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Effects of selected xenobiotics on hepatic and plasmatic biomarkers in juveniles of *Solea senegalensis*

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ABSTRACT

In recent years, *Solea senegalensis* has increasingly been used in pollution monitoring studies. In order to assess its response to some particular widespread pollutants, juveniles of *S. senegalensis* were administered an intraperitoneal injection of the model aryl hydrocarbon receptor agonist β-naphtoflavone (βNF) and chemicals of environmental concern, such as the fungicide ketoconazole (KETO), the lipid regulator gemfibrozil (GEM), the surfactant nonylphenol (NP) and the synthetic hormone ethinylestradiol (EE_2). Two days after injection, the effect of these chemicals was followed up as alterations of hepatic microsomal activities of the cytochrome P450 (CYPs) and associated reductases, carboxylesterases (CbEs) and the conjugation enzyme uridine diphosphate glucuronyltransferase (UDPGT). In the cytosolic fraction of the liver, the effect on CbEs, glutathione *S*-transferase (GST) and antioxidant activities was also considered. Alterations on the endocrine reproductive system were evaluated by plasma levels of vitellogenin (VTG) and the sex steroids estradiol (E_2), testosterone (T), 11-ketotestosterone (11KT) and the progestin 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P). Injection with the model compound βNF induced the hydrolysis rate of the seven CYP substrates assayed. The xenobiotic GEM induced three CYP-related activities (e.g. ECOD) and UDPGT, but depressed antioxidant defenses. EE_2 induced four CYPs, more significantly ECOD and BFCOD activities. The xenoestrogens NP and EE_2 altered the activities of CbE in microsomes and catalase, and were the only treatments that induced de novo VTG synthesis. In addition, the progestin 17,20β-P, was induced in NP-injected fish. None of the treatments caused statistically significant effects on steroid plasma levels. In conclusion, the CYP substrates assayed responded specifically to treatments and juveniles of *S. senegalensis* appear good candidates for assessing xenobiotics exposure.

1. Introduction

The action of man-made chemicals (xenobiotics), including pharmaceuticals, over non-target species has experienced growing concern, mainly due to the demonstrated interaction of these chemicals over the xenobiotic as well as endogenous metabolic pathways and steroidogenesis (Celander, 2011; Corcoran et al., 2010). Despite recent efforts in fish, little is known on the metabolism of some of these chemicals, belonging to different chemical classes and with different modes of action, over the cytochrome P450 (CYPs) isozymes (Smith et al., 2010, 2012; Smith and Wilson, 2010). Some in vitro studies in fish have strongly suggested the interaction of these chemicals over CYPs (Hegelund et al., 2004; Thibaut and Porte, 2008; Thibaut et al., 2006). In mammals, most drugs are metabolized by CYP1-4 families and in the marine fish Solea spp. the most relevant forms described so far are CYP1A1 and/or CYP3A4 (Costa et al., 2012; Koenig et al., 2013; Ribecco et al., 2012; Solé et al., 2012). Despite some exceptions, fish are expected to respond to mammalian CYPs inducers/suppressors in a similar way, as they share a high degree of homology in receptor sequences (Christen et al., 2010).

The aryl hydrocarbon receptor (AhR) agonist, β-naphthoflavone (βNF), has been largely used as model in fish for CYPs characterisation (Novi et al., 1998; Pretti et al., 2001; Smith and Wilson, 2010). The xenobiotic ketoconazole (KETO) belongs to the family of azole fungicides and in fish, it is recognized to interact with CYP1A and CYP3A related activities (Hasselberg et al., 2004, 2005, 2008; Hegelund et al., 2004). Gemfibrozil (GEM) is a drug from the fibrate class of lipid regulators, which are shown to act as a peroxisome proliferator and interact in vivo and in vitro with the fish xenobiotic and endogenous metabolizing system (Mimeault et al., 2006; Thibaut et al., 2006; Smith et al., 2012; Lyssimachou et al., 2014). The xenoestrogen 4-nonylphenol (NP) is the final degradation product of alkylphenol polyethoxylates (APEs). Its use was phased out by agreement of the European Union (EU)

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members in the 1990s due to its endocrine-disrupting properties (Nimrod and Benson, 1996), but it is still present in the marine environment. The synthetic hormone ethinylestradiol (EE_2), component of the contraceptive pill, is of environmental concern due to its high estrogenic activity in fish at very low concentration (ng/l) (Larsson et al., 1999). Both, NP and EE_2 are considered endocrine disruptors, but they also interact with enzymes involved in xenobiotic metabolism (Arukwe et al., 1997; Mortensen and Arukwe, 2007). With the exception of βNF, the above described chemicals are of environmental concern, either because of their use as fungicides (KETO), persistence, bioaccumulation and estrogenic properties (NP), estrogenic effects at very low levels (EE_2) and continued and enhanced release into the environment (GEM).

The most currently selected biomarkers in pollution monitoring studies are those from the enzymatic pool involved in the metabolism of foreign (and endogenous) chemicals. Among them, the specific activity of CYP isoforms can be screened using a panel of fluorometric substrates (Smith and Wilson, 2010) and the effects on the overall cytochrome P450-dependent monooxygenase (MO) system, as alterations in NAD(P)H cytochrome c and NADH ferricyanide reductase activities (Solé and Livingstone, 2005). Hepatic carboxylesterases (CbEs) are also phase I reactions involved in endogenous and drug metabolism (Wheelock et al., 2008). Ensuing phase II reactions, such as uridine diphosphate glucuronosyltransferase (UDPGT) and glutathione *S*-transferase (GST) activities are involved in the conjugation and elimination of endogenous and exogenous chemical by-products (Gonzalez et al., 2009). Enhanced oxyradical production can result from xenobiotic's metabolism and it is buffered by the enzymatic antioxidant defenses such as catalase, glutathione reductase and glutathione peroxidase (Livingstone, 2001).

Many molecules, including some of the xenobiotics formerly described, have been reported to act as endocrine disrupting chemicals (EDCs), affecting the reproductive system of fish. The most widely used biomarker for detection of EDCs is the analysis of the egg-yolk protein vitellogenin (VTG) in the blood of male fish as an unequivocal sign of the presence of EDCs in the aquatic environment (Sumpter and Jobling, 1995). Moreover, circulating levels of sex steroids are known to be affected by many EDCs (Jalabert et al., 2000). Steroids are key reproductive hormones involved in the regulation of all processes of reproduction (i.e., gonad differentiation, puberty, gametogenesis and gonad maturation). The process of oogenesis in females is mostly estrogen-dependant (i.e., estradiol (E_2)), spermatogenesis in males is androgen-dependant (testosterone (T) and 11-ketotestosterone (11KT)) and that of final gonad maturation and gamete release is progestin-dependent (i.e. 17,20β-P) (Scott et al., 2010). Alteration of the steroid balance caused by exposure to EDCs may lead to critical dysfunctions of the fish reproductive system.

Flatfish species have been selected as sentinels in pollution monitoring studies in Northern latitudes. In Southern latitudes of Europe, the benthic flatfish *Solea senegalensis* has been chosen as sentinel in several field and laboratory studies in relation to pollution assessment (Lopez-Galindo et al., 2010; Fonseca et al., 2011; Costa et al., 2012; Oliva et al., 2012). It is also an important species in fisheries and aquaculture, due to its high market prize, good adaptability and high growth rates in captivity (Dinis et al., 1999). However, to the best of our knowledge, the effects of particular chemicals of environmental interest over its xenobiotic metabolizing system and reproductive axis have not yet been described.

To fill this gap of information, the aim of the study was to assess the interaction of chemicals of environmental concern, with different mechanisms of action, over two closely related pathways: (1) the hepatic biotransformation enzymatic system and (2) the reproductive axis, on juveniles of the marine benthic fish *S. senegalensis*. To this end, the suitability of the commercially available CYP substrates and the adequacy of this species as sentinel of environmental pollutants, including EDCs, in future monitoring field studies is discussed.

2. Materials and methods

2.1. Fish maintenance

S. senegalensis, hatched and reared under aquaculture conditions (Stolt Sea Farm SA, La Coruña, Spain), were transported to the fish facilities of the University of Valencia (Spain) for experimentation. Specimens of 150–200 g body weight (BW) were distributed in two squared 4000 L tanks and maintained for 4 months before initiation of treatments. Physical water conditions were checked daily and maintained constant: temperature 19.4 °C ± 0.6, salinity 30.5‰ ± 1.0, oxygen 97.8% ± 1.7 and pH 7.8 ± 0.1. Fish ($n=41$) were fed with Le-5-Elite trout pellets (Skretting, UK). Handling of the fish was done according to national and institutional regulations of the Spanish Council for Scientific Research (CSIC) and Directive 2010/63/EU.

2.2. Injection of fish

The use of sunflower oil as carrier was based on previous studies by Lyssimachou et al. (2014). Fish were administered a single intraperitoneal (IP) injection of either sunflower oil alone (carrier) or in solution containing the following chemicals: βNF (CAS 6051-87-2) and KETO (CAS 65277-42-1) at 10 mg/kg BW, GEM (CAS 25812-30-0) and EE_2 (CAS 57-63-6) at 1 mg/kg BW and NP (CAS 84852-15-3) at 25 mg/kg BW. EE_2 was formerly dissolved in ethanol and this solvent evaporated to dryness under a gentle N_2 stream before being dissolved in sunflower oil. Chemicals were provided by Sigma-Aldrich, Madrid, Spain. Injection volume was 1 μl/g BW in all cases. An additional control group was established with fish that were not injected but were subjected to all other manipulations. Biometrics (total weight and total length) and blood samples were taken just before sacrifice. The metric parameters were used for condition factor (CF) calculation. Sacrifice was made two days after injection by spinal cord severing and fish were immediately dissected. After the injection, and during the 48 h before sacrifice, fish were kept in separate tanks for each condition to avoid cross-contamination. Livers were weighted and flash frozen in liquid nitrogen and stored at −80 °C until analysis. Liver weight was used for hepatosomatic index (HSI) calculation. Selection of the injected doses and length of exposure was based on formerly published data on other fish species.

2.3. Sample preparation

Microsomes and cytosol fractions were prepared from individual livers of juveniles of *S. senegalensis*. Livers (≈ 1–1.5 g) were homogenised in ice-cold buffer phosphate (100 mM pH 7.4) containing 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenatroline, 0.1 mg/ml trypsin inhibitor and 1 mM ethylenediaminetetraacetic acid (EDTA) in a 1:4 (w:v) ratio using a polytron® blender. The obtained homogenate was centrifuged at consecutive steps of 10,000*g*x30 min and 100,000*g*x60 min at 4 °C. The microsomal pellet obtained was dissolved in the former homogenization buffer, containing 20% glycerol in a 1:1 (w:v) ratio. Blood (about 1 ml) was taken using heparinised syringes and dispensed on ice-cold heparinised tubes containing 1% aprotinin (Sigma-Aldrich), to avoid proteolysis. The plasma was obtained by centrifugation (3000*g* × 15 min, 4 °C) and stored at −80 °C until analyses.