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BENZO(A)PYRENE DECREASES BRAIN AND OVARIAN AROMATASE mRNA EXPRESSION IN *FUNDULUS HETEROCLITUS*

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Abstract

The higher molecular weight polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BaP) are typically associated with genotoxicity, however, newer evidence suggests that these compounds may also act as endocrine system disruptors. We hypothesized that altered expression of the P450 enzyme aromatase genes could be a target for reproductive or developmental dysfunction caused by BaP exposure. Aromatase is at least partially responsible for estrogen homeostasis by converting androgens into estrogens. In fish, there are two isoforms of aromatase, a predominantly ovarian form, CYP19A1, and a brain form, CYP19A2. CYP19 mRNA expression was measured following BaP exposure (0, 10, 100 µg/L waterborne for 10 or 15 days) in *Fundulus* adults, juveniles and embryos by *in situ* hybridization. The CYP19A1 expression was significantly decreased after BaP exposure in the 3 month old *Fundulus* immature oocytes, but BaP did not affect CYP19A1 expression at any stage in adult oocytes. In embryo brains, BaP significantly decreased CYP19A2 compared to controls by 3.6-fold at 14 days post-fertilization. In adults, CYP19A2 expression was decreased significantly in the pituitary and hypothalamus (81% and 85% of controls, respectively). Promoter regions of *Fundulus* CYP19s were cloned, and putative response elements in the CYP19A1 and CYP19A2 promoters such as CRE, AhR and ERE may be involved in BaP-mediated changes in CYP19 expression. In order to compare the mechanism of BaP-mediated inhibition with that of a known aromatase inhibitor, fish were also exposed to fadrozole (20 and 100 µg/L). Fadrozole did not significantly decrease the mRNA expression in embryos or adult *Fundulus*. However, aromatase enzyme activity was significantly decreased in adult ovary and brain tissues. These studies provide a greater molecular understanding of the mechanisms of action of BaP and its potential to impact reproduction or development.

Keywords

Benzo(a)pyrene; *Fundulus*; Aromatase; CYP19

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INTRODUCTION

The aromatase enzyme, encoded by the CYP19 gene, is responsible for the conversion of androgens (typically testosterone and androstenedione) to estrogens. It is well established that while mammals (excepting pigs) have a single form of this gene, fish have two distinct CYP19 genes (Simpson *et al.*, 2002). Teleost CYP19A1 (CYP19a) is expressed predominantly in the ovary while CYP19A2 (CYP19b) is expressed primarily in the central nervous system. Estrogen homeostasis is integral to diverse physiological processes including reproduction, development, behavior, and carcinogenesis. Therefore, the potential for environmental contaminants to impact aromatase expression and, in turn, affect these downstream physiological processes has been a target of intense recent research and is the subject of two recent reviews (Sanderson, 2006; Cheshenko *et al.*, 2008). Structurally diverse environmental contaminants including tributyltin (Cooke, 2002), methylmercury (Hinfray *et al.*, 2006), triazole and imidazole fungicides (Zarn *et al.*, 2003) have been suggested as aromatase inhibitors, while estrogenic compounds such as nonylphenol and ethynylestradiol induce aromatase expression (Kazeto *et al.*, 2004; Contractor *et al.*, 2004). The mechanisms by which these chemicals interact with aromatase, however, are diverse including inhibition or induction of message and/or protein levels or directly inhibiting enzyme activity. Furthermore, many reported effects are at the tissue level (e.g. whole brain or gonad), and not the specific regions or cell types which may be physiologically important in reproduction and development. The objective of our study was to investigate the potential for benzo(a)pyrene (BaP), a representative carcinogenic polycyclic aromatic hydrocarbon (PAH) and fadrozole, a therapeutically used non-steroidal aromatase inhibitor, to affect CYP19A1 and CYP19A2 mRNA expression in *Fundulus heteroclitus*. By using *in situ* hybridization and testing effects of exposure at different developmental stages, we aimed to highlight some of the stage and cell-type specific effects of these two compounds on CYP19 mRNA expression.

PAHs are ubiquitous environmental contaminants derived from incomplete combustion of carbon. In the United States, PAHs as a mixture (#7), BaP (#9) and benzo(b)fluoranthene (#10) are recognized in the top ten on CERCLA's Priority List of Hazardous Substances (<http://www.atsdr.cdc.gov/cercla/05list.html>). In contrast to halogenated hydrocarbons such as PCBs and chlorinated pesticides which have shown downward trends in US sediment cores, trends in sediment PAH concentrations have been increasing particularly near urban land use (van Metre & Mahler, 2005). Benzo(a)pyrene is the most studied high molecular weight PAH. BaP induces aryl hydrocarbon receptor (AhR)-mediated CYP1 enzymes which, in turn, metabolize BaP to mutagenic and carcinogenic metabolites (ATSDR, 1995; Piskorska-Pliszczyńska *et al.*, 1986). While their role in carcinogenesis is accepted, PAHs also have been suggested as endocrine disrupting compounds (EDCs) by negatively impacting reproduction. In various *in vitro* and *in vivo* fish systems PAH exposure has resulted in decreased: egg output, ovarian somatic index, 17 β -estradiol levels, and vitellogenin production (Hoffman & Oris, 2006; Monteiro *et al.*, 2000; Nicolas, 1999; Thomas, 1990; Afonso *et al.*, 1997). Three studies have specifically investigated BaP's effects on fish CYP19A1 and CYP19A2 mRNA expression using RT-PCR with mixed results. In zebrafish (*Danio rerio*) exposed up to 3 μ g/L BaP for 56 days, CYP19A2 mRNA was increased but no effect on CYP19A1 was observed (Hoffman & Oris, 2006). Similarly, CYP19A2 induction was found when 17 dpf juvenile zebrafish were exposed for 3 days to 10 μ M BaP (2.5 mg/L) (Kazeto *et al.*, 2004). In contrast, in our previous studies with *Fundulus* exposed to waterborne BaP up to 10 μ g/L for 15 days, we did not see a significant effect on expression of either CYP19 message (Patel *et al.*, 2006). In each of these cases mRNA was extracted from either whole tissues or organisms and very sensitive PCR methods were used.

Previous studies exposing fish to fadrozole have reported aromatase inhibition correlated with reduced fecundity and decreased plasma estrogen and vitellogenin concentrations in females

(Ankley *et al.*, 2002; Panter *et al.*, 2004), gonadal masculinization (Uchida *et al.*, 2004; Fenske & Segner, 2004) and decreased courtship behaviors in males (Hallgren *et al.*, 2006). Exposure of fadrozole to salmon ovarian and brain tissue reduced estradiol production *in vitro* and these types of experiments have been suggested as a rapid screening method for other environmental aromatase inhibitors (Afonso *et al.*, 1997; Lee *et al.*, 2006). While fadrozole is a well established competitive inhibitor of aromatase enzyme activity, its effects at the CYP19 message level are less clear (Villeneuve *et al.*, 2006; Villeneuve *et al.*, 2007). To our knowledge, none of the studies in fish have addressed cellular expression of CYP19 mRNAs following fadrozole exposure.

The utility of *Fundulus* as a “premier teleost model in environmental biology” has been highlighted in a recent review (Burnett *et al.*, 2007). *Fundulus* are estuarine fish broadly distributed along the Atlantic coastline, but they maintain very small home ranges (Lotrich, 1975). Their reproductive and developmental biology has been well established (Taylor, 1999; Armstrong & Child, 1965). They have also been extensively used in both laboratory and field settings to further understand molecular mechanisms of environmental toxicants including, but not limited to: PAHs (Wassenberg & Di Giulio, 2004; Meyer *et al.*, 2003; Willett *et al.*, 1995; Wang *et al.*, 2006; Stine *et al.*, 2004), PCBs (Greytak *et al.*, 2005; Greytak & Callard, 2007; Powell *et al.*, 2000), dioxins (Prince & Cooper, 1995), metals (Zhou *et al.*, 2000; Roling *et al.*, 2007) and effluents (Dube & MacLachy, 2001).

As introduced above, effects of both BaP and fadrozole on CYP19 expression in fish have shown mixed results. In previous studies in our laboratory, we found no statistically significant effect of BaP on CYP19A1 or CYP19A2 message by RT-PCR of whole brain or gonads. However, BaP exposure caused an inhibition of ovarian aromatase activity while it induced female brain activities (Patel *et al.*, 2006). To more rigorously define constitutive CYP19 mRNA expression in *Fundulus* embryos, juvenile and adult fish, we developed *in situ* hybridization probes for both CYP19A1 and CYP19A2 mRNA (Dong & Willett, 2008). In this study, we have found that the PAH, BaP, alters local expression of CYP19 mRNA but in an age- and tissue-dependent way. In contrast, fadrozole inhibition was exclusively detected at the enzyme level. Putative response elements in the *Fundulus* CYP19A1 and CYP19A2 promoters were identified that may be playing a role in the BaP-mediated dysregulation of the CYP19 genes.

MATERIALS AND METHODS

Fish source, care and handling

A parental population of *F. heteroclitus* collected from an uncontaminated site at the New River inlet near Beaufort, NC was raised under the University Institutional Animal Care and Use Committee approved conditions. Sexually mature fish were bred and kept in salt water (20–25 parts per thousand [ppt]). The fish were maintained at 14:10 light-dark cycle in summer and 10:14 light-dark cycle in the winter. Adult fish were fed twice daily with tropical flake fish food (Tetramin, Tetra Werke, Germany) and live brine shrimp. First generation offspring, from wild parents, were used for the studies described here.

Embryo exposure

Pooled oocytes and sperm stripped from parental population of around 50 fish were mixed together for *in vitro* fertilization. Fertilized eggs were randomly sorted into seven treatment groups, namely untreated, control dimethylsulfoxide (DMSO), (0.1 µg/ml), BaP (10 and 100 µg/L), and aromatase inhibitor fadrozole (1, 5 and 10 µg/L). Fadrozole (4-(5,6,7,8-tetrahydroimidazole[1,5-a]pyridin-5-yl)-benzonitrile monohydrochloride; CGS16949A) was kindly provided by Novartis Pharma AG, (Basel, Switzerland). Eggs were placed in 10 ml of

water in clean glass bottle (n=10 embryos/bottle) and exposure began at approximately 4.5-hpf (hours postfertilization) until 10 dpf (days postfertilization). 1-Phenyl-2-thiourea (PTU, 0.003%) was added to the culture water from 5 to 10 dpf to prevent pigmentation of *Fundulus* embryos. At 10 dpf, eggs were removed from the exposure and placed in a culture plate on top of a piece of damp filter paper where they continued to develop until 14 dpf when they were collected for *in situ* hybridization.

Immature and adult exposure

Fish were exposed in 5 or 6-L tanks containing two male and two female for adult fish or four 3 month old fish (1.5 L water per adult fish, 1.25 L water per 3 month old fish). Fish were kept in the tanks 7 days for acclimatization prior to the beginning of the exposure. The *Fundulus* (n = 6–16/treatment) were exposed to the following treatment for 15 days (complete reproductive cycle): control (100 µl/L ethanol), BaP (10 µg/L (40 nM) and 100 µg/L (400 nM)) (Sigma, St Louis, MO, stock solution 1 mg/ml in 100% ethanol) or fadrozole (20 and 100 µg/L). Stock solutions of fadrozole were prepared in ethanol at concentrations of 20 and 100 mg/ml. The ethanol concentration in all treatments was 0.01%. Exposure conditions were 17.5–20.5°C, 14:10 light-dark period, and 20–25 ppt. Fish were fed Tetramin flakes twice daily. The tanks were checked twice every 24 h, and water was changed (85–100% static water renewal) daily at 15:00. On the 15th day of exposure, the fish were anesthetized by placing them into salt water that contained 3-aminobenzoic acid ethyl ester, MS222 (Sigma). The 3 month old fish were then weighed, measured, and fixed in 1 ml Bouin's solution prior to paraffin embedding, sectioning and *in situ* hybridization. Adult fish were weighed and measured, and dissected to retrieve the gonads and brains which were also weighed. Gonads and brains were fixed in 4% (w/v) paraformaldehyde for paraffin sectioning or whole mount *in situ* hybridization from the BaP-treated fish and half of the fadrozole-treated fish. Brains and gonads from the other half of the fadrozole-treated fish were placed into phosphate buffer (10 mM potassium phosphate, 100 mM potassium chloride, 1 mM EDTA, pH 7.4) that equaled 4 times the organ weight. The tissues were homogenized and centrifuged for 10 minutes at 10,000 × g at 4°C. The protein concentration was quantitated by the Bradford assay, and samples were stored at –80°C. These tissues were used for aromatase enzyme activity determination.

Whole mount in situ hybridization

Fundulus embryos were fixed in 4% (w/v) paraformaldehyde in phosphate saline solution (pH 7.4) overnight, stored in –20°C freezer after the membranes were removed by watchmaker's forceps. Whole mount *in situ* hybridization was carried out according to (Dong & Willett, 2008). Embryos or adult brains were hybridized with an antisense probe of 717 base pairs for *Fundulus* CYP19A2, of which the cDNA was cloned previously (Patel *et al.*, 2006). Following hybridization overnight at 65°C, embryos or adult brains were washed with 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) and 0.2× SSC twice for 30 min, respectively. After blocking with 2% blocking reagent (Roche, Mannheim, Germany), embryos or brains were incubated overnight with 4000× diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) at 4°C. The color reaction was carried out by incubation with BM-purple substrate (Roche).

Paraffin sectioning and in situ hybridization

Fundulus gonads were fixed in 4% (w/v) paraformaldehyde in phosphate saline solution (pH 7.4) overnight, followed by dehydration in increasing gradients of ethanol (70%, 80%, 90%, 95%, and 100% twice). After cleared in Clearify™ (American Master Tech Scientific, Inc., Lodi, CA), tissues were embedded in molten paraffin (Paraplast embedding media paraplast X-tra, Sigma). Sections of 7 µm thickness were made using a microtome (OLYMPUS CUT 4055, Olympus American Inc., San Jose, CA). The sections were cleared with a gradient of

ethanol (100%-70%) and hybridized with a 1660 nt antisense probe of *Fundulus* CYP19A1. Following hybridization overnight at 50°C, sections were washed with 2× SSC and 0.2× SSC twice for 20 min, respectively. After blocking with 2% blocking reagent (Roche), sections were incubated overnight with 4000× diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) at 4°C. The color reaction was carried out by incubation with BM-purple substrate (Roche).

Expression quantification

CYP19 expression was recorded with a digital camera (OPTRONICS) and light microscope (BX40, Olympus). For quantifying the intensity of CYP19A1 or CYP19A2 expression, stained regions were outlined by means of KODAK 1D Image Analysis Software by creating regions of interest, (ROIs) and calculating mean intensity (= sum intensity divided by ROI pixel area). For quantification of CYP19A2 mRNA expression in the embryonic *Fundulus*, conventional image software (Photoshop 7.0; Adobe) was used. The expression area was circled on the Photoshop image at the same magnification, and the expression area was divided by the embryo's total head area.

Aromatase assay

Aromatase activity was quantified by the (Lephart & Simpson, 2002) tritiated water release assay as described by (Patel *et al.*, 2006) with minor modifications. Briefly, tissue homogenates were incubated in a shaking water bath for 3 h at 28°C/100 rpm with substrate, 3.0 nM (for gonad) or 30 nM (for brain). The substrate ³H-androst-4-ene-3, 17-dione (specific activity 25.9 Ci/mmol (Perkin-Elmer Life Sciences, Boston, MA, USA)) was in the presence of a reaction buffer (10 mM potassium phosphate (dibasic), 100 mM potassium chloride, 1 mM EDTA, 1 mM dithiothreitol, 1 mM NADPH, 10 mM glucose-6-phosphate and 1 U/mL glucose-6-phosphate dehydrogenase), in 200 µL total volume of reaction. Reactions were terminated, and extracted as previously described, and 50 µL of the ³H₂O was quantified by counting in 200 µL of premixed scintillation cocktail (Ultima Gold TMXR, Packard Biosciences, Boston, MA) with a liquid scintillation analyzer (Topcount™ microplate scintillation & Luminescence counter, Packard Meriden, CT). The specificity of the assay was determined by using 20 µM 4-hydroxyandrostenedione (Sigma), an irreversible inhibitor of the catalytic activity of aromatase. Aromatase activity (pmol/h/mg) was calculated by correcting for the tritium estimated in the blank tubes (containing substrate but no protein, dpm range 77–101), the dilution factor = 3, the ³H distribution of the [¹β – ³H] androstenedione (75% at the 1β-position and 25% at the 1α-position), protein concentration (150 µg for brain and 500 µg for gonad) and the specific activity of the substrate (dpm/mass; 14,302 ± 935).

Statistics

Results are presented as mean ± SE. Data was tested for homogeneity of variance with the Bartlett's test. If variances were not significantly different, experimental significance was determined by one-way ANOVA followed by Neuman-Keulls posthoc test (p < 0.05). When data failed the Bartlett's test, the non-parametric test, Kruskal-Wallis and Dunn's Multiple comparison test was used (p < 0.05).

Promoter region cloning

In order to clone the promoter regions of both CYP19A1 and CYP19A2 reverse primers were designed based on the previously published CYP19 cDNA sequences (Patel *et al.*, 2006). Primers were synthesized by Invitrogen. Primers 1 and 2 were specific to the CYP19A1 gene: Primer 1: 5'-GCTACGCTGAGATTGCTTTGAACCTCC-3'; Primer 2: 5'-CTTCGGGTTTTGAGGAGCTACG-3'. Primers 3–6 were complementary to the CYP19A2 gene: Primer 3: 5'-GGTCAGCAGAGGAGAAGAGAGCGAAC-3'; Primer 4: 5'-

GAGTGGAGGGAGTTCTGCAGCT-3'; Primer 5: 5'-CAGGTGTGTGACAGTAAAGTTTGTGACAG-3'; Primer 6: 5'-GTGGCACAAGAAATCTGTAAGGAGGC-3'. (See also Supplementary Figure for primer locations). An adult female *Fundulus* liver GenomeWalker library (Universal GenomeWalker Kit; Clontech, Mountain View, CA) was constructed according to the manufacturer's protocol in order to use a PCR-based method for the isolation of the 5'-termini of the two *Fundulus* CYP19 genes. For CYP19A1, the first PCR was carried out using Primer 1 (and the kit's adapter-specific primer) followed by a nested second amplification with Primer 2 using the library generated from *Dra*I, *Eco*RV, *Pvu*II, or *Stu*I restriction-digested genomic DNA. For CYP19A2, Primers 5 and 6 were used with the same library for the nested PCR amplification of the 5'-flanking region of CYP19A2. To isolate amplicons corresponding to the entire 5'-flanking region, PCRs were carried out with the following sets of primer pairs: Primers 2 and 7 (Primer 7: 5'-GAATTCCTGAAATGGTTTTTCAG-3'), and Primers 4 and 8 (Primer 8: 5'-TAGTAAAGAAGGTCATGGAC-3'), for CYP19A1 and CYP19A2, respectively. The putative transcription initiation sites for each promoter region were determined using the Neural Promoter Predictions Program (<http://www.fruitfly.org/cgi-bin/seqtools/promoter.pl>). Potential transcription factor binding sites were identified by MatInspector (<http://www.genomatix.de>) and TFSEARCH (<http://www.cbrc.jp>).

RESULTS

Effects of BaP and fadrozole on CYP19A2 mRNA expression in *Fundulus* embryos

In 14 dpf *Fundulus* embryos, CYP19A2 expression was detected by *in situ* hybridization in the brain especially the hypothalamus and adrenal-like cells in the kidney. BaP strongly decreased expression of CYP19A2 gene in the brain but had no effect on interrenal expression (Fig. 1, note adrenal cells not in same focal plane as brains, expression best shown in panel 1E). When the expression area in brains was traced and quantitated, 10 and 100 µg/L BaP significantly decreased CYP19A2 expression by 3.61- and 3.66-fold compared to controls, respectively (Table 1). In contrast, embryos treated with 10 µg/L fadrozole for ten days had no change in CYP19A2 expression (Fig. 1E and Table 1). We found very weak expression of CYP19A2 in the hypothalamus and almost no expression in the pituitary in the 3 month old fish brain by *in situ* hybridization (Dong & Willett, 2008), so in this study we did not investigate BaP or fadrozole effects in 3 month old brains.

Effects of BaP and fadrozole on CYP19A2 mRNA expression in adult *Fundulus*

CYP19A2 mRNA is constitutively expressed in pituitary, hypothalamus, ventral telencephalon and olfactory bulb in adult *Fundulus* (Dong & Willett, 2008). Furthermore, CYP19A2 expression between the genders was not significantly different except in the olfactory bulb. In this study, we did not find any differential constitutive expression in any of the brain regions between males and females. Adult *Fundulus* exposure for 15 days to BaP significantly downregulated CYP19A2 expression by approximately 15–20% in the hypothalamus and pituitary compared to controls, but decreases found in the olfactory bulb and ventral telencephalon were not significant (Fig. 2 and Table 2). Fadrozole exposure did not significantly alter CYP19A2 expression in adult brain regions.

Effects of BaP on CYP19A1 gene expression in *Fundulus*

CYP19A1 mRNA is not detectable with *in situ* hybridization in any of the embryo stages that we investigated. However, CYP19A1 mRNA is expressed in the ooplasm of early stage I oocytes (primary growth stage) as strong and evenly distributed signals in the ovaries of 3 month old *Fundulus*. The effects of BaP and fadrozole on CYP19A1 gene expression in juvenile fish are shown in Fig. 3. CYP19A1 expression was clearly decreased in the early stage I oocytes by BaP but not in the fadrozole treated group (Fig. 3). To quantitate the CYP19A1

expression, early stage I oocytes were divided by size into stage Ia1 (diameter less than 0.07 mm) and stage Ia2 (diameter about 0.1 mm). CYP19A1 expression in the BaP exposed groups was approximately 71% and 72% of controls, respectively, in the 10 and 100 µg/L group in the stage Ia1. In the stage Ia2, CYP19A1 mRNA expression was significantly decreased by 46% and 42% by 10 and 100 µg/L BaP, while fadrozole had no effect on CYP19A1 at either oocyte stage (Table 3)

In adult *Fundulus*, constitutive expression of CYP19A1 mRNA also was detected in the ooplasm of early stage I oocytes (Fig. 4B1, B2, B3 and B4). After early stage I, normal expression of CYP19A1 mRNA expression moves to the vitellogenic follicle then to the vitelline envelope of membrane. Neither BaP nor fadrozole affected CYP19A1 expression in adult *Fundulus* (Fig. 4).

Effects of BaP and fadrozole on adult *Fundulus* GSI

BaP did not affect gonad somatic index (GSI) in either male or female fish compared to controls (Table 4). Fadrozole exposure increased male adult GSI at the highest dose, but had no effect on female GSI following the 15 day waterborne exposure.

Effects of fadrozole on adult *Fundulus* brain and ovary enzyme activity

Exposure to fadrozole (20 and 100 µg/L) for 15 days did not significantly affect adult fish or mean brain weight, size or survival (data not shown). While fadrozole treatment did not alter CYP19A1 or CYP19A2 mRNA expression, *in vivo* exposure to 20 and 100 µg/L fadrozole inhibited ovarian aromatase activity by 80% and 73%, respectively ($p < 0.05$, ANOVA) (Fig. 5A). Activity was not detectable in testis (data not shown). Fadrozole exposure decreased female brain activity 9.7- and 4.3-fold compared to control treated animals (Fig 5B). As expected when 2.5 µg/L fadrozole was added *in vitro* to control brain preparations, aromatase activity was also decreased (6.5-fold). However, male brain aromatase activity was not statistically affected by either *in vivo* or *in vitro* fadrozole exposure partially because of high replicate variability (Fig. 5C). Aromatase activity was decreased by 99% in the presence of 4-hydroxyandrostenedione, an irreversible inhibitor, validating the activity to be due to aromatase enzyme (data not shown).

Isolation of the cDNAs encoding *Fundulus* CYP19s

To further understand the regulation of the *Fundulus* CYP19A1 and CYP19A2 genes, we isolated and sequenced both promoters (Table 5 and supplementary figure). The CYP19A1 promoter was 525 nt to the putative start site while the CYP19A2 promoter was 1257 nt long. The putative transcription factor binding sites in CYP19A1 included: a consensus TATA box, steroidogenic factor-1 (SF1/Ad4BP), cAMP responsive element (CRE), estrogen responsive element (ERE), aryl hydrocarbon responsive element (AhR/Arnt), peroxisomal proliferator-activated receptor/retinoid X receptor (PPAR/RXR) element, nuclear factor kappa β (NF-kappaβ), and two GATA binding factor sites (Supplementary Figure and Table 5, GenBank Accession no. EU045309). In the CYP19A2 5'-flanking sequence, the putative transcription factor binding sites were: a TATA box, SF1/Ad4BP, progesterone receptor (PR), AhR/Arnt, ERE, two androgen receptor (AR), and a GATA-binding factor 3 site (Supplementary Figure and Table 5, GenBank, Accession no. EU045310).

DISCUSSION

BaP decreased CYP19A1 expression in the ovary of immature but not adult *Fundulus*

We have previously investigated the effects of BaP on both *Fundulus* CYP19A1 and CYP19A2 mRNA expression using quantitative RT-PCR. Because whole brains or gonads were

homogenized and there was a lot of interindividual variability, we were unable to measure a significant treatment-related effect on mRNA expression (Patel *et al.*, 2006). Similarly, when zebrafish were exposed to up to 3 µg/L BaP for 56 days there was no detectable effect on CYP19A1 expression in mid-vitellogenic oocytes by PCR (Hoffman & Oris, 2006). To better understand the tissue and cellular expression of CYP19A1 mRNA, we have more recently developed *in situ* hybridization probes and have described constitutive expression of this gene in *Fundulus* during development (Dong & Willett, 2008). CYP19A1 expression was not detectable by *in situ* hybridization until fish were 3 months old when it is expressed strongly and evenly throughout the ooplasm of early stage I oocytes. BaP exposure did significantly decrease this expression by about 50% in immature stage I oocytes, but had no effect on adult oocyte expression where CYP19A1 is expressed in the follicular membrane and vitelline envelop in more mature oocytes. Follicular growth in *Fundulus* ovaries is asynchronous (Selman & Wallace, 1983), and during the reproductive season there are oocytes present at each stage of oocyte maturation. While we saw no effect on CYP19A1 message expression in adult oocytes, we have reported a ≥ 2.5 -fold decrease in ovarian aromatase activity following a similar 15 day 10 µg/L waterborne BaP exposure (Patel *et al.*, 2006).

What is the potential significance of the decreased CYP19A1 expression in immature oocytes in juvenile *Fundulus*? One could hypothesize that decreased circulating estrogen concentrations would increase the time it takes for fish to reach reproductive maturity. However, this would be difficult to test experimentally in *Fundulus* especially if continuous exposure to BaP was necessary to sustain the inhibition of CYP19A1 expression. Furthermore, research to date on this life-stage of *Fundulus* (i.e. non-adult) and associated normal steroid levels or reproductive development is very limited.

In contrast, adult *Fundulus* oogenesis and steroidogenesis have been elegantly described (Wallace & Selman, 1978; Wallace & Selman, 1980; Selman & Wallace, 1983; Petrino *et al.*, 1989b; Petrino *et al.*, 1989a; Petrino *et al.*, 1990; Hsiao *et al.*, 1996; Cerda *et al.*, 1998; Cochran *et al.*, 1988; Bradford & Taylor, 1987). Petrino and coworkers established that *Fundulus* prematurational follicles contain the enzymes (cholesterol side chain cleavage, Δ^5 -3 β -hydroxysteroid dehydrogenase and aromatase) necessary for conversion of cholesterol to 17 α -hydroxy,20 β -dihydroprogesterone, testosterone and estrogen (Petrino *et al.*, 1989b). Furthermore, follicle cells attached to the vitelline envelope but not thecal/surface epithelial cells were capable of producing all three steroids when stimulated with either *Fundulus* pituitary extracts, 25-hydroxycholesterol or pregnenolone (Petrino *et al.*, 1989a).

Our CYP19A1 *in situ* hybridization results support this earlier work with respect to localization of expression, however, BaP exposure did not reduce CYP19A1 expression in any stage of adult oocyte development. This result presents at least two questions: 1) Why are 3 month old stage I oocytes more sensitive to inhibition than adult stage I oocytes, and 2) Why is aromatase enzyme activity significantly inhibited by 10 µg/L BaP (Patel *et al.*, 2006) but CYP19A1 message is not altered even with exposures up to 100 µg/L? Answers to these questions are speculation at this point and surely deserve more investigation, and many direct or indirect hormonal or metabolic mechanisms may be involved. For example, the age related differences could possibly be related to discrepancies in absorption, distribution, metabolism or excretion of BaP between reproductively immature and adult fish. On one hand, younger *Fundulus* may not metabolize and excrete BaP as quickly allowing for higher concentrations to accumulate in the immature oocytes, in turn, allowing BaP to have a direct effect on CYP19A expression. However, the opposite result could be predicted based on Monteverdi and Di Giulio's work wherein previtellogenic oocytes (less than 0.5 mm) accumulated low to non-detectable amounts of ^{14}C -BaP following maternal exposure. In contrast, mid vitellogenic oocytes (1.1–1.4 mm) had the highest accumulation of BaP (Monteverdi & Di Giulio, 2000) suggesting more mature adult eggs would have higher BaP concentrations associated with vitellogenin uptake.

Similarly, a number of hypotheses could explain the differences in message and enzyme inhibition. Inhibition at the message level may be mediated through promoter response elements in the CYP19A1 gene. The *Fundulus* CYP19A1 promoter is similar to zebrafish (Tong & Chung, 2003; Kazeto *et al.*, 2001) and goldfish (Tchoudakova *et al.*, 2001) wherein there are putative AhR, SF1 and CRE binding sites in the 5'-flanking region. Normal expression of CYP19A1 is believed to be transcriptionally modulated through gonadotropin induced increases in cAMP (Petrino *et al.*, 1990) which in turn stimulates the CYP19 CRE (reviewed in (Cheshenko *et al.*, 2008). The Ad4BP/SF-1 site has also been suggested as being involved in transcriptional regulation of CYP19A1 in Nile tilapia ovarian follicles (Yoshiura *et al.*, 2003). However, *in vitro* studies of zebrafish CYP19A1 SF1 and ERE putative sites have suggested that these elements are not responsible for estrogen-mediated effects on CYP19A1 expression (Cheshenko *et al.*, 2007). In fact, a number of studies have shown that CYP19A1, in contrast to CYP19A2, expression is not inducible by estrogens (Kishida & Callard, 2002; Kazeto *et al.*, 2004; Sawyer *et al.*, 2006). With respect to the inhibition of CYP19A1 expression by BaP, a hypothesis has been suggested (Patel *et al.*, 2006; Hoffman & Oris, 2006), based on mammalian literature (Dasmahapatra *et al.*, 2001), that binding of BaP to the AhR on CYP19A1 may interfere with normal transactivation and expression of the gene, but this remains to be tested.

In *Fundulus* CYP19A1 mRNA expression was not detectably decreased in adult oocytes by *in situ* hybridization following 15 day BaP exposures up to 100 µg/L, but aromatase activity was decreased in adult fish exposed similarly to only 10 µg/L BaP (Patel *et al.*, 2006). Perhaps a compensatory mechanism is initiated during the 15 day continuous exposure in adults wherein oocytes can counteract aromatase inhibition so that the message levels did not appear to be decreased but the protein activity is still decreased. We have not been able to identify an effective antibody for *Fundulus* CYP19 to test whether actual protein concentrations are inhibited by BaP. Therefore, BaP also may be acting as an aromatase activity inhibitor as suggested by *in vitro* experiments with flounder ovarian tissue where 15 µM BaP inhibited steroidogenesis and estradiol production (Monteiro *et al.*, 2000). However BaP at 10 µM did not decrease rainbow trout ovarian microsomal aromatase activity *in vitro* (Hinfray *et al.*, 2006).

BaP decreased CYP19A2 expression in embryo and adult *Fundulus* brains

In contrast to CYP19A1, CYP19A2 was detectable in *Fundulus* embryo brains as early as 3 dpf. In this study we measured the effects of a 10 day exposure of BaP on CYP19A2 embryo expression at 14 dpf and found significant decreases in brain expression particularly in the hypothalamus and ventral telencephalon. The long-term developmental significance of decreased embryonic CYP19A2 expression is currently incompletely understood in teleosts and deserves more research. When protogynic fish like zebrafish were exposed to tributyltin, an aromatase inhibitor, from 0 to 70 days posthatch, a male biased population with a high incidence of fish with sperm lacking flagella resulted (McAllister & Kime, 2003). Similarly, dietary fadrozole exposure between 35 and 71 dpf can cause masculinization of zebrafish (Fenske & Segner, 2004). However, these studies both represent larval exposures in contrast to the embryo exposures reported here in *Fundulus*.

CYP19A2 was also expressed in adrenal-like cells of the head kidney in embryos but interestingly this expression was not decreased by BaP exposure (data not shown, note kidney cells are not in same focal plane as brains in Figure 1). To date, the significance of CYP19A2 expression in the kidney is unknown, but adrenocortical tissue does possess enzymes involved in steroid synthesis (Bara, 1968; Vermeulen *et al.*, 1995; Gonzles & Piferrer, 2003).

Three previous studies have investigated BaP's effects on CYP19A2 expression using qPCR. When zebrafish juveniles were exposed to 10 µM (2520 µg/L) BaP for 3 days and RNA was

isolated from whole fish, CYP19A2 expression was significantly induced (Kazeto *et al.*, 2004). CYP19A2 expression was also induced in zebrafish heads by a 56 day exposure to 3.35 µg/L (Hoffman & Oris, 2006). Work in our laboratory with *Fundulus* found no significant effect of BaP on whole brain CYP19A2 expression due to high interfish variability, but an increase in aromatase activity following winter but not summer BaP exposures was detected (Patel *et al.*, 2006). Using *in situ* hybridization, BaP-mediated inhibition of CYP19A2 was statistically significant in the pituitary and hypothalamus regions. Discrepancies in BaP's effects among all of these studies could be related to species, tissues from which RNA was isolated (whole fish vs. heads vs. brain), exposure (both concentrations and durations), and detection method sensitivities (PCR vs. *in situ*). In wild *Fundulus* collected from New Bedford Harbor, a superfund site, CYP19A2 mRNA and male vitellogenin expression was induced compared to fish collected from a cleaner site (Greytak *et al.*, 2005). However, the New Bedford Harbor is most highly contaminated with PCBs and other estrogenic contaminants (i.e. not PAHs), hence induction was consistent with the established induction of CYP19A2 by estrogenic compounds. The idea that certain BaP metabolites may be slightly estrogenic has been suggested as a reason for BaP's induction of CYP19A2 in previous studies (Kazeto *et al.*, 2004; Hoffman & Oris, 2006; Patel *et al.*, 2006). However, in this study CYP19A2 expression was decreased.

When considering the response elements in the *Fundulus* CYP19A2 promoters that might be contributing to transcriptional expression, we found putative ER, AR, PR, AhR and CAR/RXR binding sites. The ER binding site has been consistently reported in other fish CYP19A2 promoters (Tchoudakova *et al.*, 2001; Kazeto *et al.*, 2001) but the presence of a possible AhR has only been reported in zebrafish to date (Tong & Chung, 2003). However, transient transfection assays and DNA binding assays using the zebrafish CYP19A2 promoter suggested that while TCDD downregulated estrogen-induced expression, the dioxin response element sites were not functional (Cheshenko *et al.*, 2008). Similar studies are ongoing in our laboratory with truncated *Fundulus* CYP19 promoters to attempt to further understand the transcriptional regulation of BaP on CYP19A2.

Both CYP19A2 expression and brain aromatase activity are much higher than gonadal expression in fish. The physiological significance of this high expression is not completely resolved but has been implicated in control of reproduction, sexual behaviors, and estrogen neurogenesis (recently reviewed in (Cheshenko *et al.*, 2008)). BaP significantly decreased adult expression of CYP19A2 in both the pituitary and the hypothalamus but had no effect on expression in the ventral telencephalon or olfactory bulb. The inhibition was not sex dependent. As mentioned above, complicated interactive feedback loops control steroid synthesis in vertebrates and many compensatory systems may help counteract a stressors effect on one aspect of the system. While BaP significantly decreased CYP19A2 expression in two regions, whole brain aromatase activity was not decreased (Patel *et al.*, 2006), and GSI was not affected in either males or females. The MacLatchy laboratory has recently optimized a short-term *Fundulus* reproduction assay (Peters *et al.*, 2007) so in the future, we may be able to also test whether reproductive parameters are also adversely impacted by BaP exposure.

Fadrozole inhibited aromatase activity

An *in vivo* exposure to up to 100 µg/L fadrozole, a well established aromatase inhibitor, did not measurably decrease either CYP19A1 or CYP19A2 expression in these experiments. However, following exposure, whole brain or ovarian aromatase enzyme activity was inhibited as expected. Furthermore, fadrozole significantly increased male GSI but did not affect female GSI. Effects of fadrozole on GSI have also been reported in fathead minnows exposed to 100 µg/L for 21 days. Similar to our studies GSI increased in males, but a decrease in GSI was reported in females (Panter *et al.*, 2004). In another fathead minnow study where doses ranged

from 2 to 50 mg/L for 21 days, fadrozole decreased plasma estrogen and vitellogenin but had no effect on female GSI while it increased testosterone, 11-ketotestosterone and GSI in males (Ankley *et al.*, 2002). At the messenger RNA level, the effects of fadrozole as measured by PCR are more complicated. Dietary exposure of zebrafish to fadrozole during gonadal differentiation resulted in 100% masculinization, decreased CYP19A1, but increased CYP19A2 expression (Fenske & Segner, 2004). Villeneuve and colleagues found when fathead minnows were exposed to fadrozole both brain activity and CYP19A2 expression were decreased, however CYP19A1 expression was increased and ovarian activity showed an inverted U-shaped dose response (Villeneuve *et al.*, 2006). In a subsequent study the CYP19A2 inhibition was replicated, but the CYP19A1 expression also showed a U-shaped response (Villeneuve *et al.*, 2007). These studies suggest, as concluded by Villeneuve and coworkers, that there are not particularly robust relationships between brain or ovary aromatase activity and isoforms specific mRNA expression (Villeneuve *et al.*, 2006). Our work with BaP supports this conclusion and further suggests complex relationships between message expression and enzyme activity.

In conclusion, we found that 10 or 15 day exposures to BaP did decrease both CYP19A1 (in immature oocytes) and CYP19A2 (in embryo brains and adult hypothalamus and pituitary regions) mRNA expression in *Fundulus*. Importantly, these differences have not been detected previously using whole tissue homogenates. Furthermore, BaP and fadrozole worked through different mechanisms to alter aromatase. Additional studies of both promoter regulation and reproductive consequences of BaP exposure will hopefully add further insight into the physiological significance of the CYP19 inhibition that we have detected in embryonic, juvenile and adult *Fundulus*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of BaP exposure on CYP19A2 expression in the brain of developing embryos
 Embryos were untreated or exposed to DMSO, BaP (10 or 100 µg/L) or fadrozole (10 µg/L) from 4.5 hpf to 10 days. On days 5–10 PTU was added to decrease pigment and facilitate whole mount *in situ* hybridization at 14 dpf. Arrow shows CYP19A2 expression. Panel F indicates nonspecific binding using sense probe. ($n= 5$ embryos/treatment).

Figure 2. Effect of BaP- or fadrozole-exposure on CYP19A2 expression in the adult *Fundulus* brain
 The CYP19A2 transcripts were found in the hypothalamus (Hy), pituitary (Hp), ventral telencephalon (VT), and extending to the olfactory bulb (OB). A and B are Control; C and D are 10 and 100 µg/L BaP treated fish; E and F are 20 and 100 µg/L fadrozole. B shows pituitary region dissected from A. Arrows indicate expression in the pituitary. ($n = 3-8$ fish/sex/treatment; females shown)

Figure 3. Effect of BaP exposure on CYP19A1 mRNA expression in the ovary of 3 month old *Fundulus*

Fish were sectioned and visualized by section *in situ* hybridization after exposure to DMSO (control), BaP (10 or 100 µg/L) or fadrozole (100 µg/L). B, D, F and H are magnification of A, C, E and G, respectively. The CYP19A1 expression was found in ooplasm of oocytes in the control and fadrozole treated group as strong and evenly distributed signals, but the signal was decreased in the BaP (10 and 100 µg/L) treated group ($n= 4-5$ fish/treatment).

Figure 4. Effect of BaP- or fadrozole-exposure on CYP19A1 mRNA expression in the ovary of adult *Fundulus*

Fish were sectioned and visualized by section *in situ* hybridization after exposure to DMSO (control, A1-G1 and A2 -G2), BaP (100 µg/L, A3-G3) or fadrozole (100 µg/L, A4-G4). A1-G1 shows CYP19A1 mRNA expression (purple color) and Eosin contrast stain (pink color). Expression of CYP19A1 mRNA was detected in B: early stage I (primary growth stage, B1, B2, B3 and B4) in the ooplasm of oocyte; C: mid stage I (Stage Ib) in the membrane and ooplasm (previtellogenesis follicle stage, C1, C2, C3 and C4); D: stage Ic in the follicular epithelium (D1, D2, D3 and D4); E: Stage II (vitellogenesis) in the follicular epithelium of membrane (E1, E2, E3 and E4); F: Stage III (early maturational follicles) in the vitelline

envelope of oocytes (F1, F2, F3 and F4); and G: No signal was detected after stage IV (maturation follicles, G1, G2, G3 and G4) oocytes. No significant effect was detected between control, BaP or fadrozole-treated groups in each stage. ($n= 4-5$ fish/treatment).

Figure 5. Aromatase activity in *Fundulus*

Fundulus were exposed to waterborne fadrozole (20 or 100 $\mu\text{g/L}$) for 15 days. Ethanol (180 μL) was used as the solvent control. Ovary or brain homogenate (500 or 150 μg protein, respectively) was incubated with 3 nM (or 30 nM) ^3H -androstenedione for 3 h at 28°C. Aromatase activity was measured as the release of tritiated water and reported as pmol/mg/h. Each bar represents the mean $\pm$ SEM of 7–8 fish per treatment. As a positive control 2.5 $\mu\text{g/L}$ fadrozole was added to control tissues during the course of the reaction and results are the bars labeled F *in vitro*. (A) Ovarian activities. (B) Female brain activities. (C) Male brain activities. (ANOVA, * $p \leq 0.05$).

Table 1

Quantitative CYP19A2 expression in the brain/head area of BaP- or fadrozole-exposed 14 dpf *Fundulus* embryos.

Control	DMSO	BaP 10 µg/L	BaP 100 µg/L	Fadrozole 10 µg/L
0.20 ± 0.02 ^a	0.18 ± 0.02 ^a	0.06 ± 0.01 ^b	0.05 ± 0.01 ^b	0.16 ± 0.03 ^a

The CYP19A2 expression area was quantified with image analysis and was expressed in pixels/head area.

Differences in letters signify significantly different (Mean ± SEM, $n = 5$ embryos/treatment, ANOVA, $p \leq 0.05$) values.

Table 2
Quantitative CYP19A2 expression ($\times 10^4$) in brain regions in control, BaP or fadrozole-exposed adult *Fundulus*.

	n	HY	HP	VT	OB
Control	14	5.37 $\pm$ 0.10 ^a	5.79 $\pm$ 0.05 ^a	4.82 $\pm$ 0.11 ^a	4.07 $\pm$ 0.14 ^a
10 μ g/L BaP	6	4.75 $\pm$ 0.42 ^a	4.76 $\pm$ 0.24 ^b	4.20 $\pm$ 0.25 ^a	3.95 $\pm$ 0.22 ^a
100 μ g/L BaP	6	4.37 $\pm$ 0.32 ^b	4.94 $\pm$ 0.21 ^b	4.16 $\pm$ 0.17 ^a	3.73 $\pm$ 0.32 ^a
100 μ g/L Fadrozole	7	5.05 $\pm$ 0.30 ^a	5.41 $\pm$ 0.12 ^a	4.58 $\pm$ 0.29 ^a	3.86 $\pm$ 0.25 ^a

The mean intensity of CYP19A2 expression was quantified with KODAK 1D image analysis software. Fish were exposed for 15 days to ethanol control, BaP (10 and 100 μ g/L) or fadrozole (100 μ g/L). HY: hypothalamus; VT: ventral telencephalon; OB: olfactory bulb; HP: pituitary. There was no statistical difference between sexes so mean intensities were combined for males and females.

Differences in letters are significantly different with in a region (Mean $\pm$ SEM, Kruskal-Wallis, $p \leq 0.05$) values.

Table 3
Quantitative CYP19A1 expression ($\times 10^4$) in control and BaP exposed 3 month old *Fundulus* ovaries.

Con	Stage Ia (0–70 μ m)		Stage Ia (71–150 μ m)	
	B 10	B 100	B 10	B 100
4.54 $\pm$ 0.28	3.21 $\pm$ 0.25	3.29 $\pm$ 0.27	2.13 $\pm$ 0.11 *	2.29 $\pm$ 0.16 *
		4.18 $\pm$ 0.42	3.95 $\pm$ 0.29	3.75 $\pm$ 0.41

The mean intensity of CYP19A1 expression was quantified in the early stage I (stage Ia oocytes 0–70 μ m in size and stage Ia oocytes 71–150 μ m in size) of ooplasm of oocyte with KODAK 1D image analysis software after section *in situ* hybridization.

Mean $\pm$ SEM, $n = 4-5$ fish/treatment, Kruskal-Wallis

* $p \leq 0.05$ versus control.

Table 4Gonad somatic index ($\times 10^2$) of BaP- or fadrozole treated adult fish.

Sex	Control	BaP 100 $\mu\text{g/L}$	Fadrozole 20 $\mu\text{g/L}$	Fadrozole 100 $\mu\text{g/L}$
Female	4.13 ± 0.66 (13) ^a	5.20 ± 1.05 (7) ^a	5.64 ± 0.58 (3) ^a	6.23 ± 1.97 (4) ^a
Male	1.83 ± 0.24 (9) ^b	1.53 ± 0.13 (8) ^b	2.56 ± 0.50 (4) ^{bc}	3.53 ± 1.43 (3) ^c

Ovary or testis weight divided by whole fish weight. Different letters within a sex are significantly different (ANOVA, $p \leq 0.05$, Mean $\pm$ SEM (n)).

Table 5

Putative response elements and their locations in the CYP19A1 and CYP19A2 promoter regions.

Gene	Response Element	Location
CYP19A1	GATA-1	-396 to -384
	GATA-1	-373 to -361
	NF-kappaB	-360 to -349
	PPAR	-312 to -290
	AHR	-264 to -240
	ER	-221 to -203
	SF1/Ad4BP	-188 to -181
	CRE	-124 to -104
	TATA box	-100 to -84
CYP19A2	GATA-3	-1213 to -1201
	AR	-1063 to -1045
	CAR/RXR	-773 to -763
	AR	-705 to -688
	NF-kappaB	-522 to -510
	ERE/ER	-498 to -480
	AHR/ARNT	-419 to -413
	PR	-324 to -306
	SF1/Ad4BP	-182 to -177
	TATA box	-176 to -160

Locations numbered back from atg (+1) start site. GenBank Accession nos. EU045309 and EU045310, respectively. PCR primer locations, putative response elements and transcription initiation sites are depicted in supplemental figure.