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Cloning and transcription of nuclear receptors and other toxicologically relevant genes, and exposure biomarkers in European hake (*Merluccius merluccius*) after the Prestige oil spill

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ABSTRACT

In November 2002 the tanker Prestige released more than 60,000 t of a heavy fuel oil which spread over Galician waters and the Biscay Bay, affecting coastal ecosystems. Polycyclic aromatic hydrocarbons are the main components of the Prestige fuel oil and induce biotransformation metabolism and peroxisome proliferation in marine organisms. In vertebrates, this later response involves peroxisome proliferator-activated receptors (PPARs), transcription factors belonging to the nuclear receptor superfamily, that act upon heterodimerization with the retinoid X receptor (RXR). In order to assess the possible biological effects of the Prestige oil spill in the Biscay Bay, male and female juvenile and adult European hakes *Merluccius merluccius* were sampled in June and December 2004 and 2005. PCR screening of hake liver cDNA with degenerate primers resulted in cloning and sequencing of cDNA fragments of PPARα (1011 bp), PPARγ (812 bp), RXR (270 bp) and of the PPARα target gene palmitoyl-CoA oxidase (AOX1, 792 bp). Fragments of another 9 toxicologically relevant genes were also cloned and sequenced. PPARα mRNA expression was not significantly different among groups. In juvenile females transcription of PPARγ, RXR and AOX1 significantly increased in June 2005 when compared to June 2004. In adult males levels of AOX1 decreased in the same period. AOX1 and 7-ethoxyresorufin O-deethylase (EROD) activities, measured as exposure biomarkers, differed between years only in males sampled in June. EROD activity was higher in 2004 than in 2005 in adults, whereas both juvenile and adults showed higher AOX1 activity in 2005. The lack of historical data previous to the accident or in areas not affected by the accident did not allow to relate observed variations in gene transcription levels and enzyme activities to the Prestige oil spill. Reported data could be useful for comparison purposes for future studies in European hake and contributes gene sequence information relevant for future toxicological studies.

1. Introduction

In November 2002 the Prestige tanker sank 130 mi offshore of Galicia (42°16'N; 12°07'W) releasing in the following months more than 60,000 t of a heavy fuel oil that spread over Galician waters and the Biscay Bay up to Brittany. When compared to previous oil spill events, the volume of oil spilled by the Prestige, the persistence of its chemical constituents, its extent over time, and the extension of the area affected make the Prestige oil spill unique (Marigómez et al., 2006), with potentially harmful effects in affected ecosystems. In order to assess the biological effects of this oil spill in the Biscay Bay, European hakes *Merluccius merluccius* were collected in the ICES fishing area VIIIb. According to early estimations (March–April 2003) of fuel deposited in the sea bottom, the East Cantabrian Sea of the Biscay Bay (ICES VIIIb area) received a lesser amount of fuel (31.5 t recorded on platform) than Galicia (176.4 t in Miño–Finisterre platform) but more than the West Cantabrian area (0 t in Estaca–Peñas platform) (Sánchez, 2003).

M. merluccius was selected for the study because it is both commercially and ecologically one of the most important demersal marine fish species in the area (Sánchez and Gil, 2000). Its diet is mainly composed of finfish and bony fish, but also of zoobenthos and other benthic crustaceans, zooplankton and nekton (Guichet, 1995). The ICES fishing area VIIIb constitutes the largest nursery of European hake (Sánchez and Gil, 2000), where it has long been suffering from over-fishing (Muus and Nielsen, 1999). Thus, its spawning biomass has been in recent years at a historical low level and outside safe biological limits (ICES, 2007). Hakes studied in the present work ranged between 17 and 63 cm in length. Thus, based on the works of Lucio et al. (2000) and Murua (2006) on the relationship between mean length and maturity in *M. merluccius*, hakes studied here were juveniles and/or young adults during the Prestige oil spill. Studies during the first year after the Prestige oil spill (2003) indicated that hakes and their prey in the Biscay Bay were in contact with the fuel oil (Sánchez et al., 2006).

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The Prestige oil is a heavy fuel type M-100 (according to Russian terminology), number 6 (UK classification) or number 2 (French classification). It is a residual product from oil distillation containing saturated hydrocarbons, resins, and nearly 50% polycyclic aromatic hydrocarbons (PAHs) (Alzaga et al., 2004). Fish are vulnerable to PAHs that are uptaken by respiration, ingestion or dermal contact, individual PAHs or mixtures of PAHs having been reported to induce a wide range of adverse lethal and sublethal effects (Meador et al., 1995). Some observed effects include activation of phase I metabolism (Whyte et al., 2000), peroxisome proliferation (Cajaraville et al., 2003) and endocrine disruption (WHO/IPCS, 2002) that can be used as biomarkers.

The peroxisome proliferator-activated receptor (PPAR) could bind to PAHs and trigger the process of peroxisome proliferation in fish, as in mammalian cells some PAHs have been reported to act as PPAR agonists (Kim et al., 2005). The retinoid X receptor (RXR) is the obligate heterodimerization partner of PPAR, allowing binding to peroxisome proliferator response elements (PPRE) that regulate target genes involved in peroxisome proliferation (Bardot et al., 1993; Liu et al., 2005). Three different PPAR subtypes (α, β and γ) encoded by separate genes and showing different tissue distribution patterns have been described in different vertebrate species including fish *Salmo salar* (Andersen et al., 2000; Leaver et al., 2007), *Sparus aurata* and *Pleuronectes platessa* (Leaver et al., 2005), *Danio rerio* (Robinson-Rechavi et al., 2001), *Fugu rubripes* (Maglich et al., 2003; Kondo et al., 2007), *Dicentrarchus labrax* (Boukouvala et al., 2004), *Salmo trutta* (Batista-Pinto et al., 2005), *Chelon labrosus* (Raingeard et al., 2006, 2009), *Carassius auratus* (Mimeault et al., 2006), and *Tetraodon nigroviridis* (Metpally et al., 2007). PPAR target genes, like those of the peroxisomal β-oxidation pathway in the case of PPARα (Lee et al., 1995), are mainly involved in lipid homeostasis (Wahli et al., 1995). For RXR, three major subtypes (α, β and γ) exist in vertebrates (Ross et al., 2001). RXRs have been reported in fish species such as *D. labrax* (Villeneuve et al., 2004), *D. rerio* (Robinson-Rechavi et al., 2001; Waxman and Yelon, 2007), *C. labrosus* (Raingeard et al., 2009), *Sebastiscus marmoratus* (He et al., 2009) or many others directly submitted to the GenBank. Recently, we described peroxisome proliferation and differential expression of PPARα, PPARγ and RXRα mRNAs of the thicklip grey mullet *C. labrosus* exposed to Prestige-like fuel oil (Bilbao et al., submitted for publication). Palmitoyl-CoA oxidase (AOX1) enzymatic activity (the first and rate-limiting enzyme of the peroxisomal β-oxidation pathway) as well as both PPARα and RXRα mRNA expressions were up-regulated in fish exposed to the fuel for 2 days, whereas PPARγ was down-regulated.

In order to assess the biological effects of the Prestige oil spill in the Biscay Bay, the objective of the present study was to clone fragments of PPARα, PPARγ, RXR and AOX1 to perform a semi-quantitative determination of their transcription levels in liver of juvenile and adult male and female European hakes in spring (June) and winter (December) 2004 and 2005. Other toxicologically relevant genes related to peroxisomal β-oxidation (multifunctional protein 2 — MFP2- and Δ^2,Δ^4-dienoyl-CoA reductase 2 — DECR-), cytochrome P450s (subfamilies 1, 2 and 3), the tumor suppressor gene p53, estrogen receptor α (ERα), vitellogenins (VTGA and VTGB) and housekeeping genes (18S rRNA and β-actin) were cloned in order to increase the basic toxicologically relevant gene sequence knowledge on this ecologically and economically important species. Additionally, AOX1 and 7-ethoxyresorufin O-deethylase (EROD) activities were measured in the same samples as exposure biomarkers of pollution by PAHs.

2. Materials and methods

2.1. Fish and tissue collection

M. merluccius were sampled by trawling in the East Cantabrian Sea of the Biscay Bay (ICES VIIIb water area between latitudes 43°30'N and 45°09'N, and between longitudes 1°35'W and 1°46'W). Juvenile and adult female and male hakes were collected at 70–120 m depth by personnel of the Marine Research Division of AZTI-Tecnalia Foundation in June and December 2004 and 2005. The sex of individuals was assigned *in situ* during sampling by visual inspection of gonads. Fish captured ranged between 17 and 63 cm length. Above 38 cm for males and 47 cm for females fish were considered adults (Lucio et al., 2000; Murua, 2006).

For semi-quantitative RT-PCR and enzyme activity studies, juvenile and adult males and females were collected in June 2004 and 2005 (5 individuals of each group), and 5 juvenile females and 5 juvenile males in December 2004 and 2005. Every fish was dissected out to collect liver that was immediately frozen in liquid nitrogen. Once in the laboratory, 50–100 mg of liver was excised and immediately placed in RNA later (Sigma-Aldrich, St. Louis, USA), to be conserved at −80 °C until RNA isolation was performed.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from each individual fish liver using TRIzol (Invitrogen, Carlsbad, USA) and cDNA was obtained (SuperScript™ First-Strand Synthesis System, Invitrogen, Leek, The Netherlands) from 3 μg of total RNA.

2.3. PCR amplification, molecular cloning and sequence characterization

cDNAs were PCR amplified (standard PCR procedure for targets up to 4 kb with Taq DNA Polymerase, Invitrogen) using degenerate primers designed after aligning (ClustalX software, at EMBL-EBI, Cambridge, UK) known teleost and other phyla sequences to amplify conserved regions of PPARα, PPARγ, RXR, AOX1, MFP2, DECR, CYP1A, CYP2K, CYP3A, p53, ERα, VTGA and VTGB as well as β-actin and 18 S rRNA as housekeeping genes. The sequences for primer design (Table 1) were retrieved from the National Center for Biotechnology Information (NCBI, USA). After determination of the exponential phase for each set of primers, PCR amplifications were performed using an iCycler thermocycler (Bio-Rad, San Diego, California, USA) under the conditions depicted in Table 1. Amplified fragments were visualized after electrophoresis in ethidium bromide stained 1.5% agarose gels.

PCR products showing unique amplicons matching the expected amplification length were purified using a PCR purification kit (Qiagen, Hilden, Germany) for posterior sequencing in the Sequencing and Genotyping Service available in the University of the Basque Country, using in each gene case the specific degenerate primers described above. PCR products showing multiple amplicons were cloned in pCR2.1-TOPO vector using the TOPO-TA cloning kit (Invitrogen). Plasmids were isolated and purified using the S.N.A.P miniprep kit (Invitrogen) and automatically sequenced using vector primers M13FW and M13RV. Alignments and similarity matrices were calculated using Blastn, Blastx and ClustalW. The structure of deduced amino acid sequences of cloned fragments was predicted by comparison with known structures and by models generated by Swiss-model (Kopp and Schwede, 2004).

2.4. Semi-quantitative RT-PCR

Semi-quantitative RT-PCRs were run by monoplex-PCR using the Taq DNA polymerase (Invitrogen). PCR mixture contained 1 μl of the cDNA (1 μg/μl), 0.2 μl of the Taq polymerase (5 U/μl), 0.5 μl of dNTPs (10 mM), 0.5 μl of each primer (10 μM), 1× reaction buffer and water in a final volume of 25 μl. All PCR primers were chosen within the target sequences previously cloned to generate specific products around 200 bp; FW-5′-CTTGACGACAGCGACCTG-3′ and RV-5′-GCTCACGCAGGTCGGCCAGC-3′ for PPARα; FW-5′-AGCTTGTTTACG-ACCACTGC-3′ and RV-5′-CTCCGGGTGCACGTGGTCCA-3′ for PPARγ; FW-5′-CCATCGCTCCATCTCCGTG-3′ and RV-5′-CATCTGGGTTGAAGAGGAC-3′