, as substrates for sex inversion. These fish are exposed to marine pollution from oil spills during cargo handling. Polycyclic aromatic hydrocarbons (PAHs), such as phenanthrene (Phe), are the main crude oil components and are toxic to fish, acting as endocrine disruptors (ED). This is the first study that investigated impacts of Phe as an ED in *E. marginatus* juveniles. An *in vivo* sublethal exposure (96 h) to Phe was carried out at two concentrations (0.1 mg/L and 1 mg/L); exposure to the vehicle (ethanol; ETOH) was also performed. Plasma levels of 17β-estradiol (E_2), testosterone (T) and 11-ketotestosterone (11-KT) were measured by ELISA. Gonads, liver and spleen were processed for histological analysis. In an *in vitro* bioassay, gonad fragments were incubated with Phe (8.91 mg/L) or ETOH. Steroid levels in the culture media were measured by ELISA. The *in vivo* exposure to Phe triggered an increase of the area of the hepatocytes, increased number of melanomacrophagic centers and hemosiderosis in the spleen; ETOH induced similar effects on spleen. E_2 and T levels did not change in plasma or *in vitro* media. In plasma, ETOH decreased 11-KT levels. Phenanthrene sharply reduced 11-KT levels *in vitro*. Although *in vivo* bioassay results were not unequivocal owing to ethanol effects, Phe might disrupt steroidogenesis in juvenile grouper, possibly causing dysfunctions during sex change and gonadal maturity, considering the importance of 11-KT in developing ovaries.

1. Introduction

The dusky grouper, *Epinephelus marginatus* (Serranidae: Perciformes), is a marine protogynous hermaphrodite, typical of rocky bottoms and widely distributed throughout the Southwest Atlantic Ocean, from the British Isles to South Africa and the Southwest Indian Ocean in Southern Mozambique and Madagascar (Rodrigues-Filho et al., 2009). This species presents great commercial value and their overfishing combined to its complex reproductive strategies and environmental degradation has placed this species on the Red List of Threatened Species of the International Union for the Conservation of Nature (IUCN) (Figueiredo and Menezes, 1980; Heemstra and Randall, 1993; Cornish and Harmelin-Vivien, 2004; Rodrigues-Filho et al., 2009).

Protogynous hermaphroditism is a reproductive strategy in which all fish mature first as females and later in adult life some individuals pass through sexual inversion, when ovaries are replaced by testes, becoming reproductive males (Figueiredo and Menezes, 1980; Lo Nostro et al., 2003). This process is modulated mainly by steroid pattern modification, started when 17β-estradiol (E_2) levels decrease whereas 11-ketotestosterone (11-KT) levels increase, promoting masculinization (Bhandari et al., 2003; Garcia et al., 2013). Significant levels of reproductive steroids have been detected in protogynous juveniles as black sea bass, *Centropristis striata*, gag, *Mycteroperca microlepis* and also *E. marginatus* (Borg, 1994; Heppell, 2005; Rodrigues-Filho, 2010) and the high levels of androgens observed in young females could be related to a physiological demand of a biochemical structure triggered to respond promptly to any changes that request sexual inversion.

E. marginatus is possibly the most common benthopelagic predator from shallow waters to 100 m deep (Heemstra and Randall, 1993; Barreiros, 1998; Harmelin and Harmelin-Vivien, 1999). Individuals' distribution varies according to fish size and age, therefore, juveniles are usually limited to infralittoral areas and afterwards adults disperse throughout the whole rocky bottom profile (Harmelin and Harmelin-Vivien, 1999). Thus, juveniles are more frequently exposed to anthropogenic actions and may be

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threatened by most activities over marine coastal habitats (Sluka et al., 1994).

It is estimated that 2.95 million tons of oil are released by anthropogenic activities in marine environments every year (Doerffer, 2013). Crude oil is mostly composed of hydrocarbons (98%), oxygen, nitrogen and sulphur compounds (Evans and Rice, 1974; Zagatto & Bertoletti, 2008). Polycyclic aromatic hydrocarbons (PAHs) present a hydrophobic/lipophilic structure responsible for their high toxicity levels since, even in minor concentrations, they may bioaccumulate in tissues and affect biota physiology (Evans and Rice, 1974; Meador et al., 1995; Camus et al., 2002; Veintemilla, 2006; Zagatto & Bertoletti, 2008).

Phenanthrene (Phe) is the main hydrocarbon in crude oil (achieving 98% of its composition, according to Salvo et al., 2016) and is one of the 129 contaminants of overriding concern by USEPA (*United States Environmental Protection Agency*) (U.S. EPA) due to their distribution and toxicity (Karami et al., 2016). In a survey taken along the Atlantic Ocean coastline, Phe was found in almost every sample analysed and presented the greatest concentrations (170 pg/L average) reaching up to 1400 pg/L on the US coast (Lohmann et al., 2013). According to Maskaoui et al. (2002) Phe concentrations reached 1.37 μg/L in surface water and 11 ng/g in sediments from de Juliong River Estuary and Western Xiamen Sea in China. In São Sebastião Channel, site of Brazil's greatest oil port (Terminal Almirante Barroso – TEBAR), Medeiros and Bícego (2004) found 16.2 ng/g of phenanthrene in sea sediments and, in the same year, environmental monitoring of the area registered up to 79 mg/L of oil waste in water (CETESB, 2004).

As is the case for other PAHs, Phe may represent an "*Endocrine Disrupting Chemical*" (EDC) (Nicolas, 1999; Monteiro et al., 2000a,b; Han et al., 2010; Geraudie et al., 2014). These compounds may alter hormonal biosynthesis and metabolism, mimic natural steroids, inducing or blocking physiological responses and impacting reproduction and/or other endocrine processes (Tyler et al., 1998; Nicolas, 1999; Matthiessen and Johnson, 2007; Geraudie et al., 2014; Kennedy and Smyth, 2015; Senthilkumaran, 2015; Maqbool et al., 2016). Additionally, Phe can affect fish growth, induce DNA damages, disturb behavior (Jee et al., 2004; Carvalho et al., 2008; Machado et al., 2014) and cause histopathological lesions (Veintemilla, 2006; Karami et al., 2016).

The liver is responsible for detoxification and biotransformation, consequently, it is a highly susceptible organ to accumulation and, if overcharged, could develop many hepatic injuries (Takashima and Hibiya, 1995). The spleen is a very important organ for fish immunology. In this organ, melanomacrophage centers (MMCs) are usually observed. They consist of pigmented macrophage aggregates (containing melanin, lipofuscin and hemosiderin, the lattera result of erythrocyte degradation) which carry out phagocytosis. The number, size and distribution of MMCs can be altered when an individual is exposed to contaminants, representing a chemical stress biomarker (Wolke, 1992; Agius and Roberts, 2003).

The aim of this study was to investigate –*in vivo* and *in vitro*- the effect of Phe exposure on steroid levels and balance, as well as histopathology of liver, spleen and gonads of *E. marginatus* juveniles. Up to our knowledge, this is the first study to assess the effect of anthropogenic contamination by this hydrocarbon in this species of ecological concern.

2. Materials and methods

2.1. Phenanthrene half-life

Aiming to understand Phe behaviour in sea water and its real concentration, phenanthrene half-life was estimated under the experimental setup. A stock solution of Phe was obtained by dissolving 0.25 g of Phe in 0.01 L of ethanol (ETOH) and then in 0.04 L of sea water. From this stock solution, 0.024 L were added to a 120 L sea water tank. At the following times: 0 h, 12 h, 24 h, 36 h and 48 h, 1 L samples were analysed by *Görtler Laboratório do Brasil LTDA* using the GCMS method USEPA-8270D (protocol USEPA-3510C). Phe half-life was estimated by Microsoft Excel 2010 and the rate of real/nominal concentration is shown in Fig. 1.

2.2. Experimental animals

E. marginatus juveniles were captured in São Sebastião coast, SP, Brazil (23°51′50″S and 45°46′55″W) and held in fiberglass tanks (2000 L) with filtered running sea water (dissolved oxygen 6.5 ± 1.0 ppm, flow rate 80% tank volume/day) at natural

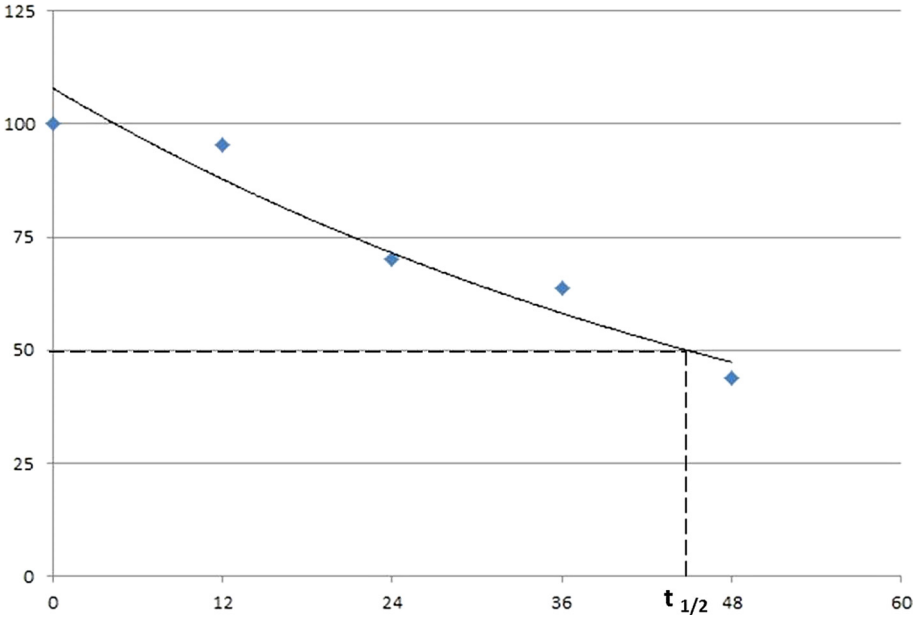

Fig. 1. Phenanthrene decay. Bioassay conditions in function of time. Data is presented in percentage of initial real concentrations. Dotted line shows phenanthrene half-life time of 45.25 h (C = 107.92e−0.017t).

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temperature (temperature ± SD = 24.0 ± 2.5 °C) and photoperiod (12:12) for at least 15 days before the start of the bioassays. Fish were fed three times a week with eviscerated headless sardines (*Sardinella brasiliensis*). All experimental procedures took place at CEBIMar/USP, SP, Brazil.

2.3. LC_{50} determination

To establish the sub-lethal Phe concentrations a 96 h bioassay was carried out to determine median lethal concentration (LC_{50}) to these individuals. Thirty fish (mean weight ± SD = 229.8 ± 43.2 g) were randomly distributed into six 120 L covered glass aquaria (five fish per tank; density = 9.57 g/L) three days before the start of the bioassay to get accustomed to this new environment (temperature ± SD = 22 ± 2 °C, dissolved oxygen ± SD = 6.5 ± 1.0 mg/L, natural photoperiod (12:12) of the season the assay was performed). Throughout this period, fish were fed once a day and 75% of water was renewed daily. A stock solution of Phe 98% (Sigma-Aldrich P11409) was prepared dissolving it first in ETOH and then in filtered sea water. Fish were exposed to Phe at nominal concentrations of 0.47, 0.94, 1.88 and 3.76 mg/L (based on Veintemilla, 2006) by adding the necessary volume of stock solution to each tank (vehicle 0.004% per aquaria). A control group without any addition and a group subjected only to the vehicle (ETOH 0.004%) were carried out in parallel. During the experiment fish were not fed and 75% of water was renewed daily, together with chemical solutions. Mortality was registered daily and the LC_{50} was estimated by Probit transformation of the mortality dose curve (Finney, 1971).

2.4. In vivo sub-lethal bioassay

To evaluate Phe effects, a 96 h sub-lethal bioassay was performed. Forty fish (mean weight ± SD = 252.6 ± 48.1 g) were transferred to aquaria 72 h before exposure, following the same procedures taken previously for the LC_{50} determination. Fish were exposed to Phe at nominal concentrations of 0.1 mg/L (PHE1) and 1 mg/L (PHE2). Control treatments were also carried out, with ETOH (0.004%) and without vehicle (Control). Each experimental condition was tested in duplicate with 5 individuals per 120 L aquaria (n = 10). During this experiment fish were not fed and 75% of media was daily renewed together with chemical solutions (temperature ± SD = 22 ± 2 °C, dissolved oxygen ± SD = 6.5 ± 1.0 mg/L, natural photoperiod). No mortality was observed during the experiment.

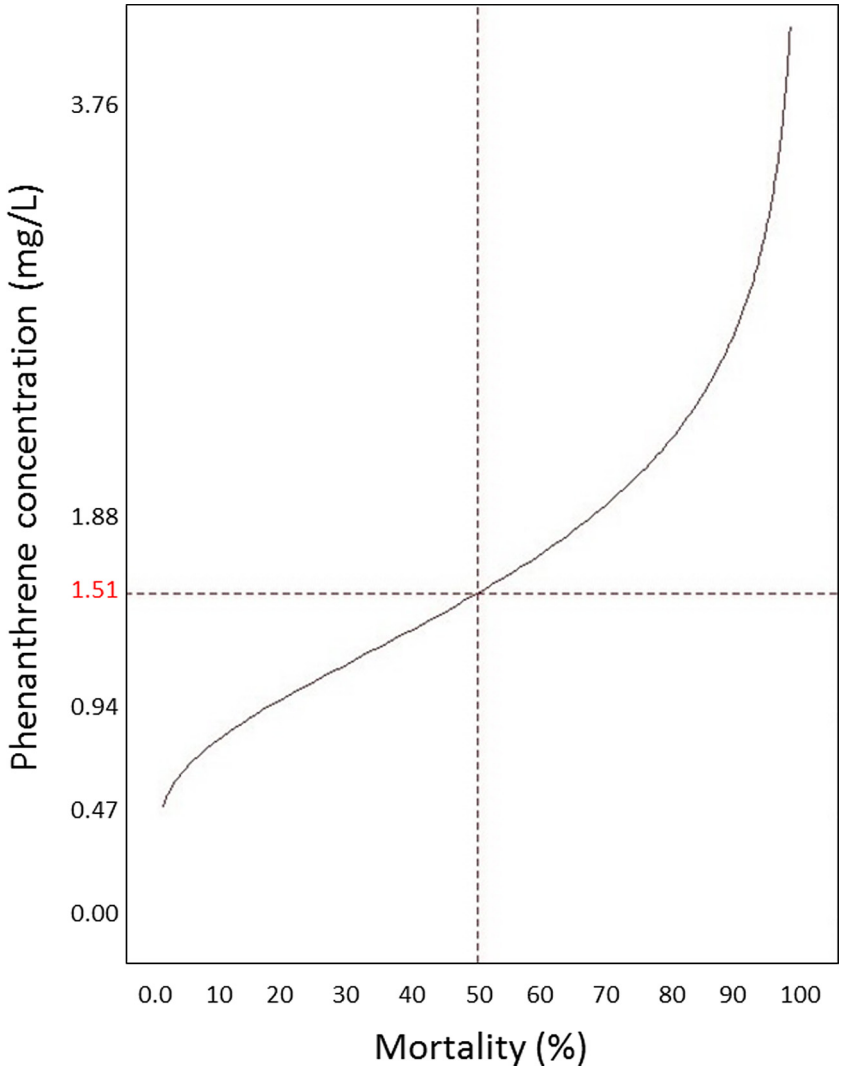

Fig. 2. *Epinephelus marginatus*. Phenanthrene concentration-response curve after 96 h exposure. Crossing dotted lines point to the LC_{50} value (1.51 mg/L). Probit statistical analysis.

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After the exposure period, fish were desensitized in ice-chilled sea water, peripheral blood was collected by caudal vein puncture, using a heparin-coated (5000 UI/Roche®) 22G needle attached to a 5 mL syringe. Blood samples were centrifuged for six minutes at 587×*g*, plasma was transferred to heparin-coated cryotubes, and immediately placed at −80 °C until sex steroids quantification. Hereupon, fish were decapitated; gonads, liver and spleen were collected and fixed for histological analysis. The animals were euthanized by sectioning their spinal cord, as approved by the Animal Ethics Committee of the Institute of Biosciences, University of São Paulo (Protocol number 206/2014).

2.5. In vitro bioassay

To analyse Phe effects strictly on gonadal steroidogenesis, an *in vitro* bioassay was carried out. Six *E. marginatus* juveniles (mean weight ± SD = 335.8 ± 58.9 g) were anesthetized (benzocaine 0.1 g/L), decapitated and their gonads were immediately collected. Gonads were fractionated in three similar fragments (≤10%; mean weight ± SD = 0.034 ± 0.012 g) and each fragment was placed into 2 mL glass vials and pre-incubated in 1 mL of Krebs-Ringer-HEPES-glucose buffer (KRHG; 120 mM NaCl, 49 mM KCl, 1.2 mM $MgSO_4$, 8.3 mM KH_2PO_4, 10 mM glucose and 10 mM HEPES, pH 7.4) for 30 min at 26 °C on a mixer (*Thermomixer compact – Eppendorf*). Buffer was discarded and replaced with three experimental incubation media (one to each fragment from every fish): 1000 μL KRHG (control); 250 μL ethanol stock solution and 750 μL KRHG (etoh 0.4%) and 250 μL Phe stock solution and 750 μL KRHG (phe 8.91 mg/L). The incubation was performed for four hours at 26 °C mixer, forthwith media were transferred to cryotubes and placed into a −80 °C freezer until processing.

2.6. ELISA for steroids

ELISA commercial kits were used to determine E_2, T (IBL International) and 11-KT (Cayman Chemicals®) levels in plasma and incubation media. Assays readings were performed with a Spectra MAX 2μ microplate reader at 450 nm wavelength for E_2 and T, and at 405 nm for 11-KT. 11-KT validation for *Epinephelus marginatus* has already been done by Garcia et al. (2013); Estradiol and Testosterone validation was determined by calculations of inter and intra-assays variation. The detection limit of the assay was 10 pg/ml for E2, 50 pg/ml for T and 1.3 pg/ml for 11-KT. The coefficients of variation (CVs) intra and interassay found for E2 were 12.43% and 15.33%; 11.67% and 6.31% for T and 9.2% and 8.6% for 11-KT, respectively.

2.7. Histological analysis

Gonads were fixed in Bouin's solution for 24 h at room temperature, then transferred to ethanol 70°GL. Liver and spleen were fixed in 10% buffered formaldehyde. Gonads, liver and spleen were dehydrated, embedded in paraffin (Erv-Plast, *Easy Path*) and sectioned at 5 μm (Leica RM225 microtome); sections were stained with Haematoxylin-Eosin. Histological analysis was performed in a Leica DM 1000 microscope coupled with *Leica DFC 295* camera for photograph capture. Number of MMCs was quantified in spleen sections and normalized to organ section area. Hepatocytes area was also measured (*Leica Application Suite* V3).

2.8. Statistical analysis

Determination of the LC_{50} value was achieved by the PROBIT (*probability unit*) method (Finney, 1971) (Minitab 17.). One way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons were used to establish differences in plasma steroids between experimental groups. Nested one-way ANOVA was used to establish differences in MMCs numbers and hepatocyte areas between groups, using the *Holm-Sidak* test for multiple comparisons (SigmaStat 3.0). When data did not meet homogeneity of variance and normality assumptions, it was log-transformed before analysis. Steroids secretion in "*in vitro*" incubation media were relativized to gonadal fragment weight due to individual variation in the size of the gonads. Steroid levels were analysed using a randomized blocks design (Statistica 7). Values were considered significantly different at $p \leq 0.05$. Data are presented as mean ± SEM.

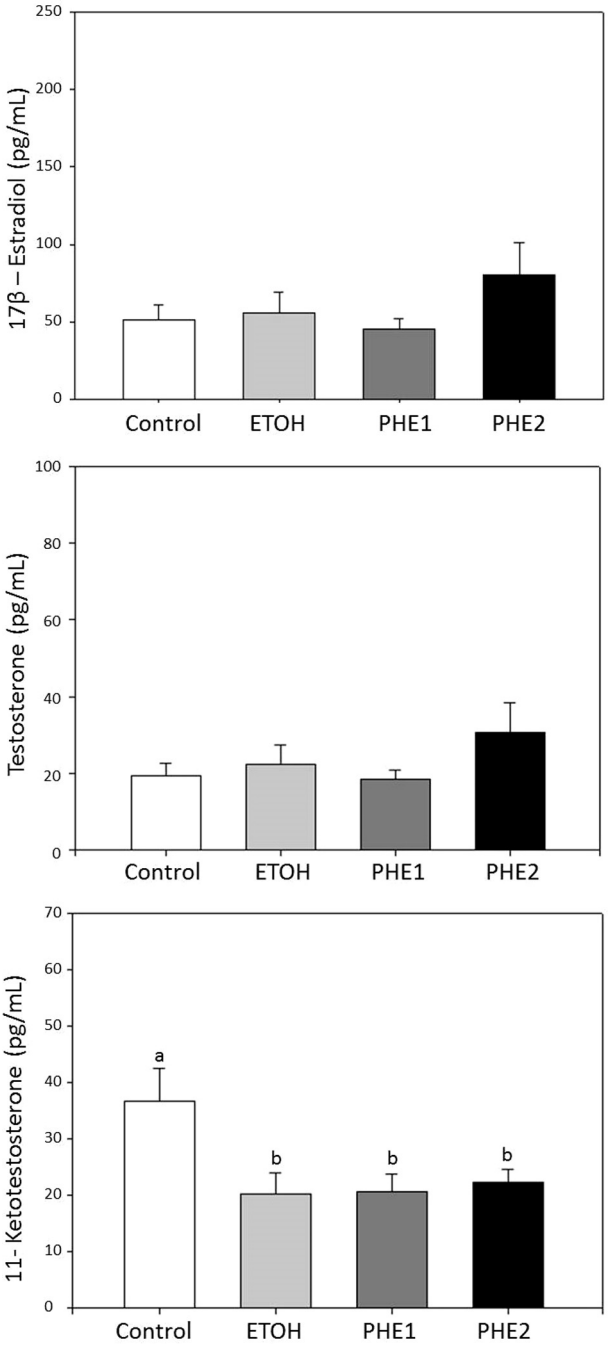

Fig. 3. Sex steroids concentrations in plasma of *Epinephelus marginatus* exposed to waterborne Phe. A) 17β –Estradiol, B) Testosterone, C) 11-Ketotestosterone. Data expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$; *One-way* ANOVA and *Tukey* test). Control Group; ETOH Group (Ethanol 0.004%); PHE1 Group (Phenanthrene 0.1 mg/L); PHE2 (Phenanthrene 1 mg/L).

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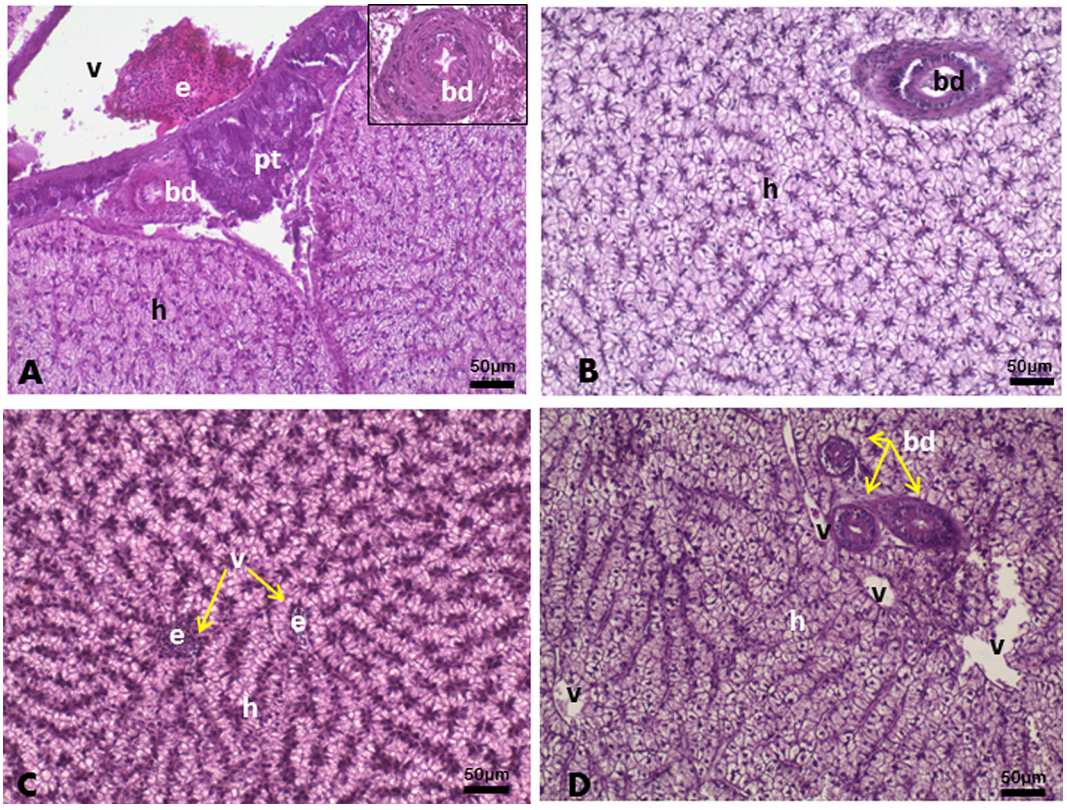

Fig. 4. Liver histological sections of *Epinephelus marginatus*. A) Control group, where a typical hepatocyte organization is found; scattered pancreatic tissue and biliary ducts are also observed; Detail) Biliary duct. B) ETOH group (ethanol 0.004%). C) PHE1 group (phenanthrene 0.1 mg/L); D) PHE2 group (phenanthrene 1 mg/L). Bars represent 50 μm; 10 μm for detail. Haematoxylin-eosin. e, erythrocytes; h, hepatocyte; v, blood vessel; bd, biliary duct; pt, pancreatic tissue.

3. Results

3.1. 96-h LC_{50} determination

After a 96 h exposure, LC_{50} for Phe on *E. marginatus* juveniles was established as 1.51 mg/L (Fig. 2). This value was used to establish the sub-lethal Phe concentrations used thereafter.

3.2. In vivo bioassay

3.2.1. Plasma steroids

No significant differences were found in plasma T ($P = 0.335$) or E_2 ($P = 0.763$) between experimental groups. However, a significant decrease in 11-KT in plasma was observed ($P = 0.015$) for groups ETOH, PHE1 and PHE2 relative to control (Fig. 3).

3.2.2. Histology

3.2.2.1. Gonads. All individuals showed a typical protogynous juvenile structure (Rodrigues-Filho, 2010). Oocyte primary growth (perinucleolar) was observed in ovarian lamellae, as well as oogonia cell-nests (data not shown). No anatomic or morphological differences were observed between experimental groups.

3.2.2.2. Liver. Hepatic sections from the control group showed a typical liver structure composed of hepatocytes with low lipid vacuolation, intertwined with thin pancreatic tissue layers (Fig. 4A). Animals from the ETOH group presented great individual variation, since some sections presented a vacuolation increase and others had a high carbohydrates deposition in their cytoplasm (Fig. 4B). Sections from the PHE1 group livers (Fig. 4C) showed a significant increase in hepatocyte area in comparison to the other groups ($P \leq 0.001$) (Fig. 5). Although a trend to this increase was also observed in the PHE2 group (Fig. 4D), it was not possible to determine a significant difference, possibly due to the high individual variation observed which might be caused by ethanol in individual fish.

3.2.2.3. Spleen. Spleen sections from the control group showed intertwined red and white pulp, typical for Teleosts, and scarce MMCs (Fig. 6A). Individuals exposed to Phe presented pigmentation typical of hemosiderosis (Hibiya et al., 1982), especially those

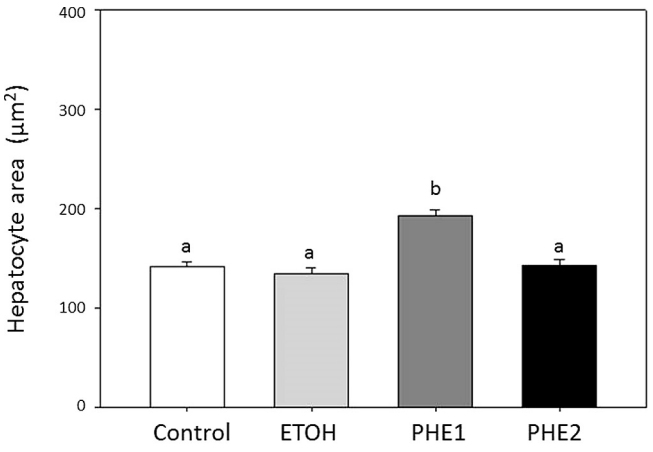

Fig. 5. Hepatocyte mean area from *Epinephelus marginatus* juveniles exposed to sub-lethal Phe concentrations (mean ± SEM). Different letters indicate significant differences ($P \leq 0.001$; *One-way* ANOVA and *Holm-Sidak test*). Control group, ETOH group (ethanol 0.004%), PHE1 group (Phe 0.1 mg/L), PHE2 group (Phe 1 mg/L).

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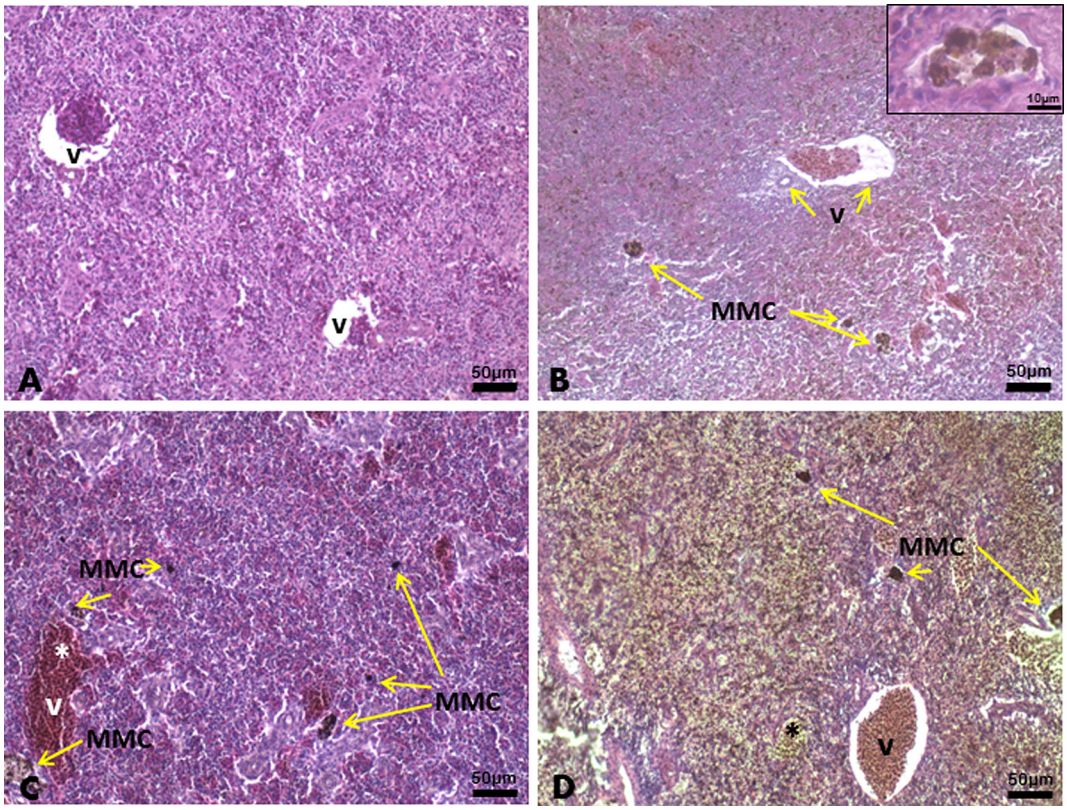

Fig. 6. Spleen histological sections from *Epinephelus marginatus* juveniles from *in vivo* assays. A) Control group, where the typical red and white pulp organization is found; B) ETOH group (ethanol 0.004%); Detail) Melano-macrophage aggregate. C) PHE1 group (Phe 0.1 mg/L); D) PHE2 group (Phe 1 mg/L). See the golden yellow-brown pigment typical of hemosiderosis in spleens of animals exposed to Phe (asterisk). Bars represent 50 µm; 10 µm for detail. Haematoxylin-eosin. v, blood vessel; MMC, Melanomacrophage center.

from the PHE2 group (Fig. 6B–D). The number of MMCs increased significantly from control animals in the other experimental groups ($P \leq 0.001$) (Fig. 7).

3.3. In vitro bioassay

No significant differences were found in culture media T ($P = 0.5979$) or E_2 ($P = 0.310486$) levels between experimental groups. However, gonadal exposure to Phe resulted in an 11-KT decrease when compared to the control group ($P = 0.033$) (Fig. 8).

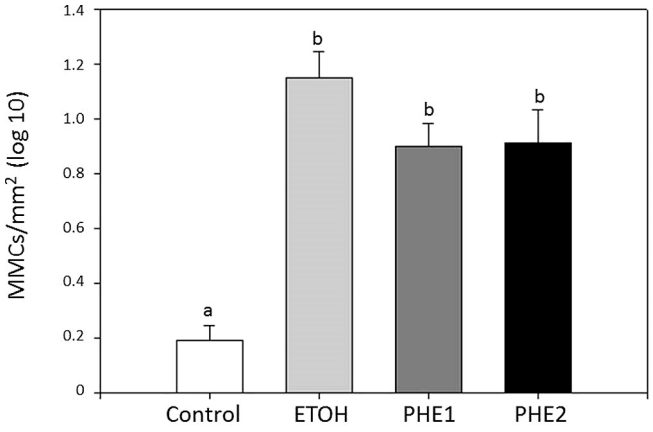

Fig. 7. Number of MMCs per section area from *Epinephelus marginatus* exposed in sub-lethal bioassays (mean ± SEM). Different letters indicate significant differences ($P \leq 0.001$; *One-way* ANOVA and *Holm-Sidak test*). Control group, ETOH group (ethanol 0.004%), PHE1 group (Phe 0.1 mg/L), PHE2 group (Phe 1 mg/L).

4. Discussion

The present study is the first to investigate Phe effects on steroid balance of *E. marginatus* juveniles, showing a significant reduction in 11-KT levels *in vitro*, which suggests its action as ED to this species. Although this decrease might not appear so alarming to protogynous juveniles, considering its primary role in spermatogenesis, some studies demonstrated that 11-KT is also involved in the control of oocyte growth (Lokman et al., 2002; Endo et al., 2011; Setiawan et al., 2012) and numerous *in vitro* and *in vivo* bioassays have shown the fundamental role of 11-KT in morphological sexual characteristics development and physiology of reproduction in several species (Lokman et al., 1998; Rohr et al., 2001; Lokman et al., 2007; Divers et al., 2010; Setiawan et al., 2012). Additionally, in protogynous species this steroid is central to early sexual inversion phases and could even be responsible for triggering this process (Bhandari et al., 2003; Garcia et al., 2013).

Several studies describe PAHs as fish endocrine disruptors, however, no consistent pattern is observed on these physiological responses (Thomas, 1988; Goodbred et al., 1997; Monteiro et al., 2000b; Roy et al., 2003; Martin-Skilton et al., 2006; Lister et al., 2008; Han et al., 2010; Geraudie et al., 2014; Kennedy and Smyth, 2015). For example, decreases in T levels are reported in goldfish, *C. auratus* (males and females), and turbot, *Scophthalmus maximus,* juveniles exposed to crude oil leavings (Lister et al., 2008; Martin-Skilton et al., 2006), whereas no differences in this steroid levels were observed in cod, *Gadus morhua,* exposed to crude oil (Martin-Skilton et al., 2006). Moreover, E_2 levels did not change in males and females of Artic cod, *Boreogadus saida,* exposed to PAHs mixture, while a significant decrease in its level

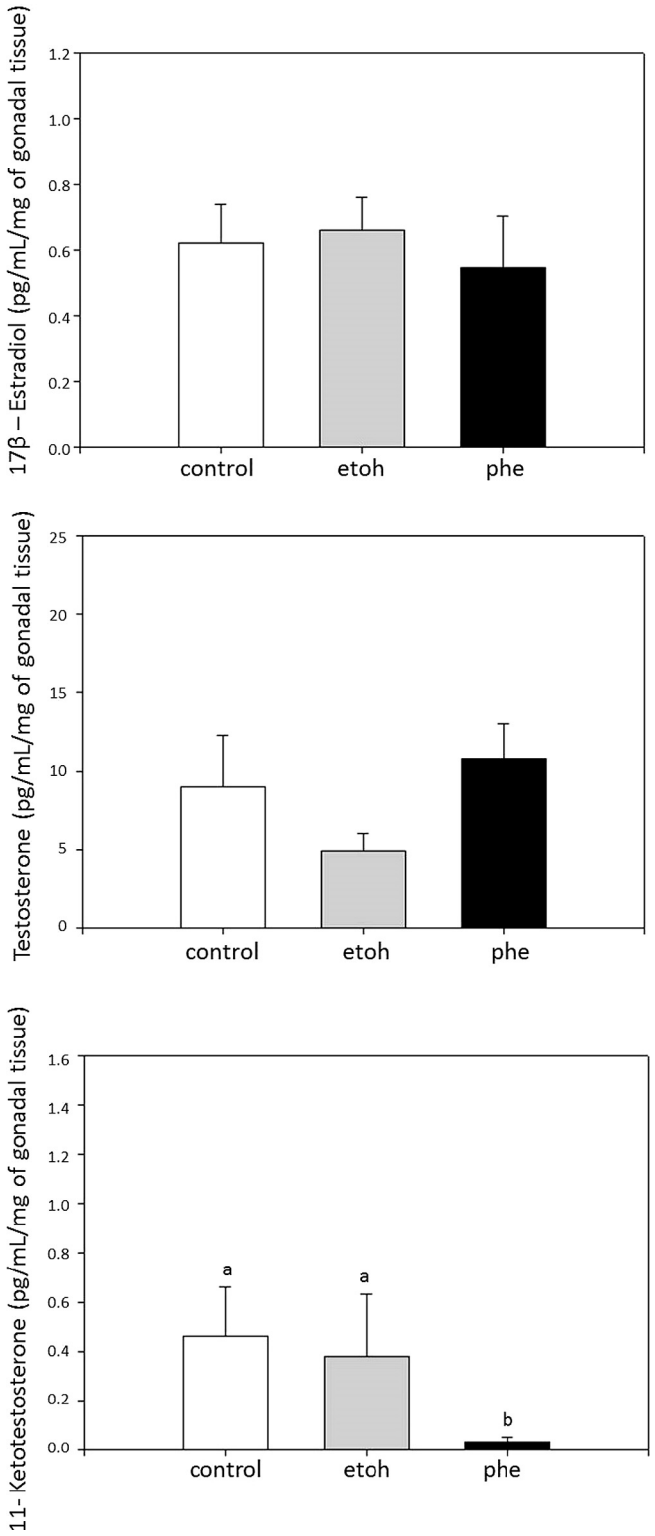

Fig. 8. Sex steroid levels in culture media from *Epinephelus marginatus* gonads (mean ± SEM). A) 17β –Estradiol, B) Testosterone, C) 11-Ketotestosterone. Different letters indicate significant differences ($P < 0.05$; DBC ANOVA and *Tukey* test). Control group, etoh group (ethanol 0.4%); phe group (Phe 8.91 mg/L).

was reported for hornyhead turbot, *Pleuronichthys verticalis,* subjected to sediments contaminated with these compounds (Roy et al., 2003).

The present study did not find significant differences in plasma sex steroid concentrations in *E. marginatus* juveniles exposed to Phe. A significant decrease in plasma 11-KT was observed upon ETOH exposure, and therefore in PHE1 and PHE2 groups when compared to the control group, meaning that the acute exposure to ETOH seems to have some influence on 11-KT plasma levels. The effect of Phe on 11-KT *in vivo*, if any, could be masked by this effect of ETOH.

On the other hand, the results of the *in vitro* bioassay showed a decrease in 11-KT levels only in the Phe group, which suggests an effect as a gonadal endocrine disruptor. Since detoxification of PAHs involves action of cytochrome P450 enzymes, which are also deeply related to steroids biosynthesis and regulation, it is plausible to assume that they can interfere on steroidogenic enzymes activities or expression, resulting in steroids hormones unbalance (Stegeman and Lech, 1991; Nicolas, 1999; Martin-Skilton et al., 2006; Kennedy and Smyth, 2015). Liu et al. (2012) demonstrated that inhibition of CYP17 and CYP19A gene transcription in fish gonads was related to steroid disruption in zebrafish, *Danio rerio.* However, disruption of these genes usually impacts other steroids levels too. As 11-KT was the only steroid affected by Phe, another pathway of disruption could be responsible for 11-KT decrease.

Studies regarding the effects of ETOH on fish physiology are scarce (Bhanu and Philip, 2011). Roberts et al. (1995) verified that *in vivo* exposure to ETOH can induce activity of several cytochrome P450s in rats, which could prove of importance in steroid balance considering their central role on steroidogenic biosynthesis. On that matter, it has been demonstrated that high acute exposure to alcohol can result in gonadal disturbances in male rats due to pathway competition between ETOH oxidation and T production (Pasqualotto et al., 2004). Additionally, stress by itself can cause a decrease in androgens plasma levels (Pankhurst and Van Der Kraak, 1997). Thus, chemical stress induced by exposure to ETOH could interfere on steroid conversions. The enzyme 11β-hydrosteroid dehydrogenase (11β-HSD) converts 11β-hydroxysteroids to 11-KT and also takes part on corticosteroids pathways so that high levels of cortisol might cause a decrease in 11-KT levels (Ozaki et al., 2006). Another possibility for decreased levels of 11-KT in ETOH exposed fish is a higher metabolic and/or excretion rate.

The Phe 96-h LC_{50} value of 1.51 mg/L determined in the present study for *E. marginatus* juveniles was higher than those established for rainbow trout, *Salmo gairdneri*, largemouth bass, *Micropterus salmoide* and tambaqui, *Colossoma macropomum* (0.04, 0.18 and 0.94 mg/L, respectively) (Black et al., 1983; Veintemilla, 2006). Although marine fish are considered to be more susceptible to PAHs due to their larger amount of unsaturated fatty acids (directly related to cell membrane fluidity), the juveniles used in this study were less sensitive than *S. gairdneri* and *M. salmoide* embryos and larvae. Also, tambaqui juveniles weighted about a 10% of *E. marginatus* juveniles weight, presenting a greater surface-volume ratio and thus a higher exposure area to the contaminant (Walker and Peterson, 1991).

In what concerns the histopathological analysis, a subtle trend towards cytoplasmic vacuolation was observed in experimental groups in spite of extensive interindividual variation. Hepatocyte area from fish of the PHE1 group showed a significant increase when compared to control animals, whereas PHE2 and ETOH individuals had a heterogeneous response. To understand more clearly the effect of Phe and ETOH on hepatic cells, a longer (chronic or sub chronic) exposure would be more appropriate.

Since teleost fish lack lymph nodes, the spleen, being the main hematopoietic organ, is essential for their immunologic system (Genten et al., 2009). Within it, MMCs sequester products of pathological tissue degradation and aid in removal of phagocytized material. Although MMCs can increase in number in head kidney and liver, a higher number of splenic MMCs is a histopathological bioindicator of stress and unbalance of homeostatic mechanisms, indicating an alteration of fish health or environmental degrada-

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tion (Fournie et al., 2001). In the present study, a significant increase in the number of MMCs was detected in all experimental groups compared to the control. This increase is probably related to a tissue injury caused by ETOH exposure or the effect of chemical stress on fish physiology.

Hemosiderosis in organs such as spleen, liver and kidney is often related to organic environmental contamination (Thiyagarajah et al., 1998). In this study, hemosiderosis was observed in fish spleen from both Phe groups, however it was emphatically more pronounced in animals subjected to the highest Phe concentration (PHE2), which suggests a concentration related relationship between Phe and accelerated erythrocyte degradation.

It is fundamental not to infer that an absence of consistent responses to a contaminant, especially on an acute exposure, corresponds to the environmental reality, since individuals are usually subject to a chronic exposure and physical-chemical interactions between several substances and variables (Zagatto & Bertoletti, 2008). Moreover, cellular effects are frequently, but not always, determinant to adverse effects on higher biological levels. Individuals might possess homeostatic mechanisms, which, depending on environmental conditions, could generate different physiological responses.

5. Conclusions

The Phe 96-h LC_{50} for *E. marginatus* juveniles was established as 1.51 mg/L. Incubation with Phe caused a significant decrease in 11-KT levels in gonads *in vitro*, whereas the *in vivo* bioassay also showed a similar effect in animals exposed only to ETOH, thus it was not possible to establish an unequivocal role of Phe as an endocrine disruptor. An increase in splenic MMCs number was observed in animals subjected to ETOH, as well as those exposed to Phe at both concentrations. Hemosiderosis in spleen seems to be dose related to Phe exposure. Although a trend towards an increase in hepatocyte area was observed in exposure groups, it was not possible to establish a clear pattern for liver histopathology due to interindividual differences. A subchronic exposure could be enlightening to resolve these issues.

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References

Agius, C., Roberts, R.J., 2003. Melano-macrophage centres and their role in fish pathology. J. Fish Dis. 26, 499–509.
Barreiros, J.P., 1998. Sexual inversion in *Epinephelus marginatus* (Lowe, 1834) (Pisces: Serranidae, Epinephelinae). Rev. Port. Zoot. 5, 81–90.
Bhandari, R.K., Komuro, H., Nakamura, S., Higa, M., Nakamura, M., 2003. Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (*Epinephelus merra*). Zoolog. Sci. 20, 1399–1404.
Bhanu, S.V., Philip, B., 2011. Effect of ethanol on branchial adenosine triphosphatases in *Oreochromismossambicus*(Peters). Toxicol. Int. 18, 27–30.
Black, J.A., Birge, W.J., Westerman, A.G., Francis, P.C., 1983. Comparative aquatic toxicology of aromatic hydrocarbons. Fundam. Appl. Toxicol. 3, 353–358.
Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. 109C, 219–245.
Camus, L., Jones, M.B., Borseth, J.F., Grosvik, B.E., Regoli, F., Depledge, M.H., 2002. Total oxyradical scavenging capacity and cell membrane stability of haemocytes of the Arctic scallop, *Chlamysislandicus*, following benzo(a)pyrene exposure. Mar. Environ. Res. 54, 425–430.
Carvalho, P.S., Kalil, D.D.C.B., Novelli, G.A., Bainy, A.C., Fraga, A.P.M., 2008. Effects of naphthalene and phenanthrene on visual and prey capture endpoints during early stages of the dourado *Salminus brasiliensis*. Mar. Environ. Res. 66, 205–207.
CETESB, COMPANHIA DE TECNOLOGIA DE SANEAMENTO AMBIENTAL, 2004. Monitoramento de águas costeiras: Litoral Norte em Relatório de Qualidade das Águas Litorâneas no Estado de São Paulo. Balneabilidade das Praias, pp. 273–331.
Cornish, A., Harmelin-Vivien, M., 2004. *Epinephelus marginatus*.IUCN 2007.IUCN Red List of Threatened Species. www.iucnredlist.org.
Divers, S.L., Mcquillan, H.J., Matsubara, H., Todo, T., Lokman, P.M., 2010. Effects of reproductive stage and 11-ketotestosterone on LPL mRNA levels in the ovary of the shortfinned eel. J. Lipid Res. 51, 3250–3258.
Doerffer, J.W., 2013. Oil Spill Response in the Marine Environment. Elsevier, Pergamon.
Endo, T., Todo, T., Lokman, P.M., Kudo, H., Ijiri, S., Adachi, S., Yamauchi, K., 2011. Androgens and very low density lipoprotein are essential for the growth of previtellogenic oocytes from Japanese eel, *Anguilla japonica*, *in vitro*. Biol. Reprod. 84, 816–825.
Evans, D.R., Rice, S.P., 1974. Effects of oil on marine ecosystems: a review for administrators and policy makers. Fish. Bull. 72, 625–638.
Figueiredo, J.L., Menezes, N.A., 1980. Manual de peixes marinhos do sudeste do Brasil III. Teleostei (2). Museu de Zoologia da Universidade de São Paulo, São Paulo, p. 90.
Finney, D.J., 1971. Probit Analysis. Cambridge University Press, London, p. 333.
Fournie, J.W., Summers, J.K., Courtney, L.A., Engle, V.D., Blazer, V.S., 2001. Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. J. Aquat. Anim. Health 13, 105–116.
Garcia, C.E.O., Araújo, B.C., Mello, P.H., Narcizo, A.M., Rodrigues-Filho, J.A., Medrado, A.T., Zampieri, R.A., Floeter-Winter, L.M., Moreira, R.G., 2013. Involvement of pituitary gonadotropins, gonadal steroids and breeding season in sex change of protogynous dusky grouper, *Epinephelus marginatus* (Teleostei: Serranidae), induced by a non-steroidal aromatase inhibitor. Gen. Comp. Endocrinol. 190, 170–180.
Genten, F., Terwinghe, E., Danguy, A., 2009. Atlas of Fish Histology. Science Publishers, Enfield, NH, USA.
Geraudie, P., Nahrgang, J., Forget-Leray, J., Minier, C., Camus, L., 2014. In vivo effects of environmental concentrations of produced water on the reproductive function of polar cod (*Boreogadus saida*). J. Toxicol. Environ. Health 77A, 557–573.
Goodbred, S.L., Gilliom, R.J., Gross, T.S., Denslow, N.P., Bryant, W.L., Schoeb, T.R., 1997. Reconnaissance of 17B-estradiol, 11-ketotestosterone, vitellogenin, and gonad histopathology in common carp of United States streams: Potential for contaminant-induced endocrine disruption. US Geological Survey Open-File Report, Sacramento, CA, USA. 96-627.
Han, Z.X., Wang, J.H., Lv, W.Z., Min, L., 2010. Multibiomarker approaches in common carp to assess the effects of phenanthrene. Synth. React. Inorg. Metal-Org. Nano-Metal Chem. 40, 257–265.
Harmelin, J.G., Harmelin-Vivien, M., 1999. A review on habitat, diet and growth of the dusky grouper *Epinephelus marginatus* (Lowe, 1834). Mar. Life 9, 11–20.
Heemstra, P.C., Randall, J.E., 1993. FAO species catalogue. Groupers of the world (Family Serranidae, Subfamily Epinephelinae): an annotated and illustrated catalogue of the grouper, rockcod, hind, coral grouper, and lyretail species known to date. FAO Fish Synops. 16 (125).
Heppell, S.A., 2005. Seasonal fluctuations in androgen levels in females of the hermaphroditic gag, *Mycteroperca microlepis*, with an emphasis on juvenile animals. Comp. Biochem. Physiol. 142A, 84–91.
Hibiya, T., Yokote, M., Oguri, M., Sato, H., Takashima, F., Aida, K., 1982. An Atlas of Fish Histology. Normal and Pathological Features, p. 147.
Jee, J.H., Kim, S.G., Kang, J.C., 2004. Effects of phenanthrene on growth and basic physiological functions of the olive flounder, *Paralichthys olivaceus*. J. Exp. Mar. Biol. Ecol. 304, 123–136.
Karami, A., Romano, N., Hamzah, H., Simpson, S.L., Yap, C.K., 2016. Acute phenanthrene toxicity to juvenile diploid and triploid African catfish (*Clarias gariepinus*): molecular, biochemical, and histopathological alterations. Environ. Pollut. 212, 155–165.
Kennedy, C.J., Smyth, K.R., 2015. Disruption of the rainbow trout reproductive endocrine axis by the polycyclic aromatic hydrocarbon benzo[a]pyrene. Gen. Comp. Endocrinol. 219, 102–111.
Lister, A., Nero, V., Farwell, A., Dixon, D.G., Van Der Kraak, G., 2008. Reproductive and stress hormone levels in goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Aquat. Toxicol. 87, 170–177.
Liu, X., Ji, K., Choi, K., 2012. Endocrine disruption potentials of organophosphate flame retardants and related mechanisms in H295R and MVLN cell lines and in zebrafish. Aquat. Toxicol. 114, 173–181.
Lo Nostro, F., Grier, H., Andreone, L., Guerrero, G.A., 2003. Involvement of the gonadal germinal epithelium during sex reversal and seasonal testicular cycling in the protogynous swamp eel, *Synbranchus marmoratus* Bloch 1795 (Teleostei, Synbranchidae). J. Morphol. 257, 107–126.
Lohmann, R., Klanova, J., Pribylova, P., Liskova, H., Yonis, S., Bollinger, K., 2013. PAHs on a west-to-east transect across the tropical Atlantic Ocean. Environ. Sci. Technol. 47, 2570–2578.
Lokman, P.M., Vermeulen, G.J., Lambert, J.G., Young, G., 1998. Gonad histology and plasma steroid profiles in wild New Zealand freshwater eels (*Anguilla dieffenbachia* and *A. australis*) before and at the onset of the natural spawning migration. I. Females. Fish Physiol. Biochem. 19, 325–338.
Lokman, P.M., Harris, B., Kusakabe, M., Kime, D.E., Schulz, R.W., Adachi, S., Young, G., 2002. 11-Oxygenated androgens in female teleosts: prevalence, abundance, and life history implications. Gen. Comp. Endocrinol. 129, 1–12.

Lokman, P.M., George, K.A., Divers, S.L., Algie, M., Young, G., 2007. 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis, in vitro*. Reproduction 133, 955–967.

Machado, A.A.S., Hoff, M.L.M., Klein, R.D., Cordeiro, G.J., Avila, J.M.L., Costa, P.G., Bianchini, A., 2014. Oxidative stress and DNA damage responses to phenanthrene exposure in the estuarine guppy *Poecilia vivipara*. Mar. Environ. Res. 98, 96–105.

Maqbool, F., Mostafalou, S., Bahadar, H., Abdollahi, M., 2016. Review of endocrine disorders associated with environmental toxicants and possible involved mechanisms. Life Sci. 145, 265–273.

Martin-Skilton, R., Thibaut, R., Porte, C., 2006. Endocrine alteration in juvenile cod and turbot exposed to dispersed crude oil and alkylphenols. Aquat. Toxicol. 78, S57–S64.

Maskaoui, K., Zhou, J.L., Hong, H.S., Zhang, Z.L., 2002. Contamination by polycyclic aromatic hydrocarbons in the Jiulong River Estuary and Western Xiamen Sea, China. Environ. Pollut. 118, 109–112.

Matthiessen, P., Johnson, I., 2007. Implications of research on endocrine disruption for the environmental risk assessment, regulation and monitoring of chemicals in the European Union. Environ. Pollut. 146, 9–18.

Meador, J.P., Stein, J.E., Reichert, W.L., Varanasi, U., 1995. Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. Rev. Environ. Contam. Toxicol. 143, 79–165.

Medeiros, P.M., Bícego, M.C., 2004. Investigation of natural and anthropogenic hydrocarbon inputs in sediments using geochemical markers. II. São Sebastião, SP––Brazil. Mar. Pollut. Bull. 49, 892–899.

Monteiro, P.R.R., Reis-Henriques, M.A., Coimbra, J., 2000a. Polycyclic aromatic hydrocarbons inhibit *in vitro* ovarian steroidogenesis in the flounder (*Platichthys flesus*L.). Aquat. Toxicol. 48, 549–559.

Monteiro, P.R.R., Reis-Henriques, M.A., Coimbra, J., 2000b. Plasma steroid levels in female flounder (*Platichthys flesus*) after chronic dietary exposure to single polycyclic aromatic hydrocarbons. Mar. Environ. Res. 49, 453–467.

Nicolas, J.M., 1999. Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. Aquat. Toxicol. 45, 77–90.

Ozaki, Y., Higuchi, M., Miura, C., Yamaguchi, S., Tozawa, Y., Miura, T., 2006. Roles of 11β-hydroxysteroid dehydrogenase in fish spermatogenesis. Endocrinology 147, 5139–5146.

Pankhurst, N.W., Van Der Kraak, G., 1997. Effects of stress on reproduction and growth of fish. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Cambridge Univ. Press, Cambridge, pp. 73–93.

Pasqualotto, F.F., Lucon, A.M., Sobreiro, B.P., Pasqualotto, E.B., Arap, S., 2004. Effects of medical therapy, alcohol, smoking, and endocrine disruptors on male infertility. Rev. Hosp. Clín. Med. São Paulo 59, 375–382.

Roberts, B.J., Shoaf, S.E., Song, B.J., 1995. Rapid changes in cytochrome P4502E1 (CYP2E1) activity and other P450 isozymes following ethanol withdrawal in rats. Biochem. Pharmacol. 49, 1665–1673.

Rodrigues-Filho, J.A., 2010. Alterações morfológicas e hormonais das gônadas e da hipófise da garoupa Epinephelus marginatus (Teleostei: Serranidae) durante a inversão sexual (Master's dissertation). University of São Paulo, São Paulo.

Rodrigues-Filho, J.A., Sanches, E.G., Garcia, C.E.O., Pannuti, C.V., Sebastini, E.F., Moreira, R.G., 2009. Threatened fishes of the world: *Epinephelus marginatus* (Lowe, 1834) (Serranidae: Epinephelinae). Environ. Biol. Fish. 85, 301–302.

Rohr, D.H., Lokman, P.M., Davie, P.S., Young, G., 2001. 11-Ketotestosterone induces silvering-related changes in immature female short-finned eels, *Anguilla australis*. Comp. Biochem. Physiol. 130, 701–714.

Roy, L.A., Steinert, S., Bay, S.M., Greenstein, D., Sapozhnikova, Y., Bawardi, O., Leifer, I., Schlenk, D., 2003. Biochemical effects of petroleum exposure in hornyhead turbot (*Pleuronichthysverticalis*) exposed to a gradient of sediments collected from a natural petroleum seep in CA, USA. Aquat. Toxicol. 65, 159–169.

Salvo, L.M., Severino, D., De Assis, H.C.S., Da Silva, J.R.M., 2016. Photochemical degradation increases polycyclic aromatic hydrocarbon (PAH) toxicity to the grouper *Epinephelus marginatus* as assessed by multiple biomarkers. Chemosphere 144, 540–547.

Senthilkumaran, B., 2015. Pesticide-and sex steroid analogue-induced endocrine disruption differentially targets hypothalamo–hypophyseal–gonadal system during gametogenesis in teleosts – a review. Gen. Comp. Endocrinol. 219, 136–142.

Setiawan, A.N., Ozaki, Y., Shoae, A., Kazeto, Y., Lokman, P.M., 2012. Androgen-specific regulation of FSH signalling in the previtellogenic ovary and pituitary of the New Zealand shortfinned eel, *Anguilla australis*. Gen. Comp. Endocrinol. 176, 132–143.

Sluka, R., Chiappone, M., Sullivan, K.M., 1994. Comparison of juvenile grouper populations in southern Florida and the central Bahamas. Bull. Mar. Sci. 54, 871–880.

Stegeman, J.J., Lech, J.J., 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ. Health Perspect. 90, 101–109.

Takashima, F., Hibiya, T., 1995. An Atlas of Fish Histology: Normal and Pathological Features. Kodanska, Tokyo, Japan.

Thiyagarajah, A., Hartley, W.R., Abdelghani, A., 1998. Hepatic hemosiderosis in buffalo fish (*Ictiobus* spp.). Mar. Environ. Res. 46, 203–207.

Thomas, P., 1988. Reproductive endocrine function in female Atlantic croaker exposed to pollutants. Mar. Environ. Res. 24, 179–183.

Tyler, C., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28, 319–361.

U.S. EPA. United States Environmental Protection Agency. https://www3.epa.gov.

Veintemilla, C.A.C., 2006. Impactos do fenantreno sobre o tambaqui Colossoma macropomum Cuvier, 1818: CL50, crescimento e hematologia (Master's dissertation). Instituto Nacional de Pesquisa da Amazônia (INPA), Manaus.

Walker, M.K., Peterson, R.E., 1991. Potencies of polychlorinated dibenzo-p-dioxin, dibenzofuran, and biphenyl congeners, relative to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, for producing early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 21, 219–237.

Wolke, R.E., 1992. Piscine macrophage aggregates: a review. Annu. Rev. Fish Dis. 2, 91–108.

Zagatto, A.P., Bertoletti, E., 2008. Ecotoxicologia Aquática - Princípios e Aplicações São Carlos, Brazil.

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