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Steroid Hormones Protect against Fluoranthene Ethoxyresorufin-O-Deethylase (EROD) Activity Inhibition

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Abstract: The physiological conditions of an organism may influence its ability to cope with environmental stressors, such as contaminants. Biotransformation and the endocrine system interact with each other to promote animal's fitness. However, little is known regarding the interaction between hormones and response to pollutants such as polycyclic aromatic hydrocarbons (PAHs). In this *in vitro* study, we aimed to increase the knowledge regarding the effects of steroid hormones on ethoxyresorufin-O-deethylase (EROD) activity inhibited by contaminants. The effects on *in vivo* induced EROD activity of *Anguilla anguilla* were assessed by conducting single and combined exposures to fluoranthene (FL) and to physiological levels of two major steroid hormones (cortisol and 17ß-estradiol). Hepatic microsome exposure to the lowest concentrations of FL (0.1 and 0.3 µM), as well as to cortisol and 17ß-estradiol (E_2), led to significant EROD activity induction. However, the highest tested concentrations of FL (0.9 and 2.7 µM) significantly inhibited this enzymatic activity. When microsomes were simultaneously exposed to 0.9 µM FL and one of the hormones, both cortisol and E_2 were able to decrease the inhibitory effects, with the former completely reverting EROD activity inhibition. These findings support the idea that cortisol and E_2 can help prevent the inhibitory effects of PAHs over biotransformation enzymes, highlighting the physiological relevance of these hormones.

Keywords: organic contaminants; endocrine regulation; biotransformation enzymes; *in vitro* effects

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1. Introduction

Despite the importance of water resources to all forms of life, they have been affected for decades by anthropogenic activity, continuously subjected to releases of xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAHs) [1,2]. This group of substances includes highly toxic contaminants that can reach the environment as a result of anthropogenic activities such as fossil fuel combustion, petroleum spills, industrial effluents, and highway runoff, although they can also be released into the environment from natural sources (e.g., forest fires and volcanic activity) [3]. Their persistence and bioaccumulation, along with their carcinogenic and mutagenic properties [1,4], are among the highest concerns associated with their environmental presence. In the last several years, environmental PAHs levels in aquatic and terrestrial ecosystems, as well as in air and in food [5], have increased due to rapid urbanization and increasing industrialization [3,6], being detected in significant concentrations. Among PAHs, fluoranthene (FL) is among the most abundant in human food and marine compartments. This substance, considered a priority pollutant in the European Water Framework Directive (2000/60/CE), has genotoxic potential [7].

After uptake by aquatic organisms, such as fish, PAHs undergo a biotransformation process, mainly in the liver [8], by phase I enzymes and cytochrome CYP1A monooxygenase enzymes, such as ethoxyresorufin-O-deethylase (EROD) [3,9], in an attempt to produce more hydrophilic compounds that are easier to excrete. CYP1A activity is regulated by

aryl hydrocarbon receptor (AhR) transcription factor, which is involved in the regulation of the genes that code for the enzymes involved in biotransformation [7]. EROD activity is frequently used as a biomarker of exposure to organic contaminants such as PAHs and structurally related compounds (e.g., ß-naphthoflavone (BNF)), which have been used to induce EROD activity in several studies [3,9,10]. However, EROD activity may be influenced in the natural environment by, for example, organisms' endogenous factors, including physiological status and endocrine levels [11]. Hormones play an essential role in CYP1A expression, either directly by influencing CYP1A gene expression or AhR function or indirectly via crosstalks with other transcription factors [12].

The physiological condition of a given organism may influence its ability to respond to environmental stressors (e.g., contaminants). The endocrine system plays an important role in animal fitness because exposure to pollutants often results in an endocrine stress response, which ensures the organism's survival by helping restore homeostasis. Primary stress, such as short-term exposure to contaminants, stimulates the hypothalamic–pituitary–inter-renal (HPI) axis, leading to the production of cortisol, a major stress hormone in fish that mobilizes energy required by increased metabolic rates [13–15]. Cortisol, the main glucocorticoid in fish, is also a key hormone for behavioral responses to recognized threats (e.g., predation threat) [16]. 17ß-estradiol (E_2), the most potent estrogen, is a key steroid hormone in both sexes, involved in growth, development, morphological differentiation, sexual behaviors, and cycles [17].

Thus, biotransformation and the endocrine system are expected to interact to promote the animal's fitness. Steroid hormones have the ability to cross the hepatocyte's cell membrane and may have a direct effect on microsomes. However, in fish, the relation between EROD activity and plasmatic and cellular levels of certain steroid hormones, such as cortisol and E_2, is poorly known. Such knowledge may be vital to a better understanding of the potential link between endocrine regulation, stress response to pollutants, and the ability to metabolize them. The protective effect of steroid hormones (cortisol and E_2) on EROD activity against benzo[a]pyrene-induced inhibition was recently reported [18]. However, its protection potential against other PAHs such as FL is unknown. Additionally, the data on the effects of FL on biotransformation enzymes are scarce. The available *in vitro* studies have reported FL's inhibitory effect on the hepatic EROD activity of fish [7,19].

In vitro methods are increasingly being recognized as valuable tools to assess the toxic effects of xenobiotics, as they allow the assessment of effects under controlled experimental conditions [20], are cost effective, less time consuming, and generate a smaller amount of toxic waste [21]. Furthermore, *in vitro* approaches allow reduction in the number of animals used for scientific purposes, in line with European Directive 2010/63/EU [19].

In this study, we aimed to assess the individual effects of FL (0.1, 0.3, 0.9, and 2.7 µM), cortisol, and E_2 (5.997 ng/mL) *in vitro*, as well as their combined effects on *Anguilla anguilla* liver microsomal EROD activity.

2. Materials and Methods

2.1. Animals

European eels (*Anguilla anguilla*) weighing approximately 650 (±36) g were captured at the Aveiro lagoon and transported to the laboratory. The fish were allowed to depurate and acclimate to laboratory conditions for 2 weeks, and kept in 80 L tanks with aerated, dechlorinated, filtered water (dissolved oxygen levels of 7.6 (±0.3) mg/L and pH 7.2 (±0.4)), at room temperature and natural photoperiod. The fish were not fed during acclimation or during the experimental period.

2.2. Liver Microsomes Isolations

All procedures involving fish were performed following the European directive 2010/63/EU legislation for the protection of animals used for scientific purposes. After the acclimation period, 4 female eels were intraperitoneally injected with ß-naphthoflavone (Sigma-Aldrich, Madrid, Spain) dissolved in DMSO (Sigma-Aldrich, Madrid, Spain) (4 mg

per kg of body weight) to induce high EROD activity. After 24 h, the eels were sacrificed and the liver was excised, frozen in liquid nitrogen, and stored at −80 °C. Liver microsomes were obtained according to previous reports [22,23], adapted by Pacheco *et al*. [24].

2.3. In Vitro Exposure Protocol

Exposures were performed in a quartz cuvette according to [19]. Stock solutions of FL (Sigma-Aldrich, Madrid, Spain) were prepared in DMSO. Cortisol (Merck) and 17β-estradiol (E_2) (Sigma-Aldrich, Madrid, Spain) solutions were prepared in Tris-HCl 0.1 M pH 7.4 with KCl 0.15 M and 20% glycerol (assay buffer) containing 0.5 µM ethoxyresorufin (substrate) (Roche) (Table S1). Assays were performed in a total volume of 1.1 mL. The final concentration of cortisol and E_2 in the test cuvette was 5.997 ng/mL, with concentrations based in previously reported fish plasmatic E_2 levels [15,25,26] and plasmatic cortisol levels [5,8,15]. For each condition, 5 µL of hepatic microsomes was used and the incubation period was set to 3 min. The description of the *in vitro* exposure protocol is presented in Table S2.

2.3.1. Single Exposures

Single exposures consisted of adding to 5 µL of microsomes: (a) 1090 µL of buffer substrate solution (BS) and 5 µL of buffer solution (B), used as a control; (b) 1090 µL of BS and 5 µL of dimethyl sulfoxide (DMSO), used as a DMSO control; (c) 1090 µL of BS with cortisol (at a final concentration of 5.997 ng/mL; BSC) and 5 µL of DMSO, to assess the effects of cortisol on EROD activity; (d) 1090 µL of BS with 17β-estradiol (at a final concentration of 5.997 ng/mL; BSE_2) and 5 µL of DMSO, to assess the effects of E_2 on EROD activity; and (e) 1090 µL of BS and 5 µL of fluoranthene (FL) solutions (to obtain the final concentrations of 0.1, 0.3, 0.9 and 2.7 µM), to assess the effects of FL on EROD activity.

2.3.2. Combined Exposures

Combined exposures consisted of adding to 5 µL of hepatic microsome suspension: (f) 1090 µL of BSC (at a final concentration of 5.997 ng/mL) and then 5 µL of FL solutions (to obtain the final concentrations of 0.1, 0.3, 0.9 and 2.7 µM) to assess the effects of cortisol on EROD activity challenged with each tested concentration of FL; (g) 1090 µL of BSE_2 (at a final concentration of 5.997 ng/mL) and then 5 µL of FL solutions (to obtain the final concentrations of 0.1, 0.3, 0.9, and 2.7 µM), to assess the effects of E_2 on EROD activity simultaneously exposed to each tested concentration of FL (Tables S1 and S2).

2.4. EROD Activity Determination

EROD activity was determined following the Burke and Meyer (1974) [27] protocol. The reaction, performed at 25 °C, was initiated by adding 10 µL of NADPH (Roche) (10 mM final concentration) to the incubating suspension in the cuvette, as described in Table S2. The resorufin formation was measured for 3 min (excitation wavelength 530 nm and emission wavelength 585 nm using a Jasco FP 750 spectrofluorometer) and EROD activity was expressed as picomoles per minute per milligram of microsomal protein. Liver microsomal protein concentrations were determined according to the Biuret method [28], using bovine serum albumin as the standard.

2.5. Statistical Analysis

Data were tested for normality and homogeneity of variance. Student's *t*-test was used to compare the results of the control with the solvent control (DMSO). One-way ANOVA, followed by a post hoc Tukey test, was performed to assess significant effects between the different treatment groups [29]. Results are expressed as mean ± standard error (SE). Sigmastat 2.03 was used for statistical analysis. Differences between groups were considered significant at $p < 0.05$.

3. Results

EROD activity in the solvent control did not significantly differ from control conditions (EROD activity of *A. anguilla in vivo* exposed to 4 mg/kg BNF (control) ($p > 0.05$, Student's *t*-test)). The average of the activities of controls in both assays was 7.02 ± 0.22 pmol/min/mg of protein, whereas for the solvent control, activity was 7.33 ± 0.16 pmol/min/mg of protein. Thus, the latter was used as the control. In the presence of FL, a biphasic EROD activity response was observed, as the addition of 0.1 and 0.3 µM FL significantly increased EROD activity (64% and 26%, respectively), whereas 0.9 and 2.7 µM FL led to significantly lower activities (14% and 35%, respectively) (Figure 1).

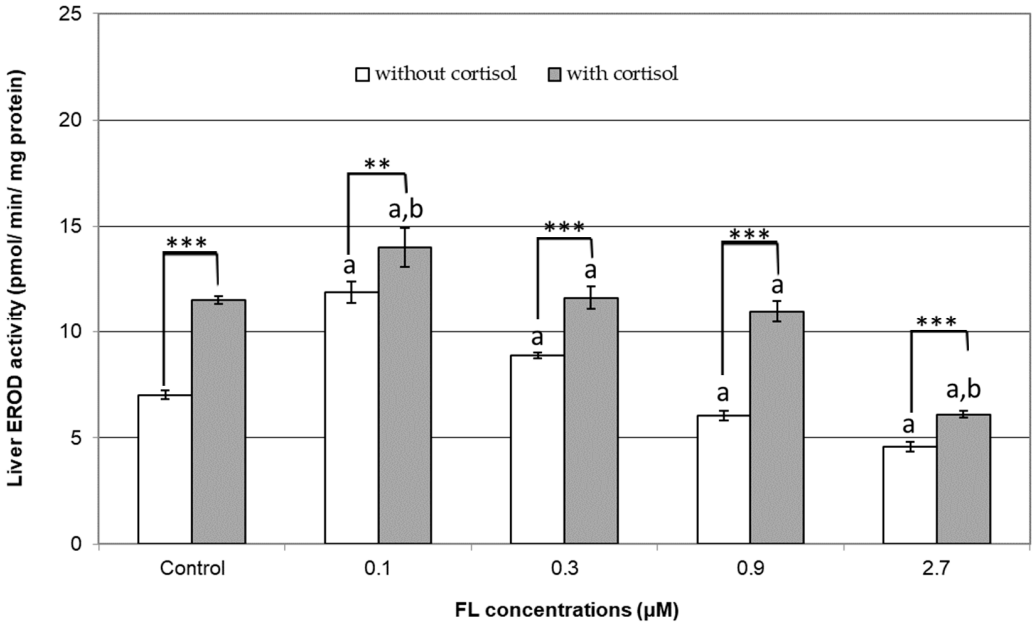


Figure 1. *In vitro* effects of fluoranthene (FL) on *A. anguilla* liver EROD activity in the presence and absence of cortisol (5.997 ng/mL). Bars represent mean ± SE ($n = 4$). Asterisks (*), significant differences between the presence and absence of cortisol (** $p < 0.01$; *** $p < 0.001$); a, differences from control without cortisol; b, differences from control with cortisol. One-way ANOVA followed by Tukey's post hoc test.

Exposing liver microsomes to cortisol significantly increased EROD activity, corresponding to a 64% increase compared to control levels (Figure 1). The presence of cortisol protected EROD activity from inhibition by FL. In the presence of cortisol, microsomes exposed to the tested concentrations of FL showed significantly higher EROD activities than those of individual exposures to FL. A complete recovery of this enzymatic activity to control (with cortisol) levels was observed for 0.3 and 0.9 µM FL, with the highest increase (86%) being achieved for 0.9 µM FL.

17β-estradiol (E_2) significantly increased hepatic EROD activity (Figure 2). 17β-estradiol (E_2) showed no interaction with co-exposure to lower concentrations of FL (0.1 and 0.3 µM), with enzymatic activities presenting levels very close to those with FL individual exposure. However, E_2 was able to partially revert the *in vitro* inhibition caused by 0.9 and 2.7 µM FL. The highest protection was observed at 0.9 µM FL, resulting in a 45% increase in activity when compared to FL alone. Despite this increase, it was not sufficient to reach the respective control levels (Figure 2).

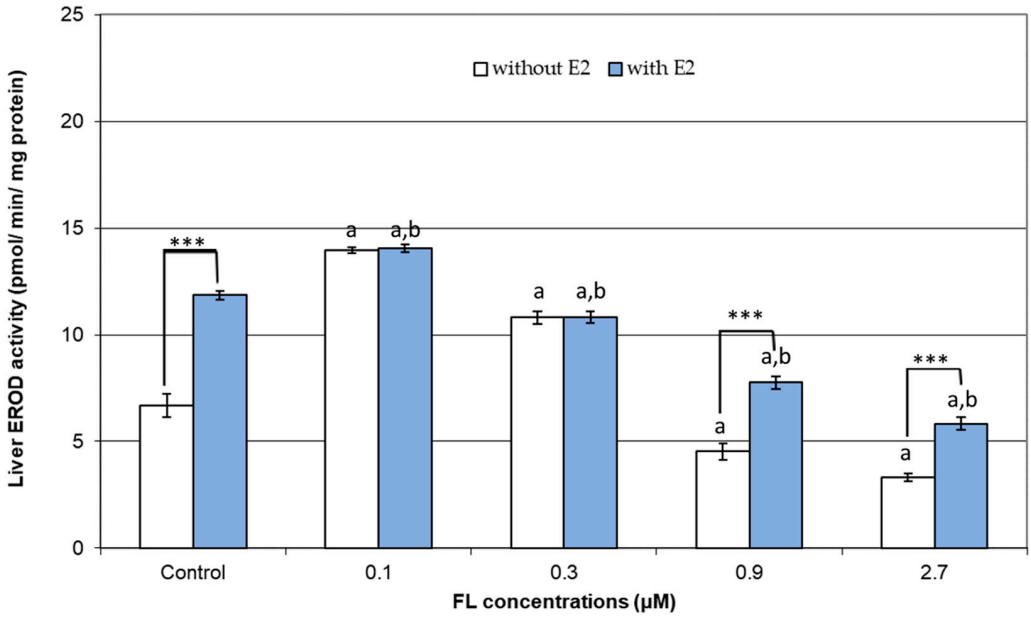


Figure 2. *In vitro* effects of fluoranthene (FL) on *A. anguilla* liver EROD activity in the presence and absence of E_2 (5.997 ng/mL). Bars represent mean ± SE (n = 4). Asterisks (*), significant differences between the presence and absence of E_2 (*** $p < 0.001$); a, differences from control without E_2; b, differences from to control with E2. One-way ANOVA followed by Tukey's post hoc test.

4. Discussion

Liver EROD activity is considered a valuable biomarker of exposure to organic contamination in fish [9] and is frequently used to assess phase I biotransformation. However, few studies have considered the effects of individual-related factors such as endocrine or hormonal individual status on biotransformation. The interaction between steroid hormones (cortisol and E_2) and the PAH benzo[a]pyrene (B[a]P) on EROD activity was studied by Ferreira *et al*. [18], who found that these hormones are, to some extent, able to protect EROD activity from inhibition caused by B[a]P. We aimed to investigate whether the protective effect of cortisol and E_2, observed for B[a]P, was also observed for other PAHs. FL was selected based on its reported ability to inhibit EROD activity *in vitro* [7,19]. Environmentally relevant concentrations of FL (0.1, 0.3, 0.9, and 2.7 µM) [18,30] and a physiologically relevant concentration of cortisol and E_2 (5.997 ng/mL) [18] were selected to assess the potential protective effects of these hormones on EROD activity challenged with FL. The data revealed that liver microsomal EROD activity can be increased *in vitro* in the presence of low concentrations of FL, up to 0.3 µM. However, higher concentrations elicit an opposite response, leading to significant activity inhibition as observed with 0.9 and 2.7 µM concentrations. Other studies have also demonstrated *in vitro* [7] and *in vivo* [19] inhibition of liver EROD activity by FL in *Solea solea* hepatocytes and in *Fundulus heteroclitus* isolated microsomes, respectively, although no significant effects on this enzymatic activity after *in vitro* exposure to FL have been reported [30]. The present study's results support the idea that FL, though able to induce EROD activity in low concentrations, can have a strong inhibitive power in high concentrations. This is most likely associated with an occurrence of non-competitive enzyme inhibition, as suggested by Willett and co-workers [19]. This possible mechanism for EROD inhibition is supported by FL IC50 and respective kinetic analysis [19] along with the lower affinity of FL for the AhR [7] function.

This inhibitory potential of FL agrees with other reports showing that PAHs such as B[a]P, which are typically CYP1A inducers, can also act as inhibitors [18,31], especially when occurring in high concentrations [7,18]. The ability of PAHs with a structure and weight similar to FL, such as phenanthrene, to induce EROD activity *in vivo* was previously reported by Oliveira *et al*. [32] in *Liza aurata* after short-term exposure to 0.3, 0.9, and

2.7 μM. However, the available data concerning CYP1A induction in fish by small PAHs (e.g., naphthalene, phenanthrene, pyrene) provided inconclusive and species-dependent results [7,19,33,34]. The present results showed that exposure to environmental levels of FL (0.9 and 2.7 μM) can affect EROD activity, which may lead to impaired biotransformation and detoxification. This may limit the relevance of its use as a biomarker in environments with high PAH levels.

Steroid hormones are endogenous factors with reported functions in regulating the expression of CYP1A [12]. However, there is scarce knowledge on the potential modulatory effect of steroid hormones such as cortisol and E_2 on EROD activity. In this *in vitro* study, cortisol and E_2 proved to be able to increase EROD activity and decrease the inhibition induced by 0.9 and 2.7 μM FL. These results are in line with those of previous *in vitro* studies that reported enhanced EROD activity after the addition of cortisol to the liver organ culture or microsomal suspension in *A. anguilla* [35]. Using the same *in vitro* approach as in the present study, cortisol and E_2 (5.997 ng/mL) were able to decrease the EROD activity inhibition by B[a]P [18], thus highlighting the protective and modulatory effect of these two steroid hormones on EROD activity compromised by exposure to PAHs. To the best of our knowledge, no *in vitro* study has assessed the impact of the presence of E_2 on EROD activity.

The other available studies with this hormone and EROD focused on the *in vivo* effects of this natural estrogen as an aquatic contaminant [15,36–38], reporting, in this context, E_2 as a suppressor of EROD activity [15,38].

Overall, the present study results showed that cortisol and E_2 have the potential to revert the inhibitory effects of 0.9 and 2.7 μM FL on EROD activity. Cortisol was able to fully recover hepatic EROD activity inhibited by 0.9 μM FL, whereas E_2, although not promoting a full reversion of the effects, lead to EROD activity that was 45% higher than in the condition with FL alone. However, neither cortisol nor E_2 were able to fully revert the inhibitory effect of 2.7 μM FL. The data suggest that cortisol may have a stronger protective role on this enzymatic activity.

A modulatory and protective effect of steroid hormones over biotransformation enzymes was demonstrated in this study, emphasizing the potential relevance of the organisms' hormonal levels on this enzymatic activity and thus on its reliability as a biomarker of exposure to organic contamination.

Further studies are needed to explore other physiologically relevant conditions, such as distinct mixtures of hormones and different hormone concentrations, able to fully recover hepatic EROD activity inhibited by other contaminants (e.g., pharmaceuticals and metals) in order to provide a better understanding of the relation between endocrine regulation and its effective protective role against the toxic mechanism of action of pollutants towards EROD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12063098/s1, Table S1. Buffers, solvents and solutions used during Experimental Setup, Table S2. Liver microsomal EROD activity assay procedures to assess effects of cortisol, 17β-estradiol (E2) and fluoranthene (FL), alone or in combination.

Author Contributions: Conceptualization, supervision, resources, methodology, and funding acquisition, M.A.S., M.P., and M.O.; investigation, data curation, visualization, and writing—original draft preparation, C.S.S.F.; formal analysis, C.S.S.F. and M.O.; validation, C.S.S.F., M.A.S., M.P. and M.O.; writing—review and editing, M.A.S., M.P., and M.O. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experimental procedures followed International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63) and were previously approved by the ethics committee and the responsible national legal authority "Direção Geral de Alimentação e Veterinária" (authorization N421/2013).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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