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Effects of Exposure to PAHs on Brain AChE in Gilthead Seabream, *Sparus aurata* L., Under Laboratory Conditions

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Abstract The purpose of this research was to assess the effects of exposure to selected PAHs (phenanthrene, pyrene, and fluorene) on brain acetylcholinesterase (AChE) in juvenile gilthead seabream (*Sparus aurata*) in short term in vivo experiments. The measured brain AChE activity was within the same range (110–200 nmol min^{-1} mg $prot^{-1}$) in all the considered treatment conditions and in the control. The only statistically significant difference in AChE activity with respect to the control group was its induction in response to the lowest tested pyrene concentration in one of two replicated studies. These results suggest that exposure to PAHs does not apprreciably affect brain AChE activity.

Keywords AChE · Seabream · PAH · Experiment

Polynuclear aromatic hydrocarbons (PAHs) are commonly occurring environmental contaminants, many of which are known to be carcinogenic. However, their accumulation in individual vertebrates is limited because they are quickly metabolized; therefore, biomarkers must be used in order to assess the exposure to PAHs. Biomarker responses to petroleum PAHs, dominated by two- and three-ringed aromatics, can be quite different from the response to pyrogenic PAHs, dominated by four- and five-ringed aromatics. Therefore, in order to provide results most relevant from the point of view of ecotoxicology, in our research we focussed on a selection of those "less toxic" PAHs: phenanthrene (Phe), pyrene (Pyr), and fluorene (Fluo). Phe, Pyr and Fluo occur in fossil fuels and are present in products of incomplete combustion. Fluo has 2 benzene rings, Phe has 3 benzene rings, while Pyr has 4 benzene rings, and all three are considered non-carcinogenic PAHs, in contrast to most high-molecular-weight PAHs. Nevertheless, their potential impact is such that they are included in the U. S. Environmental Protection Agency's (EPA) priority pollutant list.

Acetylcholinesterase (AChE) is a ubiquitous serine hydrolase which physiologically removes acetylcholine (ACh) from the synaptic cleft; for this reason, inhibition of brain AChE must be considered a potential adverse toxicological effect. Inhibition of AChE is typically associated with the exposure of organisms to organophosphate (OP) and carbamate (CB) compounds. However, inhibition of AChE has been recently reported, under both laboratory and field conditions, in reaction to metals (e.g. Guilhermino et al. 1998; Bocquené and Galgani 1998), detergents (Guilhermino et al. 1998) and algal toxins (e.g. Bocquené and Galgani 1998). The effects of PAHs on AChE activity has been investigated by some authors, even though it is known that PAHs cause toxicity via narcosis and would not, apriori, be predicted to affect AChE activity directly. Indeed, in various kinds of studies (field studies or laboratory in vivo and in vitro exposures) either AChE inhibition (Zapata-Pérez et al. 2004; Vieira et al. 2008; Kang and Fang 1997) or positive correlation with PAH exposure was found (Buet et al. 2006). Some authors set forth a well-justified hypothesis that AChE inhibition observed in their field studies might have been caused by combustion-type hydrocarbons originating from exhaust soots, used engine oil, etc. (Payne et al. 1996). Other authors observed no effects of PAHs on AChE (e.g. Jifa et al. 2006).

This study was conducted to address the possible role of selected PAHs upon AChE activity. AChE activity is used

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in biomonitoring studies in estuarine and coastal ecosystems contaminated with petrochemical products (e.g. Monteiro et al. 2007), and thus comprehensive investigation of the influence of PAHs on AChE is necessary for making correct risk assessmens in field studies. The goal of our study was to measure the response of brain AChE to the selected individual PAHs and to their mixture at environmentally relevant concentrations, in short term laboratory in vivo experiments, in order to provide evidence to support or refute the results from other studies, and to contribute towards determining the Lowest Observed Adverse Effect Level (LOAEL) and the No Observed Adverse Effect Level (NOAEL) for this stressor-biomarker combination.

Materials and Methods

Juvenile gilthead seabream (*Sparus aurata*) was used as a model species in this study. This is a protandrous hermaphroditic species widely cultured in Europe and often used as a model organism in academic experiments. Juvenile fish of this species are preferred for laboratory use in order to rule out the influence of their sex on biomarkers. About 5 month old fish were obtained from TIMAR Lda. (Setúbal, Portugal). Until the start of the exposures, the fish were kept in 2,200 L fiber aquaria supplied with filtered seawater (35 ± 2 ppt) and fed with food pellets (Aquasoja, Portugal). Fish used to conduct independent exposure studies had an average body length around 2 ± 0.3 cm and weight around 6 ± 0.3 g.

Waterborne exposures were conducted in 17 L glass aquaria (filled to 9 L) under semi-static conditions. The experiments were conducted at a temperature of 16 ± 1°C in filtered seawater (35 ± 2 ppt) under a photoperiod of 12 h light:12 h dark. Five randomly chosen fish were acclimatized to each test aquarium for 24 h with aeration (pre-exposure phase). Afterwards, fish were exposed to the test chemicals for 4 days. Five aquaria per treatment and solvent control (acetone) were used in each experiment in order to account for inter-aquarium variability. Food was not provided during the acclimation and in the course of exposures, a generally accepted practice in this kind of short-term experiments (see e.g. Vieira et al. 2008). After 4 days of exposure to the tested chemicals, fish were killed and brains from 3 fish per tank were collected and immediately frozen in cryovials in liquid nitrogen. All samples were then stored at −80°C until the analyses.

Master stock solutions (25 mL each) of chemicals were prepared in acetone (analytical grade) and stored at −20°C. Exposures were renewed daily in natural filtered seawater (50% of total volume). The chemical solutions were regularly prepared from aliquots of the master solution, diluted in a final volume of 126 µL acetone (an equal amount of acetone was used for the control aquaria) and 4.5 L of the water, and administrated directly into the aquaria at water renewal. Dissolved oxygen saturation (>80%) and total ammonia concentrations were (<0.5 mg L^{-1}) monitored every 2 days.

Each individual compound was tested at two exposure concentrations in the first and in the second study, with one concentration being retested in the second study. Due to laboratory and staff constraints, we were only able to conduct two exposure tests at a time, thus splitting the entire experiment into two separate studies. One of the concentrations was repeated in order to determine variation between the experiments; inconsistent results would indicate that other random factors affected AChE activity more considerably than the PAH exposure (indeed, different results were obtained in some cases, as illustrated in Fig. 1). Three concentrations were tested for each PAH, based on environmental relevance and test results from the literature.

Two consecutive studies with each PAH or PAH mixture were conducted with the following nominal concentrations (solvent-control was run in parallel for each study):

- Phe – 1st study: 0.11 µM (20 µg L^{-1}) and 0.56 µM (100 µg L^{-1}); 2nd study: 0.11 µM (20 µg L^{-1}) and 0.28 µM (50 µg L^{-1});
- Pyr – 1st study: 0.02 µM (5 µg L^{-1}) and 0.12 µM (25 µg L^{-1}); 2nd study: 0.02 µM (5 µg L^{-1}) and 0.25 µM (50 µg L^{-1});
- Fluo – 1st study: 0.30 µM (50 µg L^{-1}) and 1.20 µM (200 µg L^{-1}); 2nd study: 0.12 µM (20 µg L^{-1}) and 0.30 µM (50 µg L^{-1});
- PAH mix (Phe, Pyr, Fluo) – 1st study: 0.15 µM (26 µg L^{-1}) and 0.44 µM (77 µg L^{-1}); 2nd study: 0.15 µM (26 µg L^{-1}) and 0.22 µM (39 µg L^{-1}).

At the daily renewal of the 50% of the water, it was assumed that no measurable residue was remaining; however, the chemical analyses, conducted with a standard method using a Varian gas chromatograph, revealed the remaining concentrations to range from 6–12% for Phe, 2–9% for Pyr, and 10–22% for Fluo, which implies that the actual concentrations in the experiments were slightly higher than the nominal ones (see Gonçalves et al. 2008 for the description of the chemical analysis method and for the exact results).

Appropriate test concentrations of individual chemicals were chosen on the basis of reported short-term toxicity data, taking also into consideration the actual concentrations found in the environment (Neff et al. 2005). In their paper, the chronic and acute toxicities of the three PAHs were given as: Phe – 55 and 367 µg L^{-1}, Pyr – 12 and 61 µg L^{-1}, and Fluo – 150 and 730 µg L^{-1}, respectively

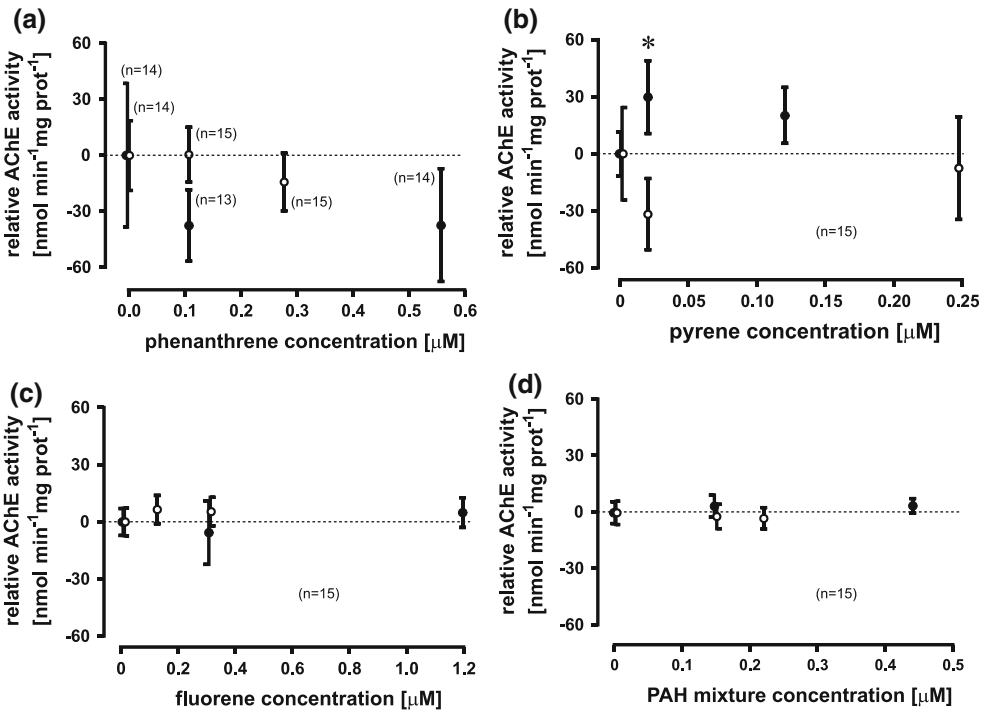

Fig. 1 Relative average activity (normalized to control values) of brain AChE in juvenile seabream exposed to **a** phenanthrene (0.11, 0.28, and 0.56 μM), **b** pyrene (0.02, 0.12, and 0.25 μM), **c** fluorene (0.12, 0.30, and 1.20 μM) and **d** the mix of PAHs (0.15, 0.22, and 0.44 μM). The number of replicates n = 15, except where indicated otherwise. *Error bars* show the mean with the 95% confidence belts, with *closed circle* indicating the first, and *open circle* the second study. The only statistically significant difference found ($p < 0.01$) is indicated with an *asterisk*, the other results are not statistically significant ($p > 0.05$)

(as in Table 3 in that paper). In two porewater samples from sediments collected from Eagle Harbor, Washington, USA, they found the following concentrations of the three PAHs of our interest: Phe – 100 and 9 μg L^{-1}, Pyr – 420 and 52 μg L^{-1}, and Fluo – 3190 and 420 μg L^{-1}, respectively (see Table 5 in their paper for details).

The proportions of the three PAHs in the mixture were taken as 34% Phe, 10% Pyr, and 56% Fluo, as recommended by Gonçalves et al. (2008) on the basis of a concentration addition model applied to the analysis of fish behavior for the individual components.

Phe (≥97% purity), Pyr (98% purity) and Fluo (98% purity) were purchased from Sigma–Aldrich (St. Louis, USA) and were of analytical grade. All other chemicals were of analytical grade and obtained from Sigma Chemical, Boehringer (Germany), and E. Merck-Darmstadt (Germany).

For measurement of AChE activity, about 30–35 mg wet weight of brain tissue was sampled from each fish, and homogenized on ice in 0.05 M phosphate buffer KH_2PO_4/K_2HPO_4 (PB AChE, pH = 7.4) at a weight:volume ratio of 1:8 using an electric homogenizer. The homogenate was then centrifuged at 10,000 g in the temperature of 4°C for 30 min., and stored at −80°C (for 1 week or less) until the AChE activity measurements were conducted.

The procedure used in our study to determine AChE activity was that of Ellman et al. (1961), modified for microplate readers by Bocquené and Galgani (1998). The enzyme kinetics was monitored at $\lambda = 405$ nm for 5 min after the reactions involving AChE in 25 μL S9 (diluted 1:10), or 25 μL 0.05 M PB AChE (blank), with 150 μL 270 μM 5,5′-dithiobis-2-nitrobenzoate (DTNB), and after 2 min incubation with 50 μL 4.5 mM acetylthiocholine (ACTC).

Total protein concentration was determined in the S9 fraction with the method by Lowry et al. (1951) adapted to microplates, with bovine serum albumin (BSA) as a standard. All protein measurements were carried out using a microplate reader (BIOTEK PowerWave 340). Absorbance was read at $\lambda = 595$ nm.

Due to the fact that some cases did not have homogeneous variances as determined by Shapiro–Wilk's and Levene's tests, data were analyzed by the non-parametric Kruskall-Wallis test, followed by Dunn's non-parametric *post hoc* multiple comparison test for selected pairs in data sets with n < 30. All statistical calculations were carried out using the StatSoft Statistica® 6.0 software package, while Dunn's tests were performed using GraphPad InStat.

Results and Discussion

Relative AChE activity for each study is illustrated in Fig. 1. The values were normalized with respect to control, which was set at level 0. The measured mean activity of brain AChE in seabream was within similar range (110–200 nmol min^{-1} mg $prot^{-1}$) in each study and no specific trend (inhibition or induction) in AChE activity changes for specific PAH compounds nor the mixture was found (see Fig. 1). Only one statistically significant

difference ($p < 0.01$) in AChE activity between the fish treated by PAHs and control was recorded; it was in the experiment with Pyr at the concentration of 0.02 μM (1st study), where induction was found. However, since in the 2nd study with the same concentration of Pyr, substantially different changes in AChE activity were observed (values somewhat lower than in control, but the changes were not statistically significant, $p > 0.05$), we consider the former an odd coincidence, especially that no statistically significant differences ($p > 0.05$) were found in any other treatment conditions. In risk assessment terms, we gave evidence that all the tested concentrations of the selected PAHs are below the NOAEL (and therefore also the LOAEL), as far as the short-term effect on AChE activity is concerned.

It is known that due to their main mode of action (narcosis), PAHs should not be a priori predicted to affect AChE activity directly. However, the fact that some correlations between AChE activity and PAH concentrations were observed by some authors, suggests a possible indirect effect of PAHs on this biomarker. Unfortunately, no definitive answer to this question has been found so far, not only because of a very limited number of studies conducted in this direction, but primarily because inconsistent results were obtained by different authors. Moreover, the mechanism of this influence, if any, still remains unclear, and various conjectures or speculations can be found in the literature.

Variable results are reported in field studies. The study conducted by Buet et al. (2006) showed many positive correlations between muscle AChE activity in European eel and PAH contamination, indicating a possible unspecific enzymatic activation. On the contrary, Zapata-Pérez et al. (2004) reported negative correlation between AChE activity in the muscles of Nile tilapia (*Oreochromis niloticus*) and PAH concentration in a case study conducted in four lagoons in Mexico. This contradiction suggests that the influence of other factors on AChE activity might be much stronger than PAH exposure.

Payne et al. (1996) found lower AChE activity in female fish in the vicinity of a pulp and paper mill, in comparison to an uncontaminated site. Since they ruled out the contamination by pesticides and inorganics, they suspected either hydrocarbons from used engine oils, or substances originating from wood chips from spruce trees as factors responsible for the inhibition of AChE. They also observed distinctly higher levels of mixed-function oxygenase (MFO) in the same group of leading to their conjecture that products of contaminant biotransformation by the MFO enzyme system might be playing a role in AChE inhibition.

In vitro experiments conducted by Kang and Fang (1997) with exposure of AChE purified from electric eel to a selection of PAHs showed dose-dependent inhibition. These authors reported that PAHs with three or more aromatic rings were potent AChE inhibitors, while those with fewer aromatic rings (e.g. Fluo) showed no or slight inhibition of AChE activity.

In spite of the clear in vitro results, in vivo experiments have provided variable results. Some authors observed no effects on AChE activity. An example is Jifa et al. (2006) in an experiment with the fish *Lateolabrax japonicus* exposed to benzo[*a*]pyrene (B[*a*]P) at 2 and 20 μg L^{-1} for 6, 12, and 18 days. Other authors have reported on the inhibition of brain AChE activity. Veira et al. (2008) in their laboratory experiments with the common goby (*Pomatoschistus microps*) exposed for 96 h to B[a]P found inhibition at 2, 4, 8, and 16 μg L^{-1} (no effect was observed at 1 μg L^{-1}), to anthracene – at 4 μg L^{-1} (with no effect at concentrations 2 μg L^{-1} and less), and with the water accommodated fraction of #4 fuel–oil (#4 WAF), used as an example of a petrochemical mixture – at all the tested concentrations (7.5%, 15%, and 30%).

The results of our experiments suggest that the short-term effect of Phe, Pyr and Fluo is very weak, at least at environmentally relevant concentrations. Our results confirm that this biomarker measured in brain tissue is not a good indicator of exposure to PAHs in field studies, as we provided evidence that the lower bound for the NOAEL (and thus also for the LOAEL) is relatively high for this stressor-biomarker combination, in comparison to concentrations of PAHs that are likely to appear in the environment.

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