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Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring

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ABSTRACT

Exposure to estrogenic chemicals discharged into the aquatic environment has been shown to induce feminization in wild freshwater fish and although fish species have been reported to differ in their susceptibility for these effects, empirical studies that directly address this hypothesis are lacking. In this study, *in vitro* ERα activation assays were applied in a range of fish species used widely in chemical testing (including, zebrafish, fathead minnow, medaka) and/or as environmental monitoring species (including, roach, stickleback, carp) to assess their comparative responsiveness to natural (estrone, estradiol, estriol) and synthetic (17α-ethinylestradiol (EE2), diethylstilbestrol (DES)) estrogens. *In vivo* exposures to EE2 via the water (nominal 2 and 10 ng/L for 7 days) were also conducted for seven fish species to compare their responsiveness for hepatic vitellogenin (VTG) mRNA induction (an ER mediated response). Of the fish species tested, zebrafish ERα was found to be the most responsive and carp and stickleback ERα the least responsive to natural steroid estrogens. This was also the case for exposure to EE2 with an ERα-mediated response sensitivity order of zebrafish > medaka > roach > fathead minnow > carp > stickleback. For VTG mRNA induction *in vivo*, the order of species responsiveness was: rainbow trout (not tested in the ERα activation assays) > zebrafish > fathead minnow > medaka > roach > stickleback > carp. Overall, the responses to steroid estrogens *in vitro* via ERα compared well with those seen *in vivo* (VTG induction for exposure to EE2) showing *in vitro* screening of chemicals using fish ERα-mediated responses indicative of estrogenic responses (VTG induction) *in vivo*.

1. Introduction

Steroid hormones play important roles in the reproductive biology of vertebrates. Many of the currently reported actions of steroid hormones, including estrogens, androgens and progestogens, are mediated via specific receptors that are most often localized in the nucleus of target cells. The steroid hormone receptors, including those for progestogens, androgens, glucocorticoids, mineralocorticoids, vitamin D, and retinoic acid, form a superfamily of transcription factors (Blumberg and Evans, 1998). Mammals express two estrogen receptor (ER) subtypes, ERα, and ERβ, and ERα appears to play a more significant role in major

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reproductive physiological functions in females (Lubahn et al., 1993; Krege et al., 1998). Each ER is encoded by a separate gene with unique transcriptional activities (Cheung et al., 2003), and the proteins differ significantly in their amino acid sequence, size and ligand-binding characteristics (Pettersson and Gustafsson, 2001). Teleost fish express at least three distinct types of ER (ERα, ERβ1 and ERβ2), and ERβ2 appears to be closely related to ERβ1, reflecting a gene duplication event (Hawkins et al., 2000). Both ER isoforms are simultaneously expressed in some target tissues but in other tissues, differential expression occurs (Kuiper et al., 1997; Hawkins and Thomas, 2004), indicating the ERα and ERβ may have distinct roles in the physiological responses to estrogens.

Estradiol-17β (E2), the principle circulating estrogen, is essential for normal ovarian development in many vertebrate species (Wallace, 1985). In fish, E2 is thought to be the main estrogen stimulating the hepatic production of vitellogenin (VTG), the precursor of egg yolk proteins (von der Decken and Waters, 1993) by an ER-mediated pathway (Pakdel et al., 1991), but the relative contribution of each ER subtypes in this process is still poorly understood (Hawkins and Thomas, 2004; Leaños-Castañeda and Van Der Kraak, 2007; Davis et al., 2010; Nelson and Habibi, 2010; Chakraborty et al., 2011). A number of studies suggest that endogenous E2 acts as a natural inducer of ovarian differentiation in non-mammalian vertebrates (Devlin and Nagahama, 2002; Pieau and Dorizzi, 2004).

The occurrence of feminized responses include elevated concentrations of blood VTG in male and immature female fish, the presence of a female-like ovarian cavity in the testis of males (oviducts), and/or a high incidence of intersex in some wild fish has been associated with the proximity of these fish to point source discharges of estrogenic effluents from wastewater treatment works (WwTW) (Jobling et al., 2006; Vajda et al., 2008). A variety of estrogenic chemicals contained within WwTW effluents have been linked with these feminized responses, including the natural steroid estrogens, E2, and estrone (E1) and the synthetic estrogen 17α-ethinylestradiol (EE2), derived from the contraceptive pill hormone, as well as a variety of more weakly estrogenic, industrial chemicals. Under laboratory conditions, some of these compounds can induce feminization and reduce reproductive ability in different fish species (Metcalfe et al., 2001; Thorpe et al., 2003; Maunder et al., 2007; Lange et al., 2008; Paul-Prasanth et al., 2011).

An analysis of the literature would indicate that different species of fish may show differences in their responsiveness and thus potentially, their susceptibility to the effects of environmental estrogens. For example, the lowest effect concentration (LOEC) for VTG induction (one of the most widely used biomarkers for estrogen exposure in fish) for comparable exposure periods would indicate that salmonid fish are more responsive to estrogen compared with cyprinid fish (e.g. Tyler et al., 2009). Very few studies, however, have made any attempts to draw direct comparisons of estrogen responsiveness in different fish species and comparisons of the *in vivo* studies across studies are difficult to consolidate because of the different experimental approaches adopted, including length of exposure, and the different assays used to quantify VTG. This knowledge, however, is particularly important given that a very small number of fish species are employed in environmental risk assessment to protect all fish species and the lack of data on the responsiveness to estrogens of fish species most widely used in field monitoring.

Reporter gene assays provide a useful technique to analyze the ligand- and species-specificity of ERs (Baker, 2001). To further our understanding of the differences in responsiveness to estrogens across fish species, we analyzed ligand- and species-specificity of fish ERs using custom developed *in vitro* ERα reporter gene assays. Further, we conducted *in vivo* exposures to EE2 measuring VTG mRNA expression with a range of temperate and warm water fish species, including some of those most commonly used in the testing of chemicals in the laboratory (zebrafish, fathead minnow, medaka, rainbow trout) and species used in environmental effects monitoring (roach, carp, stickleback).

2. Materials and methods

2.1. Animals and chemical reagents

For the first *in vivo* study, fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio* – WIK strain) were supplied from stocks raised at the University of Exeter, UK, roach (*Rutilus rutilus*) were obtained from the Environment Agency Fish Farm Calverton, UK; carp (*Cyprinus carpio*) from Priory Fisheries, Cullompton, UK, rainbow trout (*Oncorhynchus mykiss*) and three-spined stickleback (*Gasterosteus aculeatus*) from Houghton Springs, Winterbourne, UK. For the second *in vivo* study, Japanese medaka (*Oryzias latipes*, orange-red strain) and zebrafish were obtained from broodstock reared in dechlorinated tap water at the Endocrine Research Laboratory, National Institute for Environmental Studies, Tsukuba, Japan, XY male carp from Niigata Prefectural Inland Water Fisheries and Experimental Station, Niigata, Japan, rainbow trout from Teikyo University, Yamanashi, Japan and three-spined stickleback from Hokkaido Prefecture, Japan.

Estradiol-17β, estrone, estriol (E3), 17α-ethinylestradiol, diethylstilbestrol (DES) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All hormones tested in the reporter gene assays were dissolved in dimethylsulfoxide (DMSO) and the concentration of DMSO in the culture medium did not exceed 0.1%.

2.2. Construction of plasmid vectors and ER transactivation assays

The full-coding regions of medaka ERα (AB033491), zebrafish ERα (AB037185), roach ERα (AB190289), stickleback ERα (AB330740), carp ERα (AB334722) and fathead minnow ERα (AY775183) were amplified by PCR with KOD DNA polymerase (TOYOBO Biochemicals, Tokyo, Japan). The PCR products were gel-purified and subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen). An estrogen-regulated reporter vector containing four estrogen-responsive elements (4xERE), named pGL3-4xERE, was constructed as described previously (Katsu et al., 2006).

To examine the ligand-sensitivities with the ERαs, transactivation assays were performed as previously reported (Katsu et al., 2007) with slight modifications. Briefly, HEK293 cells were seeded in phenol-red free Dulbecco's Modified Eagle's medium (Sigma–Aldrich Co.) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone, South Logan, UT, USA). After 24 h, the cells were transfected with 400 ng of pGL3-4xERE, 100 ng of pRL-TK (as an internal control to normalize for variation in transfection efficiency, contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter, Promega, Madison, WI, USA), and 200 ng of pcDNA3.1-ER using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. After 4 h of incubation, natural and synthetic estrogens were added to the medium at concentrations between 10^{-5} and 10^{-16} M. After 44 h, the cells were collected, and the luciferase activity of the cells was measured by a chemiluminescence assay with Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Turner Designs Luminometer TD-20/20 (Promega). Promoter activity was calculated as firefly (*Photinus pyralis*)-luciferase