Expression of Zebra Fish Aromatase cyp19a and cyp19b Genes in Response to the Ligands of Estrogen Receptor and Aryl Hydrocarbon Receptor

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Received September 15, 2006; accepted January 8, 2007

Many endocrine-disrupting chemicals act via estrogen receptor (ER) or aryl hydrocarbon receptor (AhR). To investigate the interference between ER and AhR, we studied the effects of 17β-estradiol (E2) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the expression of zebra fish cyp19a (zf cyp19a) and cyp19b (zf cyp19b) genes, encoding aromatase P450, an important steroidalgenic enzyme. In vivo (mRNA quantification in exposed zebra fish larvae) and in vitro (activity of zf cyp19b-luciferase reporter genes in cell cultures in response to chemicals and zebra fish transcription factors) assays were used. None of the treatments affected zf cyp19b, excluding the slight upregulation by E2 observed in vitro. Strong upregulation of zf cyp19b by E2 in both assays was downregulated by TCDD. This effect could be rescued by the addition of an AhR antagonist. Antiestrogenic effect of TCDD on the zf cyp19b expression in the brain was also observed on the protein level, assessed by immunohistochemistry. TCDD alone did not affect zf cyp19b expression in vivo or promoter activity in the presence of zebra fish AhR2 and AhR nuclear translocator 2b (ARNT2b) in vitro. However, in the presence of zebra fish ERα, AhR2, and ARNT2b, TCDD led to a slight upregulation of promoter activity, which was eliminated by either an ER or AhR antagonist. Studies with mutated reporter gene constructs indicated that both mechanisms of TCDD action in vitro were independent of dioxin-responsive elements (DREs) predicted in the promoter. This study shows the usefulness of in vivo zebra fish larval and in vitro zf cyp19b reporter gene assays for evaluation of estrogenic chemical actions, provides data on the functionality of DREs predicted in zf cyp19 promoters and shows the effects of cross talk between ER and AhR on zf cyp19b expression. The antiestrogenic effect of TCDD demonstrated raises further concerns about the neuroendocrine effects of AhR ligands.

Key Words: endocrine disruption; aromatase CYP19; zebra fishestrogen receptor; aryl hydrocarbon receptor; gene expression/regulation.

In recent years, diverse cases of disturbed sexual differentiation and reproductive abnormalities have been reported in fish (Eggen et al., 2003; Jobling et al., 1998; Segner et al., 2003). These findings can be linked, at least partially, to exposure to so-called endocrine-disrupting chemicals (EDCs)—natural or synthetic compounds widely present in the environment that can disrupt hormone action. Aromatase P450, encoded by cyp19 gene(s), is considered to be a potential EDC target because it catalyzes the final step of biosynthesis of estrogens (Simpson et al., 2002), important hormones involved in the control of many physiological processes, including those related to reproduction. Several indications exist that interference with the aromatase CYP19 system in fish might lead to malfunctioning of the reproductive system. For instance, treatment with aromatase inhibitors disrupts gonadal sex differentiation in fish (Fenske and Segner, 2004).

Two structurally distinct cyp19 genes, cyp19a1 (cyp19a) and cyp19a2 (cyp19b), are found in most teleosts, including model species zebra fish (Danio rerio). Zebra fish cyp19a (zf cyp19a) is predominantly expressed in gonads, and zebra fish cyp19b (zf cyp19b) is mainly found in neuronal tissues, while lower levels of the other isoform are found in both sites and in some other tissues (Chiang et al., 2001; Sawyer et al., 2006). Characterization of zf cyp19 promoters in zebra fish led to the assumption that some EDCs, for example, estrogen- and dioxin-like compounds, might interfere with the expression of these genes due to the presence of predicted responsive elements (Kazeto et al., 2001; Tong and Chung, 2003). Estrogen-responsive element (ERE) is found in the zf cyp19b promoter, half-EREs, and aryl hydrocarbon–responsive elements (also called dioxin-responsive elements, DREs) are predicted in...
Tanguay et al. reported estrogen- and dioxin-like EDCs. Characterization of intermolecular mechanisms of action and possible cross talk of reported.

Several isoforms of both ER and AhR/ARNT are found in teleost fish, including zebra fish (Andreasen et al. 2000). Ligand-activated AhR heterodimerizes with AhR nuclear translocator (ARNT) and activates transcription of target genes through binding to DREs (Schmidt and Bradfield, 1996). Several isoforms of both ER and AhR/ARNT are found in teleost fish, including zebra fish (Andreasen et al., 2002; Karchner et al., 2005; Menuet et al., 2002; Prasch et al., 2006; Tanguay et al., 1999, 2000). 17β-estradiol (E2) is a natural ligand of ER. Many EDCs, for example, ethinylestradiol (EE2), nonylphenol (NP), or bisphenol A (BPA), can also bind to and activate ER (Klotz et al., 1996). Similarly, the AhR can be activated by many environmental contaminants, including halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (Schmidt and Bradfield, 1996). The prototypic AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic compound among HAHs and also the most potent AhR activator (Schmidt and Bradfield, 1996). In addition to their direct “classical” transcriptional actions, ER and AhR signaling pathways also converge at multiple points. Both estrogenic (Lind et al., 1999; Ohtake et al., 2003) and antiestrogenic (Chen et al., 2001; Navas and Segner, 2000; Ohtake et al., 2003) effects of AhR ligands have been reported.

Zcyp19 genes may constitute a good model to study the molecular mechanisms of action and possible cross talk of estrogen- and dioxin-like EDCs. Characterization of interference of these EDC classes with Zcyp19 expression may also add to the understanding of the pathways leading to disruption of reproduction caused by these chemicals. Therefore, the aims of this study were (1) to clarify the role of AhR/ARNT in the regulation of Zcyp19 genes expression and to examine the functionality of DRE sites predicted in the Zcyp19 promoters, (2) to study the effects of AhR activators on the expression of an estrogen-regulated target gene in fish brain (Zcyp19b), and thus (3) to reveal the possible cross talk pathway between the ERs and the AhR/ARNT complex and its effects on Zcyp19 genes expression. This was performed by combining in vivo exposure of zebra fish larvae and in vitro cell-based luciferase reporter gene assays.

**Materials and Methods**

**Chemicals.** TCDD was obtained from LGC Promochem SARL (Molsheim, France). Benz[a]pyrene (B[a]P), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 17β-estradiol (E2), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). DMSO solvent was used for all chemicals.

**Zebra fish handling and exposure.** Zebra fish larvae were reared in our breeding unit as previously described (Brion et al., 2004). At the age of 17 days postfertilization (dpf), they were exposed to chemical mixtures or to solvent alone (DMSO, 0.1% vol/vol) for 72 h (in 100 ml water, 20 fish per group for mRNA quantification or 4 fish per group for immunohistochemistry). Chemical mixtures contained E2 (10nM) or TCDD (0.001–0.1nM) or B[a]P (0.01–1µM) alone or E2 (10nM) with TCDD (0.0001–0.1nM) or B[a]P (0.01–1µM) with or without 0.5µM ANF. For each treatment, half of the exposure solution was renewed every day.

**Measurement of Zcyp19a and Zcyp19b mRNA levels.** At the end of the exposure period, the larvae were immediately placed at 4°C in RNAlater (Sigma-Aldrich, St. Quentin Fallavier, France), kept overnight at 4°C, and conserved at –20°C until further processing. The levels of Zcyp19a and Zcyp19b mRNA were measured by a branched DNA assay (Quantigene, Genospectra, Fremont, CA, USA), as described previously (Hinfray et al., 2006). Briefly, for each exposure condition, two pools of 10 whole-body zebra fish larvae were constituted, lysed, and incubated in a 96-well plate coated with synthetic oligonucleotide in the presence of a specific probe set designed according to the Zcyp19a and Zcyp19b mRNA sequences (gene bank accession numbers AF183906 and AF183908, respectively). The probe set consisted of a capture probe that anchored the target mRNA to the synthetic oligonucleotide, a blocking probe that linearized the target mRNA, and of a label probe that hybridized to the target mRNA and to a branched DNA coupled with alkaline phosphatase–bound probes. Finally, a chemiluminescence substrate dioxetan that yields a luminescence signal proportional to the amount of mRNA present in the sample was added. Quantification of luminescence was made on a microplate luminometer (Wallac Victor2, Perkin Elmer, Courtaboude, France). Zcyp19a and Zcyp19b expression values were normalized to a housekeeping gene, zebra fish β-actin (gene bank accession number NM 131031). For each pool, measurements were performed in duplicate. The experiments were repeated three times on different days.

**Immunohistochemistry.** For each exposure condition, four zebra fish brains were analyzed. At the end of the exposure period, larvae were euthanized in MS-222, fixed in PBS (pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid for 24 h at 4°C, and processed for cryosections (12 µm). Immunohistochemistry was performed as previously described (Menuet et al., 2005). Briefly, tissue sections were incubated overnight at room temperature with the polyclonal zebra fish CYP19B antibody (directed against the synthetic polypeptide CNSNGETADNRTSKE corresponding to the last 15 residues of the protein sequence), diluted at 1:1000. Then the sections were rinsed and incubated with a biotinylated goat anti-rabbit IgG (1:1500) and then with a streptavidin-peroxidase complex (1:1500). Aromatase immunoreactivity was visualized using a 3,3′-diaminobenzidine (DAB)-nickel intensification protocol (Shu et al., 1988). Stained sections were observed and photographed under an Axioscope photomicroscope (Zeiss, Göttingen, Germany) equipped with a DXC-390P digital camera (Sony, Tokyo, Japan). Visilog 6 software (Noesis, Velizy Villacoublay, France) was used for image acquisition.

**Cell culture.** CHO-K1 (Chinese hamster ovary) and U251-MG (human astrocytes) cells were maintained at 37°C under a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium without phenol-red (DMEM; Sigma-Aldrich) supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 25 mg/ml of amphotericin (antibiotic-antimycotic solution, Sigma-Aldrich) and 9% inactivated fetal calf serum (PCS; Life Technologies, Carlsbad, CA). U251-MG medium additionally contained 2mM of L-glutamine (Sigma-Aldrich).
**Plasmid constructs used for transfection.** The following plasmids were obtained from Dr R. E. Peterson at the University of Wisconsin: the reporter plasmid prtlA-luc, containing a dioxin-responsive promoter from the rainbow trout cyp1a gene (Abnet et al., 1999) in front of the luciferase-coding sequence, and the expression vectors zfAhR2 (Tanguay et al., 1999) and zfARNT2b (Tanguay et al., 2000), containing the full-length zebra fish AhR2-(zfAhR2) and ARNT2b (zfARNT2b)-coding sequences, respectively, in pBK-CMV. The expression vector zfER was a plasmid that contained the coding region of zebra fish ERα (zfERα) in Topo-pCDNA3 vector (Menuet et al., 2002). An empty Topo-pCDNA3 plasmid (Topo) containing no coding sequence for zebra fish receptor was used to equalize the amount of transfected DNA in the control (no exogenous receptor expression). The control estrogen-responsive reporter plasmid pERE-TK-luc contained an ERE site and a TATA box in front of the luciferase-coding sequence. The reporter plasmid cyp19b-luc and cyp19b_DREdel-luc (Menuet et al., 2005) contained –486/+ 34 and –371/+ 34 regions of zf-cyp19b promoter/exon I region in pGL2-basic (Promega Corporation, Madison, WI, USA), respectively. Cyp19b_DREdel-luc was used as a DRE-deficient mutant (lacking predicted DRE sites with start positions –453 and –399).

The reporter plasmid cyp19a-luc contained –536/+ 37 region of zf-cyp19a promoter/exon I, cloned into pGL2-basic vector. Total genomic DNA was obtained from adult zebra fish using conventional protocol (Nuesslein-Volhard and Dahm, 2002). The desired fragment was amplified by PCR from genomic DNA using a specific primer set designed according to the sequence of the zf-cyp19a promoter previously published (Kazeto et al., 2001), cyp19a-fw 5’-GGTTACGATAATATAGGCGATCGGA-3’, with nucleotide change (noted by lower case) introduced to create a KpnI site (underlined), and cyp19a-rev 5’-ACCTGCGATAGAAGCGGTAGGAGA-3’. The PCR reaction was carried out under the following conditions: 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 65°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 10 min. The PCR product was purified and cloned into pGEM-Teasy vector (Promega), then subcloned into pGL2-basic vector with KpnI/SacI.

A QuickChange site-directed mutagenesis kit from Promega was used according to manufacturer’s instructions to produce several mutated reporter constructs on the basis of cyp19a-luc. Gene Runner (Hastings Software Inc., Hastings-on-Hudson, NY, USA) was used to reexamine promoter regions. To create the cyp19a_DREMut-luc (mutated DRE site predicted at –238 bp), 5’-GCTGTAGAATACTCGAGGAAATGCCCAGATGCTGTCAATATC-3’ primer (forward) was used (site predicted underlined, mutated bases denoted by lower case).

Mutagenesis was also carried out to create two Apal sites in the cyp19a-luc to cut at the positions –162 bp and –110 bp, for Apal[1] and Apal[2], respectively, which facilitated production of the cyp19a_cyp19a_DREdel-luc (with the deleted 52 bp region [–162/–110], containing a putative DRE site, position start –160bp [Kazeto et al., 2001] or –154 [Tong and Chung, 2003]). The primers (forward) 5’-GAAAAACCCAGAGATGACCTTGCGCAGGCAGG-3’ and 5’-GAAAAACCCAGAGATGACCTTGCGCAGGCAGG-3’ were used to produce Apal[1] and Apal[2], respectively (created Apal sites underlined, mutated bases denoted by lower case). Reverse primers used for site-directed mutagenesis were reverse complementary to forward primers. All the inserted and mutated sequences were verified by sequencing using the Big Dye Terminator protocol (Synergene Biotech GmbH, Schliren, Switzerland).

The normalization vector CMV-gal, containing a β-galactosidase-coding sequence cloned after the constitutive promoter, was used to account for transfection efficiency from well to well.

**Transfection experiments.** The cells were trypsinized and seeded in 24-well plates in fresh medium 6 h before the transfection (concentration of cells 2 × 10⁴/3ml). All transfections were performed using a FuGene6 reagent according to manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). The DNA templates for transfection contained 150 ng of reporter vector, 15 ng of expression vectors, zfERα, zfAhR2, or zfARNT2b, or the same amount of Topo as control, and 50 ng of normalization vector CMV-gal. The medium was replaced with fresh DMEM containing 2% of charcoal-stripped FCS 12 h after the transfection. Vehicle control (DMSO 0.1%) with or without treatment chemicals was added to the medium. The cells were collected for reporter gene assays 48 h later. The medium was removed by vacuum aspiration, each well was washed with PBS, and 150 µl of Reporter Lysis Buffer (Promega) was added. Plates were frozen at –80°C and assayed at least 2 h later. Luciferase activity was assayed with BrightGlo luciferase substrate (Promega). The activity of β-galactosidase was assayed with 2-nitrophenyl-β-D-galactopyranoside (Fluka Chemica-Biochemica, Buchs, Switzerland). Each experimental point was performed in triplicates, and the experiments were repeated three times on different days, unless otherwise noted.

**In vitro translation and DNA-binding assays.** ZfAhR2 and zfARNT2b proteins were produced from vectors zfAhR2 and zfARNT2b, respectively, in TNT rabbit reticulocyte lysate (Promega) according to supplier’s recommendations. Side reactions containing [35S]methionine were performed to assess relative protein production. After the 90-min incubation at 30°C, radioactive translation products were resolved on a 7.5% SDS polyacrylamide gel, dried, and detected on Hyperfilm MP (Amersham Life Sciences, Buckinghamshire, UK), exposed for 12 h. Unlabeled reactions were stored at –70°C prior to functional studies. The sequences of oligonucleotides used for DNA-binding assays are listed in Table 1. Oligonucleotide rt_DREfw was 5′-end labeled with [32P] using T4 polynucleotide kinase and annealed to a three-fold molar excess unlabeled rt_DRErev to produce an rt_DRE probe, followed by purification. Unlabeled competitor DNAs were similarly produced by annealing unlabeled rt_DREfw:rt_DRErev, zf_a160fw:zf_a160rev, zf_a238fw:zf_a238rev, zf_b222fw:zf_b222rev, zf_b399fw:zf_b399rev, and zf_b453fw:zf_b453rev. In an in vitro DNA-binding assay was performed essentially as previously described (Tanguay et al., 2000). Briefly, approximately equal amounts of in vitro produced zfAhR2 and zfARNT2b proteins were incubated in the presence of TCDD 10nM in DMSO 0.2% or DMSO 0.2% alone for 1 h at 22°C. Following incubation, 1.5 µg poly dl-dC and binding buffer (20Mm HEPES, pH 7.9, 100Mm NaCl, 1mM DTT, 6% glycerol) were added and the incubation continued for additional 20 min at 22°C before the addition of approximately 1 ng of the labeled rt_DRE probe with or without 50-fold molar excess of unlabeled rt_DRE, zf_a160, zf_a238, zf_b222, zf_b399, or zf_b453 competitor oligos. Following 20 min incubation at 22°C, complexes were resolved on a 0.5%× TBE (90Mm Tris, 64.6Mm boric acid, and 2.5 EDTA, pH 8.3) 4% acrylamide gel at 4°C. The signal was detected on Hyperfilm MP exposed for 12 h.

**Statistical analysis.** To evaluate in vivo exposure data, nonparametric ANOVA followed by Mann-Whitney U-test was used. The differences were considered significant at p < 0.05. For evaluation of in vitro data, a normalized luciferase activity was determined for each data point by dividing the luciferase activity by β-galactosidase activity and presented as a fold induction (mean ± SD) over control. The data were checked for normality (normal distribution of data was confirmed in all cases), and one-way ANOVA followed by Tukey Honestly Significant Difference Test was applied to analyze the differences.

**TABLE 1**

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Forward* sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt_DREfw</td>
<td>ACCTTTGACGCTGATCGGAAT</td>
</tr>
<tr>
<td>zf_a_160fw</td>
<td>ACAGCTGAGGTTGATTGCTAGTGA</td>
</tr>
<tr>
<td>zf_a_238fw</td>
<td>ACGTCGGAGCCTCGGAGAT</td>
</tr>
<tr>
<td>zf_b_222fw</td>
<td>GGAAAAAGATGTTGAGTGGCTAA</td>
</tr>
<tr>
<td>zf_b_399fw</td>
<td>CGCTATTCTGGACGACAACCC</td>
</tr>
<tr>
<td>zf_b_453fw</td>
<td>AATAAAACCCGATACATGGTACCC</td>
</tr>
</tbody>
</table>

*Reverse oligos were reverse complementary to forward oligos.

**Note.** Predicted DRE sites are underlined.
between data points, which were considered significant at $p < 0.01$, unless otherwise noted.

RESULTS

ZfCYP19α and ZfCYP19β Expression in Zebra Fish Larvae
In Vivo In Response to Exposure to Different Combinations of ER and AhR Ligands

No increased mortality was observed in zebra fish exposed for 3 days to 10nM E2 or to graded concentrations of TCDD or B[a]P alone or in combination with E2. Exposure to 10nM E2, 0.1nM TCDD, and 0.5μM ANF also was not toxic to the fish. However, the addition of 0.5μM ANF to 10nM E2 and 1μM B[a]P resulted in high mortality. Consequently, no data on the zfcyp19 expression could be obtained for this treatment condition. All measurements of zfcyp19 mRNA levels were performed on whole larvae extracts.

Exposure of zebra fish to 10nM E2 had no effect on zfCYP19α levels but significantly increased the expression of zfCYP19β (Figs. 1A and 1B). Exposure to graded concentrations of TCDD (0.001–0.1nM) or B[a]P (0.01–1μM) did not affect the expression of both zfCYP19 genes (Figs. 1A and 1B, only effect of highest concentration shown). Coexposure to 10nM E2 and 0.1nM TCDD (or 1μM B[a]P) had no effect on the zfCYP19α expression (Figs. 1A and 1B), regardless of whether the coexposure was done in the presence or absence of 0.5μM ANF (Figs. 1A and 1B). A different response was observed for zfCYP19β expression: the addition of 0.1nM TCDD or 1μM B[a]P to 10nM E2 led to significant downregulation of zfCYP19β expression in comparison with the expression induced by E2 alone (Figs. 1A and 1B). The downregulating effect of TCDD could partly be rescued by adding 0.5μM of ANF, a partial antagonist of AhR, to the mixture of E2 and TCDD (Fig. 1A). Furthermore, the changes in the CYP19B protein expression in the brain in response to exposure to several combinations of ER and AhR ligands were assessed by immunohistochemistry using zebra fish CYP19B antibody. In radial glial cells, 10nM E2 strongly induced CYP19B expression, but 0.1nM TCDD did not (Fig. 2). In fish, coexposed to 10nM E2 and 0.1nM TCDD, only a few CYP19B-positive cells were observed. In the group exposed to 10nM E2, 0.1nM TCDD and 0.5μM ANF, the staining was much more intense compared to the E2 + TCDD group, nonetheless, it was still less intense compared to the E2 group (Fig. 2). The expression pattern of CYP19A could not be assessed due to unavailability of specific antibody for this zebra fish protein.

ZfCYP19α and ZfCYP19β Genes Promoter Activity In Vitro in Response to Treatment with Different Combinations of ER and AhR Ligands

To gain deeper insights into the effects of E2 and TCDD on zebra fish promoters activity, appropriate luciferase reporter gene assays were performed. The concentrations of treatment chemicals used in the in vitro experiments were below the cytotoxicity thresholds. The zfcyp19b promoter was studied in U251-MG (glial) cell line, previously reported as the cell context favorable for its activity (Menuet et al., 2005). Cyp19a-luc was initially transfected in CHO-K1 (ovarian) and U251-MG cells to assess the possible influence of the cell context on the promoter activity. Basal luciferase activity produced by cyp19a-luc in CHO-K1 was about 10 times higher than that in U251-MG. Moreover, treatment with 10nM E2 in the presence of zfERz led to upregulation of zfcyp19α promoter activity in CHO-K1 cell line, while no response was observed in U251-MG (Fig. 3). Subsequent studies of this promoter were performed in CHO-K1 cells only.
Comparing the response of zf\(cyp19a\) promoter to E2 with and without cotransfected zf\(ER\alpha\), and using E2 antagonist ICI, it was possible to show that ER is needed for the upregulation of this promoter by E2 (Fig. 4). As a control, the empty pGL2-basic vector was subjected to the same analysis. Unexpectedly, it also responded to E2 treatment in the presence of zf\(ER\alpha\) although the induction magnitude was lower (Fig. 4). Basal level of luciferase activity produced by cyp19a-luc in CHO-K1 cells was 26 ± 7 times higher than that produced by pGL2-basic. It was also higher (eightfold ± twofold) in comparison to cyp19b-luc in CHO-K1 cells. Furthermore, statistical analysis showed that the differences between the response of cyp19a-luc and pGL2-basic to E2 in CHO-K1 cells are significant (\(p < 0.05\)). This allowed us to presume that the response of cyp19a-luc to E2 in CHO-K1 cells is zf\(cyp19a\) promoter specific. Since no ERE site is found in the zf\(cyp19a\) promoter, we assumed that the indirect regulation by E2 might occur through the steroidogenic factor 1 (SF1) and half-ERE sites located in close proximity (SF1 at −127 bp and half-ERE at −101 bp from the transcription start). However, studies with mutated reporter gene constructs proved that this is not the case since the E2 response of constructs bearing mutations in the putative SF1 and half-ERE sites was not significantly different from that of the wild-type promoter (data not shown).

Next, the response of the zf\(cyp19a\) and zf\(cyp19b\) promoters to TCDD was investigated. The control dioxin-responsive promoter (prt1A-luc) exhibited dose-dependent response to TCDD treatment in the presence of zfAhR2/zfARNT2b in both CHO-K1 and U251-MG cells (Fig. 5; only one TCDD concentration [10nM] effect is shown). A slight upregulation in response to 10nM TCDD also occurred in the absence of zfAhR2/zfARNT2b. However, this effect was significantly
enhanced in the presence of these receptors (Fig. 5). The functionality of DRE sites predicted in the zf*cyp19a* and zf*cyp19b* promoters was examined using cyp19a-luc and its mutants, cyp19a_DREmut-luc and cyp19a_DREdel-luc, in CHO-K1 cells (Fig. 5A) and cyp19b-luc and its deletion

FIG. 3. The response of zebra fish cyp19a promoter to treatment with E2 in CHO-K1 and U251-MG cells. Cells were transfected with the zf*cyp19a*-luciferase reporter gene (cyp19a-luc) and cotransfected with a control vector (Topo) or a vector expressing zfERα. Transfected cells were treated with DMSO 0.1% with or without E2 10nM. The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of three (in CHO-K1) or two (in U251-MG) independent experiments, where each point was performed in triplicates. The data point significantly different from the control (Topo DMSO 0.1%) is marked with “a” (p < 0.01).

FIG. 4. The effect of zebra fish ERα and its agonist (E2) and antagonist (ICI) on the activity of zebra fish cyp19a promoter and pGL2-basic vector in CHO-K1 cells. Cells were transfected with the zf*cyp19a*-luciferase reporter gene (cyp19a-luc) or with the pGL2-basic vector and cotransfected with a control vector (Topo) or a vector expressing zfERα. Transfected cells were treated with DMSO 0.1% with or without E2 10nM or E2 10nM and ICI 1μM. The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of three independent experiments, where each point was performed in triplicates. Data points significantly different from the control (Topo DMSO 0.1%) are marked with “a”; selected data points significantly different from zfERα E2 10nM are marked with “b”; p < 0.01 in all cases.

FIG. 5. The response of different reporter genes to treatment with TCDD in the presence of zebra fish AhR2 and ARNT2b in CHO-K1 and U251-MG cells. Cells were transfected with different reporter genes (control dioxin-responsive promoter-luciferase [prt1A-luc] and control estrogen-responsive promoter-luciferase [pERE-TK-luc] in CHO-K1 [A] and U251-MG [B]) cells; zf*cyp19a*-luciferase [cyp19a-luc] and its DRE-deficient mutants [cyp19a_DREmut-luc and cyp19a_DREdel-luc] in CHO-K1 cells [A]; zf*cyp19b*-luciferase [cyp19b-luc] and its DRE-deficient mutant [cyp19b_DREdel-luc] in U251-MG cells [B] and cotransfected with a control vector (Topo) or vectors expressing zfAhR2 and zfARNT2b. Transfected cells were treated with DMSO 0.1% with or without TCDD 10nM. The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of three independent experiments, where each point was performed in triplicates. Data points significantly different from the control (Topo DMSO 0.1%) are marked with “a”; selected data points significantly different from zfAhR2/zfARNT2b DMSO 0.1% are marked with “b”; p < 0.01 in all cases.
mutant, cyp19b_DREdel-luc, in U251-MG cells (Fig. 5B). The activity of the zf
CYP19A promoter and its DRE-deficient mutant was not significantly affected by treatment with 10nM TCDD in the absence of zfAhR2/zfARNT2b (Fig. 5A). However, the activity of the zfCYP19B promoter was slightly upregulated in the presence of TCDD and absence of zfAhR2/zfARNT2b (Fig. 5B). The DRE-deficient mutant of zfCYP19B promoter was upregulated similarly to wild type, and the E2-responsive promoter containing just an ERE site and a TATA box (pERE-TK-luc) responded to the TCDD treatment in the absence of zfAhR2/zfARNT2b similarly to zfCYP19B promoter (Fig. 5B). The activity of zfCYP19A and zfCYP19B promoters, as well as of the control E2-responsive promoter, was significantly decreased in the presence of zfAhR2/zfARNT2B as compared to activity without expressed exogenous receptors (Fig. 5). The activity of the dioxin-responsive promoter was, on the contrary, upregulated in the presence of zfAhR2/zfARNT2B and absence of TCDD, and the addition of TCDD caused further significant upregulation of this response (Fig. 5). However, the addition of TCDD in the presence of zfAhR2/zfARNT2B did not further affect the activity of zfCYP19 promoters or that of the control E2-responsive promoter as it neither rescued the downregulation produced by expression of zfAhR2/zfARNT2B nor did it cause further downregulating effects (Fig. 5). The general response pattern of altered constructs did not significantly differ from that of the wild

FIG. 6. The response of the zebra fish cyp19a-luciferase reporter gene to treatment with E2 or TCDD, alone or in combination, in CHO-K1 cells in the presence of zebra fish ERα, AhR2, and ARNT2b. Cells were transfected with the zfCYP19A-luciferase reporter gene (cyp19a-luc) and cotransfected with a control vector (Topo) or vectors expressing zfERα, zfAhR2, and zfARNT2b. Transfected cells were treated with DMSO (0.1%) with or without 10nM E2 or 1nM TCDD alone or in combination. The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of three independent experiments, where each point was performed in triplicates. Data points significantly different from the control (Topo DMSO 0.1%) are marked with "a" (p < 0.01).

FIG. 7. The response of different reporter genes to treatment with the ligands of ER and/or AhR in U251-MG cells in the presence of zebra fish ERα, AhR2, and ARNT2b in U251-MG cells. The cells were transfected with different reporter genes (control estrogen-responsive promoter-luciferase [pERE-TK-luc], zfCYP19B-luciferase [cyp19b-luc], and its DRE-deficient mutant [cyp19b_DREdel-luc]) and cotransfected with a control vector (Topo) or vectors expressing zfERα, zfAhR2, and zfARNT2b. Transfected cells were treated with 10nM E2 or 1nM TCDD alone or in mixture or in combination with 1μM ANF or 1μM ICI. The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of five independent experiments, where each point was performed in triplicates. In (A), “a” indicates data points that are significantly different from the control (Topo DMSO 0.1%); “b” indicates selected data points that are significantly different from zfERα/AhR2/ARNT2b E2 10nM; and “c” indicates selected data points that are significantly different from zfERα/AhR2/ARNT2b TCDD 1nM. In (B), “a” indicates data points that are significantly different from control (Topo DMSO 0.1%); “b” indicates data points that are significantly different from zfERα/AhR2/ARNT2b E2 10nM TCDD 1nM. p < 0.01 in all cases.

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FIG. 8. Electrophoretic mobility shift assay of zebra fish AhR2 and ARNT2b interactions with DRE sites predicted in zebra fish cyp19a and cyp19b promoters. In vitro translated zfAhR2 and zfARNT2b proteins were incubated with 10nM TCDD and γ°P-labeled oligo rt_DRE derived from a DRE in the rainbow trout cyp1a promoter. In lane 2, 50-fold molar excess of unlabeled competitor oligo rt_DRE was added, and 50-fold molar excess of unlabeled competitor oligos derived from putative DREs in zf cyp19a and zf cyp19b promoters, zf_a_160, zf_a_238, zf_b_222, zf_b_399 and zf_b_453, was added in lanes 3–7. The experiment was repeated two times on different days, representative picture is shown. The arrow indicates the position of specific complex. DNA-binding assays were performed to further investigate the ability of zebra fish AhR2/ARNT2b heterodimer to bind to the DRE sites predicted in the zf cyp19a and zf cyp19b promoters. Radioactively labeled probe rt_DRE designed on the basis of the rainbow trout cyp1a promoter (Tanguay et al., 1999) was used as a control. A strong complex was formed between the zfAhR2/zfARNT2b and rt_DRE probe in the presence of TCDD (Fig. 8, lane 1). This complex was reported to migrate as duplets of unknown nature (Tanguay et al., 2000). However, we have observed a single dispersed band migration. The complex was competed by a 50-fold molar excess of unlabeled rt_DRE (Fig. 8, lane 2), indicating that DNA binding is specific. None of the competitor oligos containing sequences of DREs predicted in zf cyp19a and zf cyp19b promoters were able to compete with the active complex formation (Fig. 8, lanes 3–7).

DISCUSSION

The Effects of Estrogens on the zf cyp19a Expression

To establish the cellular system suitable for examining zf cyp19a promoter activity in vitro, two cell lines were transfected with a luciferase reporter driven by this promoter. Data demonstrated that the zf cyp19a promoter is more active in the ovarian cells context (CHO-K1) in comparison to glial cells (U251-MG). This finding corresponds well to in vivo observations since gonads (and especially ovary) were shown to be the main sites of zf cyp19a expression (Chiang et al., 2001; Fenske and Segner, 2004; Goto-Kazeto et al., 2004; Hinfray et al., 2006; Sawyer et al., 2006). We have observed a significant upregulation of the zf cyp19a promoter activity in E2-treated CHO-K1 cells in the presence of zfERα, in contrast to in vivo observations in zebra fish larvae where no upregulation of zf cyp19a by E2 was observed. In vitro E2 response was dependent on the presence of zfERα and also on the cellular context, as no upregulation was observed in U251-MG cells, even in the presence of zfERα. Surprisingly, the empty pGL2-basic vector, containing no promoter in front of luciferase-coding sequence, also responded to E2 treatment in the presence of zfERα, although the induction magnitude was significantly lower. Thus, although the observed induction of the zf cyp19a promoter by E2 may be an artifact induced by the empty vector, significantly higher basal luciferase activity and response to E2 of cyp19a-luc allowed us to presume that this...
induction is zf cyp19a promoter specific. The discrepancy between our in vivo and in vitro observations on the zf cyp19a expression in response to E2 can be partially explained by the higher sensitivity of the in vitro reporter gene assays compared to the in vivo assay. Differences between the response of artificial reporter genes and endogenous target genes expression have been observed even in the same cell context (Shipley and Waxman, 2005). It is also possible that the induction of the zf cyp19a expression in vivo occurs only in specific cell types; thus, this effect is masked when measurements are performed on the whole-body homogenates. Moreover, it is well known that response to estrogens depends on several factors other than simple ligand-ER binding, such as ligand transport to target tissues or bioactivation, which may add to differences between in vitro and in vivo estrogenic responses.

Our in vivo results agree with previous studies that have shown the lack of estrogens’ effect on zf cyp19a expression in larvae (Hinfray et al., 2006). In another study, downregulation of zf cyp19a expression in larvae was observed in response to exposure to 1–100nM of EE2 (Kazeto et al., 2004), but the authors suggested that this effect is not mediated directly through the 5′-flanking region of zf cyp19a. Exposure of adult female zebra fish to 10nM of E2 for 7 days also suppressed zf cyp19a expression and aromatase activity in the ovary (Hinfray et al., 2006). On the contrary, in the protandrous black porgy (Acanthopagrus schlegeli), treatment of undifferentiated fish with 6 mg E2/kg for 3 months resulted in an increased gonadal aromatase activity (Lee et al., 2004), but it was not established if the observed increase in aromatase activity was the result of enhanced expression of cyp19a or cyp19b. Cyp19a mRNA levels in the gonad of Rivulus marmoratus were upregulated in response to BPA, but downregulated by NP (Lee et al., 2006). Cyp19a expression was also upregulated in the brain of Atlantic salmon juveniles exposed to EE2 (Lyssimachou et al., 2006). Our results together with published data show that the estrogen effects on cyp19a expression may differ depending on the biological model used (species, in vitro vs. in vivo) as well as on the experimental design employed (life stage of development, mode of fish exposure, examined tissues, and ER ligand used). Further detailed investigation of in vivo effects of estrogens on cyp19a gene expression throughout the course of development might be of interest.

**Examination of the Functionality of DRE Sites Predicted in the zf cyp19a and zf cyp19b Gene Promoters**

Exposure to TCDD had no effect on the zf cyp19a or zf cyp19b mRNA levels in zebra fish larvae in vivo. We also examined the functionality of DRE sites predicted in zf cyp19 promoters in vitro using zf AhR2 and zf ARNT2b receptor proteins. Zf AhR2 was shown to be the functional receptor form both in vitro and in vivo (Prasch et al., 2003; Tanguay et al., 1999). Zf ARNT1 splice proteins, but not zf ARNT2b, were suggested to be the preferred dimerization partners for zf AhR2 in vivo (Prasch et al., 2004, 2006). However, zf ARNT2b was shown to form a functional heterodimer with zf AhR2 in vitro that can specifically recognize DREs and induce DRE-driven transcription (Tanguay et al., 2000), which justifies the use of this form of zebra fish ARNT in the present in vitro assays. The activity of the control dioxin-responsive promoter was upregulated by TCDD (or B[a]P) treatment in the presence of zf AhR2/zf ARNT2b in the CHO-K1 and U251-MG cells, which confirmed that these cellular systems contain all the co-factors necessary for the functioning of a classical AhR pathway. A slight upregulation in response to AhR agonists also occurred in the absence of zf AhR2/zf ARNT2b. However, significantly higher upregulation in the presence of zf AhR2/zf ARNT2b suggests that the low levels of endogenous receptors expressed in CHO-K1 and U251-MG cells do not hinder the observation of specific effects of exogenously expressed receptors. In the absence of zf AhR2/zf ARNT2b, TCDD treatment did not affect zf cyp19a promoter activity. It did, however, slightly upregulate the activity of zf cyp19b promoter. The DRE-deficient mutant of this promoter was similarly upregulated under these conditions, suggesting the independence of the response observed from the predicted DRE sites. The possible mechanism of this upregulation is discussed in the next subsection. Expression of zf AhR2/zf ARNT2b led to significant downregulation of the activity of zf cyp19a and zf cyp19b promoters, their DRE-deficient mutants, and of the control E2-responsive promoter containing only an ERE site. The addition of TCDD (or B[a]P) had no further effect on this downregulation. Thus, the downregulation of promoter activity caused by expression of zf AhR2/zf ARNT2b seems to be an unspecific phenomenon, independent of DREs predicted in the zf cyp19a and zf cyp19b promoters. This could be due to the generally increased translation burden on the cells. Interestingly, the activity of the control dioxin-responsive promoter was significantly increased in the presence of zf AhR2/zf ARNT2b even in the absence of TCDD, suggesting that functional DRE elements can promote higher basal activity of the promoter in the presence of high levels of AhR and ARNT, even without the ligand. In in vitro DNA-binding assays, zf AhR2/zf ARNT2b heterodimer binds to DRE even in the absence of ligand (Tanguay et al., 2000). Thus, it is possible that in the cell culture the exogenously expressed unliganded zf AhR2/zf ARNT2b were not readily bound by histones and therefore were able to exert some transcriptional effects on the activity of promoters containing functional DREs. Overall, the pattern of zf cyp19a and zf cyp19b promoters’ response to zf AhR2, zf ARNT2b, and TCDD, which drastically differed from that of the control dioxin-responsive promoter, indicated the nonfunctionality of predicted DRE sites. We also demonstrated by electrophoretic mobility shift assays the inability of DREs predicted in the zf cyp19a and zf cyp19b promoters to bind to liganded zf AhR2/zf ARNT2b heterodimer. The unresponsiveness of zf cyp19a and zf cyp19b
to TCDD may be due to low conservation of consensus sequence.

Observed unresponsiveness of zf cyp19a to AhR agonists stands in agreement with several reports (Hoffmann and Oris, 2006; Kazeto et al., 2004). However, diverse effects of AhR agonists on aromatase in the ovary were observed in other studies. In mice, AhR cooperates with SF1 to activate cyp19 transcription in ovarian granulosa cells (Baba et al., 2005). TCDD decreases cyp19 mRNA levels in cultured rat granulosa cells, but the dependence of this effect on DRE-like sites in the promoter was not established (Dasmahapatra et al., 2000). TCDD reduced ovarian aromatase activity in adult female zebra fish (King Heiden et al., 2006). B[a]P inhibited CYP19 in ovary tissue of flounder (Platichthys flesus) in vitro (Rocha Monteiro et al., 2000) and in adult female killifish ovary in vivo, without affecting cyp19a mRNA levels (Patel et al., 2006). Thus, exposure to AhR ligands apparently may have an effect on aromatase activity in the ovary, but in respect to an effect on cyp19a expression, the evidence is equivocal.

Exposure to B[a]P was shown to increase mRNA levels of cyp19b in zebra fish larvae in 3-days exposure assay as well as in adult females exposed from immature stage for 56 days, but it was not the AhR activation but rather the weak estrogenic activity that was suggested to be primarily responsible for this effect (Hoffmann and Oris, 2006; Kazeto et al., 2004). The lack of zf cyp19b induction by B[a]P in our assay might be due to the lower concentrations used than in the study by Kazeto et al. (2004).

Altogether, these data provide evidence that TCDD and B[a]P may possibly alter the expression and/or activity of cyp19 genes via several mechanisms, which might differ between different organisms. However, our data provide strong evidence that DRE sites predicted in the zf cyp19a and zf cyp19b promoters are not functional. Consequently, it is unlikely that either the isoform of the zf cyp19 genes can be used as a biomarker of exposure to dioxin-like compounds.

Cross Talk between ER and AhR and Its Effects on the zf cyp19b Expression

E2 strongly upregulated the activity of the zf cyp19b promoter in the reporter gene assays and the expression of zf cyp19b in zebra fish larvae, leading to de novo synthesis of CYP19B protein in radial glial cells, in agreement with previous studies (Hinfray et al., 2006; Kishida et al., 2001; Menuet et al., 2005). TCDD attenuated the normal E2-induced response of zf cyp19b expression. This was either partially (in vivo) or fully (in vitro) rescued by the addition of AhR antagonist, ANF, suggesting the involvement of AhR in the downregulation mechanism. The inability to observe full rescue in vivo could be explained by the lower ANF concentration used compared to in vitro experiments (due to observed toxicity of higher concentrations in combination with TCDD). It might also reflect the complexity of in vivo system setup, including the rates of uptake, metabolism and biodegradation of the chemicals, or the fact that ANF is a partial and not a full antagonist of AhR. Another AhR ligand, B[a]P, also downregulated the zf cyp19b response to E2 both in vivo and in vitro in our study and to EE2 in the other (Kazeto et al., 2004). The effect of cotreatment with ANF on the B[a]P-induced changes in the E2 response of zf cyp19b could not be measured because the exposure to E2, B[a]P, and ANF led to high mortality of the larvae, as was also observed by others (Billiard et al., 2006). However, in vitro observations suggested the involvement of AhR in this mechanism. Induced changes in zf cyp19b mRNA levels were closely paralleled on the protein level, thus confirming the functional significance of our findings on the effects of ER and AhR ligands on the zf cyp19b expression.

In our in vitro studies, TCDD downregulated the E2-induced response driven from a single ERE site on the promoter, independent of putative DRE sites or any other transcription sites, as was shown by experiments with the DRE-deficient mutant of the zf cyp19b promoter and with the E2-responsive promoter containing an ERE site, only. Similar results were recently obtained with mammalian receptors, showing that AhR agonists attenuate E2-induced transcription from a single ERE site, without binding directly to ERs or affecting expression levels of ERs (Ohtake et al., 2003). Evidence of a similar mode of action in teleosts has also been observed in another study (Bemanian et al., 2004).

Previous studies on the antiestrogenic effect of AhR ligands in fish focused mainly on hepatic E2 target genes involved in vitellogenesis (Anderson et al., 1996; Navas and Segner, 2000). To our knowledge, the present work provides the first report showing the antiestrogenic effect of an AhR agonist on an E2-regulated gene within a glial cell context. The biological significance of this effect is not known. However, radial glial cells are known to play a crucial role in embryonic and adult neurogenesis (Götz et al., 2002), and E2 is an important neurotrophic and neuroprotective factor. Further, it has been shown that AhR is expressed in the areas of fish brain involved in neuroendocrine regulation of reproductions, such as hypothalamus and gonadotropin cells (Ortiz-Delgado et al., 2002). Therefore, it can be hypothesized that disruption of the normal E2-induced expression of aromatase (and hence of local E2 synthesis) in radial glial cells may be a significant pathway for neuroendocrine effects of AhR ligands in vertebrate.

Exposure to TCDD or B[a]P alone had no effect on the zf cyp19b mRNA levels in zebra fish larvae or on the zf cyp19b promoter–driven luciferase expression in U251-MG cell line in the presence of zf AhR2/zf ARNT2b in vitro. However, a slight upregulation of this gene’s promoter activity by TCDD was observed in the presence of zf ERα, zf AhR2, and zf ARNT2b proteins. This effect was blocked by cotreatment with an excess of either ER antagonist ICI or AhR antagonist ANF, suggesting the involvement of both ER and AhR in the process. Experiments with the DRE-deficient mutant of the zf cyp19b promoter
and the control E2-responsive promoter, containing one ERE site only, suggested that this mechanism is independent of predicted DRE sites, and only the ERE site is involved in this type of ER and AhR interaction. It was shown that liganded mammalian AhR/ARNT heterodimer can directly associate with unliganded ER, leading to stimulation of ERE-mediated transcription (Ohtake et al., 2003), in agreement with our present findings. In light of this, the slight upregulation of the zfcyp19b promoter activity and its DRE-deficient mutant as well as of the control E2-responsive promoter, observed in the presence of TCDD without any expressed zebra fish receptors, can be explained by the presence of low levels of mammalian ER, AhR, and ARNT, which were able to engage in the same kind of action. The differences between fold induction numbers observed in our study and the study of Ohtake et al. (2003) can be attributed to either the structural differences between mammalian and fish receptors, which may account for differences in relative potencies of ligands between mammals and fish (Abnet et al., 1999), or to the different cellular contexts used and the different promoters studied. We observed the weak estrogenic effect of TCDD in vitro only in the absence of the ER ligand, as was also shown by others (Ohtake et al., 2003). Thus, the inability to observe upregulation of endogenous zfcyp19b gene expression by TCDD in vivo in zebra fish larvae can be explained by the presence of endogenous estrogens, which preclude potential estrogenic actions of dioxins.

In summary, we have evaluated the effects of ER and AhR ligands on the activity of zfcyp19a and zfcyp19b genes by in vivo exposure of zebra fish larvae and by in vitro luciferase reporter gene assays. The zfcyp19a gene was not affected by treatment with E2 in vivo. However, a slight upregulation of this gene promoter was observed in vitro, the mechanism of which could not be clarified at the moment. In our study, AhR ligands, TCDD, or B[a]P, did not affect zfcyp19a and zfcyp19b expression in vivo, and in vitro experiments suggested the nonfunctionality of predicted DRE sites. However, we have shown the attenuation of normal E2-induced upregulation of zfcyp19b expression by AhR ligands both in vivo and in vitro. We confirmed the involvement of AhR in this mechanism and were able to show in vitro its independence from the putative DRE sites predicted in the zfcyp19b promoter. This inhibition of E2-induced zfcyp19b expression in radial glial cells is a novel mode of AhR ligands action, which points to the possible disruption of neuroendocrine functions of estrogens as one of the toxic effects of this compound. We have also observed a slight induction of zfcyp19b promoter activity by TCDD in vitro, which seems to function in accordance with the mechanism where liganded AhR/ARNT associates with unliganded ER and triggers transcription from the ERE site. Thus, here we show that in fish, as it has been demonstrated in mammals, the levels of ER agonist estrogen might determine the estrogenicity or antiestrogenicity of AhR agonists in relation to estrogen-responsive genes, and that ER/AhR cross talk follows several pathways, not always depending only on the presence of DRE sites in the gene promoters. These findings should be taken into account while interpreting the results of studies investigating the estrogen-related actions of AhR ligands, especially, in mixtures. Further investigation of interactions between ER, AhR, their ligands, and gene promoters, as well as characterization of coregulators of this process, might greatly facilitate the research on the estrogen-related actions of dioxins.

ACKNOWLEDGMENTS

We thank V. Nesati for help with electrophoretic mobility shift assays and A. Clarke for help with manuscript preparation. This work was part of the European Program “Endocrine disrupters: exploring novel endpoints, exposure, low-dose and mixture-effects in humans, aquatic wildlife, and laboratory animals (EDEN)” and the Swiss National Research Programme “Endocrine Disruptors: Relevance to Humans, Animals, and Ecosystems.” It was financially supported by the Swiss Federal Office for Education and Science, grant Nr. 01.0376-1, and by the Swiss National Science Foundation (NRP50, Project Xebra, 4050-66552). F.B. and N.H. are funded by the Budget Civil de Recherche et Développement (grant BCRD-DRC-10-05) and the Programme National de Recherche sur les Perturbateurs Endocriniens of the Ministère de l’Ecologie et du Développement Durable (CV n°05000194).

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