

Brain aromatase in Japanese medaka (*Oryzias latipes*): Molecular characterization and role in xenoestrogen-induced sex reversal

Adam J. Kuhl, Steve Manning, Marius Brouwer*

Department of Coastal Sciences, University of Southern Mississippi, 703 East Beach Drive, Ocean Springs, MS 39564, USA

Received 20 August 2004; accepted 31 January 2005

Abstract

In female fish estrogen is required for the development of primary and secondary sex characteristics and is derived from the aromatization of androgens by aromatase. There are two isoforms of aromatase in several teleost species, brain and ovarian. The objective of this study was two-fold: clone and sequence the coding and promoter region of brain aromatase in medaka, and determine the effects of exposure to an environmental estrogen (*o,p*-DDT) on sex determination and brain aromatase transcription and activity. The brain aromatase coding sequence was obtained by reverse transcription polymerase chain reaction (RT-PCR) and PCR-based genomic DNA walking was used to clone the promoter of the brain aromatase gene. The promoter sequence revealed potential binding sites for the estrogen receptor and for transcription factors involved in primary neurogenesis and sex determination. Medaka fry were exposed to increasing *o,p*-DDT concentrations (0–5.5 µg/L) from days 1 to 15 after hatch and brain aromatase expression and activity were measured on days 5, 9, and 14. A complete male-to-female sex reversal occurred at 5.5 µg/L *o,p*-DDT and aromatase activity and expression data showed a significant five-fold increase at this concentration at day 14. This information suggests that brain aromatase is involved in the abnormal sexual differentiation of fish treated with xenoestrogens. © 2005 Elsevier Ltd. All rights reserved.

Keywords: Brain aromatase; Medaka; Endocrine disruption; DDT

1. Introduction

Endocrine disruptors are exogenous chemicals that affect organisms in ways similar or antagonistic to those of endogenous endocrine factors. Some effects of these chemicals include skewed sex ratios of alligators in pesticide ravaged Lake Apopka, Florida [1], inter-sex fish downstream of sewage treatment plant in the United Kingdom and U.S. [2,3], and feminized testis in common carp [4] and medaka [5] treated with xenoestrogens in the laboratory.

Some of the most studied endocrine disruptors are environmental estrogens, or xenoestrogens. These are compounds that mimic estrogen in the body. Due to estrogen's importance in development of sexual phenotype, reproductive tract morphology, and sexual differentiation of the central nervous system, exposure to environmental estrogens

and anti-estrogens, especially during critical developmental stages, can have deleterious effects in the male reproductive system including decreased fertility, reduced gonad size, development of testis-ova, and complete sex reversal [6,7].

There are many genes involved in the sex-determining cascade that are influenced by xenoestrogens, but one of particular importance is *cyp19*. A member of the cytochrome P450 superfamily, *cyp19* codes for the aromatase enzyme. Aromatase converts androgens to estrogens and is expressed in a number of tissues including the brain, liver, and gonads. Aromatization of androgen is the main source of estrogen, which is an important factor in the sex-determining pathway. Inhibition of aromatase in fish, reptiles, and birds results in masculinization of females [8–11]. In rainbow trout (*Oncorhynchus mykiss*), aromatase could be detected 3 weeks before the first signs of histological sex differentiation. These observations, along with high aromatase activity associated with ovarian differentiation, implicate estrogen, and possibly

* Corresponding author. Tel.: +1 228 872 4294; fax: +1 228 872 4204.
E-mail address: marius.brouwer@usm.edu (M. Brouwer).

aromatase, performing an essential role in gonadal differentiation in lower vertebrates [8,12].

Of late, a second isoform of aromatase (*cyp19b*) has been found in the brain of several teleost species, including zebrafish (*Danio rerio*), goldfish (*Carassius auratus*) and Nile tilapia (*Oreochromis niloticus*) [13–15]. This isoform has much higher activity and mRNA levels in the brain than the ovary has of ovarian aromatase (now termed *cyp19a*) [16]. Both isoforms show greater homology to the same isoform across species than they do for the other isoform within the same species.

Both endogenous and environmental estrogens influence aromatase activity and expression. Medaka treated with the synthetic estrogen 17- α -ethinylestradiol show increased *cyp19a* expression in the testis, an organ in which aromatase is usually not present [17]. Melo and Ramsdell [18] demonstrated an increase in aromatase activity and an induction of female-specific localization of *cyp19b* activity in the brain of medaka when treated with estradiol. For these reasons, we postulate that the production of estrogen by the brain isoform of aromatase is a driving force behind early sexual differentiation and is affected by environmental estrogens which may result in sex reversal.

Analysis of the promoter region of the ovarian aromatase gene shows that the promoter contains two estrogen responsive elements (ERE) [19], which mediate transcriptional activation via the estrogen receptor. The presence of these elements suggests that xenoestrogens may influence aromatase expression and activity through binding to the estrogen receptor.

The estrogen receptor (ER) is a member of the nuclear steroid receptor family. Three isoforms of ER (α , β , and γ) have been identified in fish [20]. These multiple isoforms allow control of tissue and cell-specific expression of estrogen responsive genes. Activation of the ER begins with the binding of a ligand to the ligand-binding domain and dimerization with another ligand bound ER. The homodimer binds to a specific palindromic DNA sequence, the ERE, in the promoter region of an estrogen responsive gene, contributing to transcription activation. Unlike the DNA binding region, the ligand-binding region is non-specific, which allows non-specific ligand binding [21]. Thus, structurally diverse compounds such as pesticides, heavy metals, and PCBs can bind to the estrogen receptor causing agonistic or antagonistic responses [21–23]. The estrogen receptor in medaka is expressed from early embryonic stages at basal levels in both sexes. Expression increases upon maturity and has a positive feedback loop in which estrogen positively regulates the expression of more ER [24].

This study examines the effect of the estrogen agonist *o,p*-DDT, a synthetic pesticide constituting between 10 and 25% of manufactured DDT, on a small freshwater fish. While DDT was banned in the United States in 1973, worldwide levels of DDT and its metabolites are between 1 and 10 ng/L in estuaries and coastal areas, and between 0.1 and 1 ng/L in the open sea [25]. More importantly, it has been shown to have femi-

nizing effects on male medaka through immersion exposure [26] and direct injection into oocytes [27]. Bioaccumulation (increasing concentration in an organism that exceeds that of its environment) and biomagnification (increasing concentration as a function of trophic level) has led to DDT concentrations in higher-level organisms in Long Island sound to reach 0.5 ppm in small fish and 2.0 ppm in larger fish [28].

In this study we report the complete cDNA and promoter sequences of brain aromatase of Japanese medaka. The feminizing capacity of *o,p*-DDT is demonstrated and its effects on brain aromatase expression and activity are characterized. This study suggests that aromatase is involved in male-to-female sex reversal in response to an environmental estrogen.

2. Materials and methods

2.1. Experimental animals

Animals used in this study were drR medaka, which contain a red pigment color marker on the male Y chromosome. Fish with an XY chromosome have an orange-red phenotype and XX are white. However, if a male-to-female phenotypic sex reversal occurs, which can be distinguished from secondary sex characteristics, an orange-red female fish can be produced. drR fry were hatched from broodstock cultured and maintained at the Gulf Coast Research Laboratory, University of Southern Mississippi, Ocean Springs, MS. Animal care and experimentation were conducted in accordance with University of Southern Mississippi guidelines for animal care and use. Eggs were collected from broodstock and combined into culture bowls and microscopically confirmed as fertilized. Embryos were transferred to glass hatching jars containing approximately 4 L of hatching solution (1.00 g/L NaCl; 0.030 g/L KCl; 0.040 g/L CaCl₂; 0.162 g/L MgSO₄ in distilled water). Salinity of the hatching solution was brought to 5 g/L with NaCl to control fungus growth. Hatching jars were maintained under continuous fluorescent light in a water bath at 24 \pm 1 °C and vigorously aerated to suspend embryos and keep them separated from each other. On day 0, newly-hatched fry were removed and randomly distributed into retention chambers that were housed in each treatment aquarium.

2.2. Exposure

o,p-DDT (96% purity) was obtained from Sigma Chemical (St. Louis, MO). Exposure concentrations were selected on basis of published responses for sex reversal for *o,p*-DDT [26]. Exposures consisted of seven 2-week exposures in duplicate at various treatment concentrations. Seventy-five drR fry for each of the seven exposures were housed in three retention chambers (100 mm Petri dish bottoms with attached 475 μ m nylon collar) of 25 fish each. Fourteen test aquaria were 35 L with a water depth of 19 cm maintained by drain siphon. Compound delivery was conducted in a setup similar

to that described by Walker et al. [29] and Manning et al. [30]. A water partitioner delivered 2 L of test solution each cycle to splitter/mixing boxes that dispensed 1 L to each duplicate aquarium for between 5 and 6 cycles/h. Test concentrations were prepared each cycle by injection of appropriate stock to the splitter boxes of each treatment. Triethylene glycol (TEG) was used as a carrier for compounds due to the low solubility of DDT in water. Exposure treatment concentrations were water control, 0, 0.1, 0.5, 1, 2.5, and 7.5 µg/L. The addition of 25, 1-day-old fish to each retention chamber initiated exposure. Test aquaria were housed within a central water bath kept at 27 ± 1 °C and provided with a 16 h light:8 h dark photoperiod supplied by fluorescent bulbs. Medaka were fed a minimum of twice daily but may be fed up to four times daily to stimulate growth. Feeding consisted of microworms and brine shrimp nauplii for days 0–8 fish, and brine shrimp and commercial flake (Zigler Brothers, Santa Anna, CA) for days 8+. Survival was monitored on days 3, 5, 9, and 14. All dead fish were removed and recorded. Four fish per duplicate (eight per treatment) were sampled, weighed, and archived for molecular analysis on days 5, 9, and 14. Two sampled fish per duplicate were preserved at -20 °C in 200 µL RNAlater (Ambion, Austin, TX) for mRNA analysis, and two were preserved at -70 °C in 200 µL phosphate buffer (100 mM KCl, 10 mM KH_2PO_4 , 1 mM EDTA, 10 mM dithiothreitol, pH 7.4) for enzyme activity analysis. Water quality (pH, temperature, and dissolved oxygen) was measured twice a week and water samples were removed four times (days 0, 5, 8, and 14) for analytical analysis of the test chemical. Upon exposure completion, fry were transferred to 18.5 L grow-out aquaria until sexual maturity so secondary sex characteristics could be observed.

2.3. Analytical chemistry

Magnolia Scientific Services Inc. (Purvis, MS) measured *o,p*-DDT concentrations according to EPA method 608 (<http://www.epa.gov/waterscience/methods/guide/608.pdf>).

2.4. Cloning of brain aromatase

Specific primers (oligo 1 and 2) (Table 1) were designed from regions of *cy19b* with high interspecies homology, but low homology in the same region between *cy19b* and *cy19a*. cDNA from *cy19b* was created using brain tissue from adult fish that had been subjected to a 1-week immersion exposure to 1 ppb estradiol to stimulate aromatase expression. Nucleic acids were extracted using a Trizol procedure with glycogen as a carrier and cDNA created using Life Technologies Superscript II reverse transcriptase. A PCR reaction using Life Technologies Platinum Pfx Taq polymerase and oligo 1 and 2 as primers gave a 931 bp amplification product. Bands were visualized by ethidium bromide on a 1% agarose gel and purified with Promega (Madison, WI) DNA purification kit and T/A cloned using a Promega cloning kit into pGem-T_Easy vector according to manufacturer's protocol and sequenced.

The 3' and 5' ends of the gene were elucidated using 3'- and 5'-RACE kits from Invitrogen. Primers for 3'-RACE were oligo 3 and 4, while 5'-RACE used oligo 5, 6 and 7. Promoter sequences were determined using genomic DNA and Universal Genome Walker Kit from Clontech (Palo Alto, CA) according to manufacturer's protocols. Genomic DNA was extracted and isolated using Qiagen (Valencia, CA) Genomic tip 100/G genomic DNA extraction kit. Gene specific primers designed from the 5' end of the fully sequenced cDNA (oligo 8 and 9) were used with outer adaptor primers provided with the kit to obtain the sequence of the promoter region. PCR conditions for the primary PCR were seven cycles of 94 °C for 2 s and 72 °C for 3 min, and then 22 cycles of 94 °C for 2 s and 67 °C for 3 min. Secondary PCR cycle conditions were five cycles of 94 °C for 2 s and 72 °C for 3 min, and then 20 cycles of 94 °C for 2 s and 67 °C for 3 min. Promoter sequences were analyzed with the web-based MatInspector from Genomatix (<http://www.genomatix.de/>) and Transcription Element Search System (TESS) (<http://www.cbil.upenn.edu/tess>) to search for putative transcription binding sites.

2.5. Phylogenetic analysis

Amino acid sequences of medaka brain aromatase, brain aromatase from seven other teleost species, and ovarian aromatase from 11 species were aligned with Clustal W 1.74 [31]. The aligned sequences were used as input into the SEQBOOT algorithm from Felsenstein's PHYLIP package [32] to generate 500 data sets by bootstrap resampling [33]. The multiple data sets were used to calculate 500 most parsimonious trees with PROTPARS. For each of the 500 trees, the aromatase sequences were put in random order and the tree-building process was repeated 10 times, each with a different random order of adding sequences. The resulting tree output file, composed of the 500 best trees found among the 500×10 runs, was used as input in the program CONSENSE that calculates a majority rule consensus tree with confidence intervals. Human aromatase was used as the outgroup.

2.6. Real-time quantitative RT-PCR

Aromatase expression was measured using real-time quantitative RT-PCR. cDNA was prepared from fish that were sampled as described above. Primers were designed from the sequences of brain aromatase. Real-time PCR was accomplished using Taqman chemistry. Aromatase primers and FAM (excitation: 490 nm/emission: 520 nm)—Black Hole Quencher dual labeled Taqman probe were used to amplify brain aromatase (oligo 10, 11, and 12). 18S primers designed from published medaka 18S sequence and Texas Red (excitation: 596 nm/emission: 615 nm)—Black Hole Quencher dual labeled Taqman probed were used as internal normalization standard (oligo 13, 14, 15). Qiagen (Alameda, CA) supplied probes and Cepheid's (Sunnyvale, California) Smart-Cycler real-time PCR system was used to amplify and measure fluorescence of aromatase and 18S. Reactions contained

100 nM probe, 900 nM primer for aromatase, and 100 nM probe and 200 nM primers for 18S. Platinum[®] Quantitative PCR SuperMix-UDG from Invitrogen (Carlsbad, CA) was used according to instructions with a Smart Cycler Additive Reagent at a final concentration of 0.2 mg/ml bovine serum albumin, 150 mM trehalose, and 0.2% Tween-20. Cycle parameters were 50 °C for 120 s, 95 °C for 120 s, and 50 cycles of 95 °C for 15 s, and 55 °C for 30 s. Relative expression was calculated through the $\Delta\Delta C_t$ method. Gene expression for each sampling time point is expressed as fold increase over control.

2.7. Aromatase activity

Aromatase activity was measured by a tritiated water release assay [34] as adapted for medaka by Melo and Ramsdell [18] and Contractor et al. [35]. Whole fry sampled during exposure were homogenized in a phosphate buffer (KCl (1 M), K₂HPO₄ (0.01 M) and EDTA (0.001 M) pH 7.4) with a hand-held pestle homogenizer and centrifuged at 21,000 × g to remove insoluble material. Protein concentration of homogenate was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Homogenate containing approximately 20 mg of protein was incubated with 5 nM Androst-4-ene-3, 17-dione [1β -³H(N)] (Perkin-Elmer, Boston, MA) in 200 μ L solution of 1 mM NADPH, 10 mM glucose-6-phosphate, 1 U/mL glucose-6-dehydrogenase, 10 mM potassium phosphate (dibasic), 1000 mM potassium chloride, 1 mM EDTA, and 1 mM dithiothreitol at 37 °C for 3 h. The glucose-6-phosphate/ glucose-6-dehydrogenase NADPH regeneration system keeps the system saturated with NADPH, which is essential in providing electrons to cyt19b via cytochrome p450 reductase [36]. After incubation, reactions were terminated by immersion in ice-cold water and adding 100 μ L 30% trichloro-acetic acid, and centrifuged at 1700 × g for 10 min to remove precipitated protein. Unconverted substrate was removed by vortexing vigorously for 60 s with 1 ml chloroform

followed by centrifugation at 1700 × g for 25 min at 4 °C. Addition of a 5% charcoal/0.5% Dextran slurry followed by a 40-s vortex and 30-min centrifugation (10,000 × g) was used to remove radiolabeled Androst-4-ene-3, 17-dione. Radioactivity of tritiated water was measured in a Beckman LS6500 liquid scintillation counter and background subtracted using samples without homogenate.

2.8. Statistical analysis

In all exposures, survival and sex reversals compared to control exposures were assessed by χ^2 -test. The statistical analysis of aromatase mRNA expression and aromatase activity were determined by a Mann–Whitney non-parametric rank-sum test due to a small sample size ($n=4$). Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Isolation and characterization of medaka P450aromB cDNA

Brain aromatase in medaka was cloned using a combination of RT-PCR and 5'- and 3' RACE. Using total RNA extracted from the adult brain of medaka, and oligos 1 and 2 (Table 1) as primers, a 931 bp product was isolated and sequenced. A BLAST search revealed high homology to other teleost brain aromatase cDNA, i.e. it showed 85 and 86% identity with brain aromatase cDNA from Mozambique and Nile tilapia, respectively. 5'- and 3'-RACE yielded a complete 1905 bp transcript with a 1497 bp open reading frame (GenBank accession no. AY319970).

The deduced amino acid sequence derived from the ORF yields a 499 amino acid protein (Fig. 1) with the initiation methionine at position 106 bp and stop codon TAA at 1603 bp and molecular weight of 57.06 kDa. There is an overall ~61–64% homology with medaka and other ovarian

Table 1
Oligonucleotide sequence, location, and functions

Oligo	Sequence	Location	Function
1	ACAGGACATGACTGACCCCTCT	621–643	Brain aromatase forward
2	GTGGGATGAAGCGCATGGC	1533–1552	Brain aromatase reverse
3	AGTGGTTGATTTACAGATGCG	1171–1192	3'-RACE GSP1
4	CTGTGTGGGCAAACATATCG	1376–1396	3'-RACE GSP2
5	ACAGGCTGCTGAGACAGGTT	1492–1512	5'-RACE GSP1
6	TCATCCTCCAGAGCTTTGCG	1190–1210	5'-RACE GSP2
7	CAATCACCATCTCCAACACG	992–1012	5'-RACE GSP3
8	ACACTGTGAAACAACCTCGTCGAGGGTT	125–152	Gene walking GSP1
9	CTCTTGCTGGATCATCTTCTTGCTCTG	94–122	Gene walking GSP2
10	CAGAGACAAGAAGATGATCCAG	94–115	Aromatase forward
11	GAAGTATCAGCATCAGAAGAAG	171–192	Aromatase reverse
12	TxRed-AACCCTCGACGAGTTTTCACAGTGT-BHQ	126–152	Aromatase probe
13	CATCTAAGGGCATCACAGACC	1445–1465	18s Forward
14	GAGACTCCGGCATGCTAACT	1344–1363	18s Reverse
15	FAM-TGGCTGAACGCCACTGTCCCTCTAA-BHQ	1400–1425	18s Probe

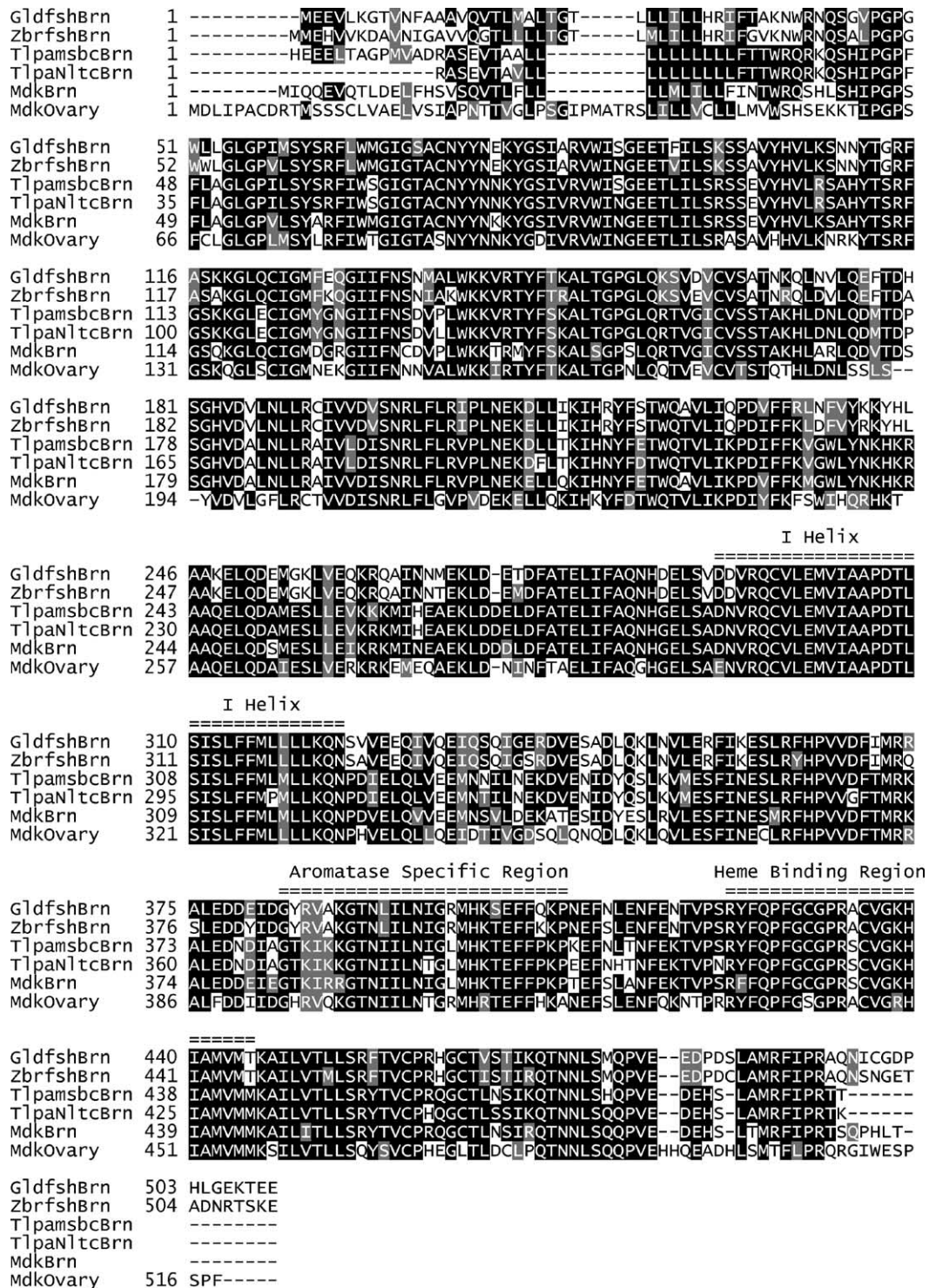


Fig. 1. Medaka brain aromatase deduced amino acid sequence showing functionally important highly conserved regions. GenBank accession nos.: goldfish brain (GldfshBrn) AB009335; zebrafish brain (ZbrfshBrn) NM_131642; tilapia Mozambique brain (TlpamsbcBrn) AF135850; tilapia Nile brain (TlpanltcBrn) AF306786; medaka brain (MdkBrn) AY319970; medaka ovary (MdkOvary) D82968.

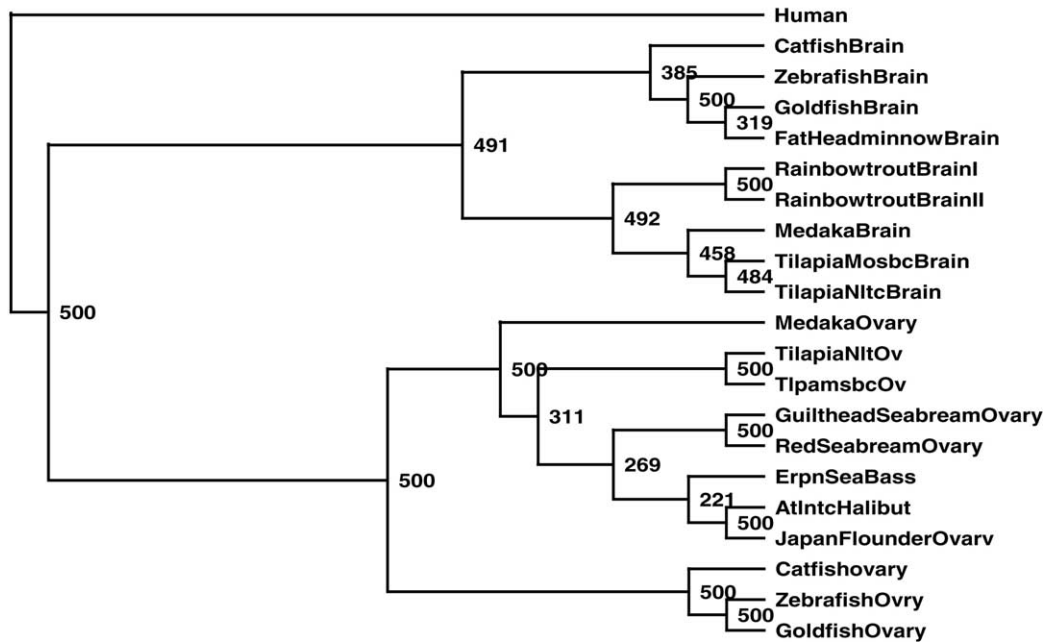


Fig. 2. Phylogenetic tree of fish aromatase. Numbers indicate the branching pattern from 500 bootstraps. GenBank accession nos.: medaka brain AY319970; medaka ovary D82968; goldfish brain AB009335; goldfish ovary AB009336; zebrafish brain NM_131642; zebrafish ovary NM_131154; tilapia Mozambique brain AF135850; tilapia Mozambique ovary AF135851; tilapia Nile brain AF306786; tilapia Nile ovary U72071; catfish brain AY325907; catfish ovary AY325908; fathead minnow brain AJ277866; rainbow trout I AJ311937; rainbow trout II AJ311938; guilthead sea bream ovary AF399824; red sea bream ovary AB051290; European sea bass AJ31177; halibut ovary AJ410171; Japanese founder ovary AB017182; human X13589.

aromatase and a ~76–83% homology with other *cyp19b*. Regions important to enzymatic function, which includes the heme-binding region, I-helix, and aromatase specific substrate binding region are highly conserved across species and isoform.

The deduced amino acid sequence from medaka brain aromatase, along with amino acid sequences from several published *cyp19a* and *b* in various teleost species, was used to infer phylogenetic relationships. Human aromatase was used as an outgroup. The resulting tree (Fig. 2) shows a distinct grouping of brain and ovarian aromatases. As expected from sequence analysis, medaka *cyp19b* clustered firmly with other brain aromatases.

3.2. Isolation and characteristics of P450aromB promoter

Using a PCR-based genomic walking technique the 5'-flanking region of the medaka P450aromB was isolated. A 1893 bp product was obtained with oligos 8 and 9 (Table 1) and sequence analysis revealed a TATA binding box at –25 bp relative to transcription start site (GenBank accession no. AY705086). Examination of the brain promoter sequences using Transcription Element Search System (TESS) and MatInspector from Genomatix revealed a palindromic estrogen responsive element, or ERE at –473, and another ERE half-site at –505. Other potential transcription factor binding sites discovered in this analysis are shown in Table 2. These include two Brn2 (–200, –554) and four Brn3

sites (–175, –507, –1503), six MyT1 sites (–246, –660, –862, –1130, –1160, 1601), four MEIS sites (–409, –564, –1000, –1647), and one Nur77 (–1037) binding site. The 5'-flanking region also contains three putative SRY/Sox elements (–152, –688, –1636) and a binding site for steroidogenic factor 1 (SF-1; –1299).

3.3. Juvenile medaka exposure to *o,p*-DDT

Juvenile medaka were exposed in a flow-through aqueous system, to *o,p*-DDT for 2 weeks beginning at hatch. Measured doses were approximately 60–73% of nominal concentrations (Table 3). Survival ranged from 79 to 94% and there is no significant difference in survival between controls and exposed groups (data not shown).

Developmental exposure to DDT resulted in a significantly ($p < 0.001$) female-skewed sex ratio (89 females and

Table 2
Potential transcription factor binding sites in the promoter regions of *cyp19a* and *cyp19b*

Factor	<i>cyp19b</i>	<i>cyp19a</i>	Function
Brn2 and 3	6	1	CNS development
MEIS	4	2	Hindbrain development
MyT1	6	3	Primary neurogenesis
DLX1	5	2	Forebrain development
Nurr7/Nur77	1	0	Neuroendocrine development
Sry/SOX9	3	3	Sex-determ Y-gene product
ER	1	1	Estrogen receptor

Table 3
Nominal and actual *o,p*-DDT exposure concentrations

Nominal ($\mu\text{g/L}$)	Mean ($\mu\text{g/L}$)	S.E.M.	Nominal (%)
0	–		
TEG only	–		
0.1	0.065	0.0065	65
0.5	0.3	0.0021	60
1	0.76	0.07	76
2.5	1.6	0.22	64
7.5	5.5	0.14	73

4 males) upon grow-out to adult at the highest (5.5 $\mu\text{g/L}$) concentration level (Fig. 3).

3.4. Aromatase expression

Gene expression was measured in individual whole fry at days 5, 9, and 14 for each treatment and quantitated using the $\Delta\Delta\text{C}_t$ method (Fig. 4). 18S was used as the internal standard and expression data for each time point are expressed as fold increase over the average of the same-day controls.

A carrier control treatment of TEG only shows no effect on gene expression. Day 5 expression shows no significant effects on gene expression at any treatment level. Day 9 expression data show that 5.5 $\mu\text{g/L}$ treatment resulted in a significant 2.33-fold increase in gene expression ($N=8$, $z=-2.021$, $p=0.043$). There is no significant increase in expression at day 9 for any other treatment levels. Day 14 also shows an increase in aromatase levels according to treatment levels. 5.5 $\mu\text{g/L}$ *o,p*-DDT resulted in a 4.55-fold increase ($N=8$, $z=-2.309$, $p=0.021$) and 1.6 $\mu\text{g/L}$ resulted in a 2.04-fold increase ($N=8$, $z=-2.309$, $p=0.021$).

3.5. Aromatase activity

Aromatase activity is expressed as relative activity compared to same-day control treatments. Only day 14 had

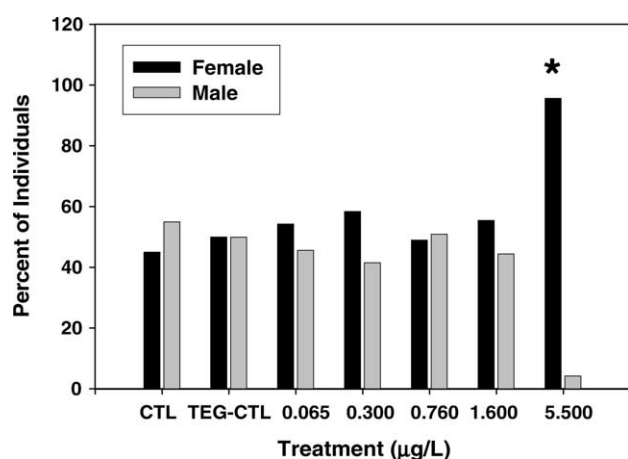


Fig. 3. Adult sex ratio of medaka after 2-week *o,p*-DDT exposure following hatch. Exposure concentrations are measured concentrations. Star indicates significant difference from a 1:1 male:female population (χ^2 (1, $N=204$) = 59.8, $p < 0.001$).

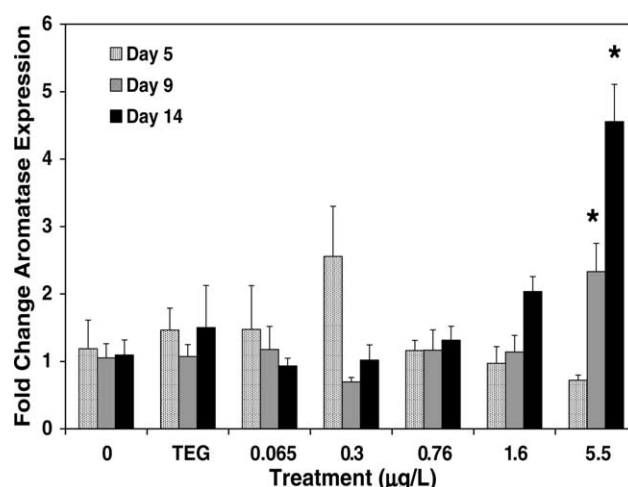


Fig. 4. Brain aromatase expression as fold change relative to same-day control treatment (\pm S.E.M.) as measured by real-time polymerase chain reaction. 18S rRNA is used as internal normalization standard. Exposure concentrations are measured concentrations. Stars indicate significant difference from control (day 9, $N=8$, $z=-2.309$, $p=0.021$); (day 14, $N=8$, $z=-2.309$, $p=0.021$).

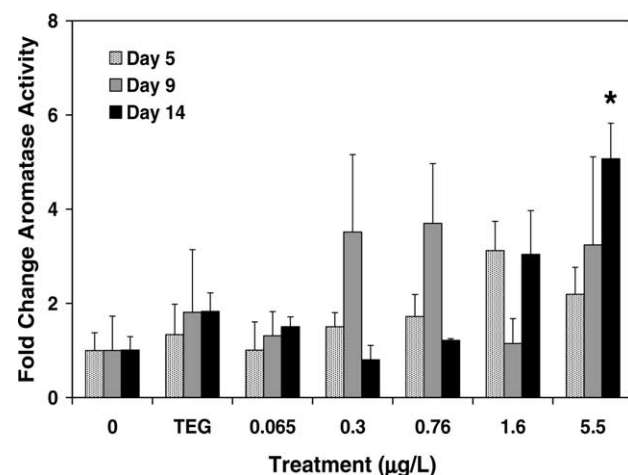


Fig. 5. Aromatase enzyme activity relative to same-day control treatment (\pm S.E.M.). Star indicates significant difference from control ($N=8$, $z=-2.309$, $p=0.021$).

consistently detectable levels of activity (Fig. 5). Activity rates follow the same pattern as gene expression at this day. There is no significant difference between any activity in any treatment level except for 5.5 $\mu\text{g/L}$ at day 14 ($N=8$, $z=-2.309$, $p=0.021$). There is also a high correlation between enzyme activity and gene expression data ($r^2=0.74$). Ovarian aromatase was not expressed in control and *o,p*-DDT-treated juvenile (days 0–14) medaka (data not shown). Therefore, measured aromatase activity is likely from brain aromatase.

4. Discussion

The purpose of this study was to examine the effects of a known endocrine disruptor on the sexual development of a

freshwater fish during a critical period of development and to examine a postulated molecular mechanism that may underlie observed effect. Exposure studies in this test confirmed previous studies that show *o,p*-DDT has feminizing effects on developing medaka [26,27]. In this study a 2-week immersion exposure to 5.5 µg/L of *o,p*-DDT resulted in a 96% female population.

In the environment, *o,p*-DDT has been found at concentrations of 0.413 µg/L in the water and 7.2 µg/L in the sediment [37]. Although our concentrations are above these measured values for DDT, it is important to point out that effective feminizing concentrations are dependent on the time of exposure, with an exposure during sexual development and puberty being 2.2 times more effective than exposure during sexual development alone [26]. DDT has been banned in the United States since 1972 and since then levels have dropped [38,39]. However, DDT is still produced and used in Mexico, China and many non-industrialized countries. Due to its persistence, detectable levels will exist for decades to come.

DDT is a very hydrophobic compound and readily dissolves in apolar solvents. This property is responsible for bioaccumulation in lipid matter in organisms. DDT concentrations in fish tissue in highly contaminated sites of the Southern California Bight have historically exceeded the Food and Drug Administration action level of 5 mg/kg wet weight [40,41]. These compounds can also be transferred maternally as they dissolve into the yolk in the eggs and are transferred to the embryo. Hence feminization can result from direct exposure from the environment in addition to maternal transfer from an exposed and contaminated mother. Edmunds et al. [27] showed a 227 ng injection into the yolk of an egg results in an 86% male-to-female sex reversal. Estrogenic responses can also occur in response to other endocrine disrupting compounds such as the alkylphenols, nonylphenol and octylphenol, the pesticide methoxychlor, ethinyl estradiol, and sewage effluent [42–46].

To understand the mechanisms behind xenoestrogen-induced feminization, how sex is determined and differentiated in fish naturally must first be examined. Sex determination refers to genetic and environmental influences on sex differentiation, while sex differentiation refers to the process of cellular development of testis and ovaries after sex has been determined. Sex differentiation is the development of primary and secondary sexual characteristics. Fish are unusual in that they have highly variable means of sex differentiation and determination. Both genetic and environmental factors can influence sex differentiation and determination and these effects can be transient or permanent.

Beginning early in sex determination and gonadogenesis, communication between nonadjacent tissues is necessary, which is accomplished through the endocrine system and the hypothalamic-pituitary-gonad axis. The endocrine system controls sex differentiation through complex pathways between the central nervous system and gonads using pituitary-derived gonadotropins and sex steroids produced in the gonad and brain [47]. While many non-steroidal hor-

mones are produced by or act on the gonads, the sex steroids have been the most extensively examined. The major sex hormone believed to be responsible for inducing and maintaining ovarian development is 17β-estradiol (E2), while the corresponding male sex hormone is 11-ketotestosterone [48]. Production of these sex steroids is very strongly linked with the early steps of gonadal differentiation and can influence long-term sex determination choices [49].

Estrogen is synthesized from testosterone by cytochrome P450 aromatase, which is expressed in the brain, gonads and liver. Recently, there has been increasing evidence that in fish multiple loci exist encoding at least two distinct isozymes of P450arom that are differentially expressed in the brain and ovary. Sexual development requires tissue-specific and temporal variations in aromatase expression. In humans alternate splicing and multiple promoters accomplish this variable expression of a single gene. In fish, multiple aromatase genes perform the same task. Separate aromatase isoforms present an extra level of regulatory intricacy to estrogen biosynthesis.

In zebrafish, Tong et al. [50] showed that the brain isoform is expressed mainly in the hypothalamus, pituitary and sensory control tissues, and production of estrogen in these regions influences neuroendocrine functions, sexual behavior, and sexual differentiation during the development of the central nervous system (CNS). In the CNS, estrogen also has a permanent developmental effect by promoting neuronal differentiation and growth. It is also associated with neuronal survival and repair [51,52]. Furthermore, there is sexually dimorphic expression of brain aromatase in developing zebrafish that is not present in ovarian aromatase expression [12]. Studies have also shown an upregulation of brain aromatase resulting from estradiol treatment occurs as early as 24 h post fertilization [16]. Additionally, preliminary work in this project shows that ovarian aromatase is not expressed in developing (days 0–14) fry. Taken together these observations suggest that brain aromatase plays a critical role in sex differentiation.

Tanaka et al. [19] sequenced the *cyp19a* in medaka. In this study, a 1905 bp product that codes for *cyp19b* was isolated and sequenced. As in other species, medaka brain aromatase shows a higher degree of homology to the brain form from other species (76–83%) than it does to the medaka ovarian form (60%). However, all aromatase sequences showed high homology in functionally important regions such as the I-helix (hydrophobic region important in substrate binding pocket formation allowing electron transport), heme-binding region, and androgen binding (aromatase specific) region [53,14]. Phylogenetic analysis of brain and ovarian aromatases confirms this pattern of relationship between the aromatases. The phylogenetic tree shows separate clustering of the two isozymes suggesting a branching of the two forms early in teleost evolution.

Estrogen regulates estrogen responsive genes by binding to and activating the nuclear estrogen receptor. This estrogen-ER complex then interacts with estrogen responsive elements (ERE) in the promoter regions of target genes

to alter transcriptional activity [17]. Tanaka et al. [19] sequenced the 5'-flanking region of the P450aromA and report a 1170 bp product with a TATA box 24 bp upstream from the transcription initiation site. Transcription factor analysis revealed half the estrogen receptor binding sites at two sites (-40 and -51) suggesting that ovarian aromatase is responsive to the ER-estrogen complex. In our study, a 1893 bp promoter region of medaka *cyp19b* was isolated and sequenced. This sequence was examined for potential transcription factor binding sites using TESS and Genomatix's MatInspector. Potential sites that had core and matrix similarity which exceeded 0.85 were further examined for sites reported in the literature to be involved in either reproductive or CNS development. Analysis revealed a TATA box at -25, a consensus ERE at -1340, and a second ERE half-site at -505. Taken together with the upregulation of aromatase in response to estrogen treatment, the presence of EREs in the promoter of *cyp19b* suggests that neuronal estrogen expression can be regulated by estrogen and the ER. Like *cyp19a*, the *cyp19b* promoter also contains potential binding site for Steroidogenic Factor 1 (SF-1) (-1299) which is involved in transcriptional regulation of P450 steroidogenic genes (*cyp11A* and *B*, steroid hydroxylases; aromatase) and in the formation of the gonads [54,19].

Three copies of SRY/SOX9 binding sites have also been found in both medaka *cyp19a* (-130, -361, -1074) and *cyp19b* (-152, -688, -1636) promoters. SRY has been suggested as the testis-determining gene, but expression is not limited to the gonadal ridge (area from which testes originate) or early development [55]. Sox9 is a gene thought to be regulated by SRY, functioning in the male sex-determination pathway [56] and its expression pattern is similar to that of aromatase [57,58]. This information suggests that aromatase is downstream of SRY in the sex-determining pathway and therefore, along with estrogen production, is important in sex-determination, sex differentiation, and sex reversal in fish [59].

Further potential binding sites in medaka *cyp19b* promoter include several factors known to be involved in primary neurogenesis and CNS development. These include Brn 2 (-200, -554) and Brn3 (-175, -507, -1503; CNS development), MEIS (-409, -564, -1000, -1647; hindbrain development), and MyT1 (-246, -660, -862, -1130, -1160, 1601; primary neurogenesis) [60–63]. Also present is Nur77 (-1037), a transcription factor important in the neuroendocrine-regulatory role of the hypothalamic/pituitary/adrenal axis [64] and which has also been implicated in neuronal differentiation [65]. Studies to determine whether these potential binding sites are functionally active are necessary. Nevertheless, the presence of these multiple factors in the regulatory region of the gene that produces estrogen strongly suggests the importance of estrogens on CNS and neuroendocrine development. The presence of EREs and neurotrophic factors in the promoter of *cyp19b* implies a convergence of the sex-determination pathway and neurogenesis pathway in the vertebrate CNS.

With this information a hypothesis on the mechanism of xenoestrogen activity can be proposed. As stated earlier, certain xenoestrogens, including *o,p*-DDT, can bind to and activate the ER [17,23,66]. This activated ER-xenoestrogen complex can bind to EREs present in the promoter region of aromatase to induce transcription and creating more aromatase. The increase in activity of aromatase might create a female level of estrogen in the developing brain, which can feminize brain neurogenesis and be the impetus behind the observed male-to-female sex reversal.

To test this hypothesis, aromatase activity and *cyp19b* mRNA levels were measured in the *o,p*-DDT treated juveniles. Ovarian aromatase was not expressed in juvenile (days 0–14) medaka (results not shown), and aromatase activity is therefore likely to be from *cyp19b*. While no significant increases were seen during early development, by day 14 significant increases in both enzyme activity and mRNA levels were observed at 5.5 µg/L. This is the same concentration that resulted in an almost complete female population, clearly establishing the link between *o,p*-DDT exposure, sex reversal and increased levels of *cyp19b* expression and activity.

Other studies have also demonstrated aromatase playing a critical role in non-genetic sex determination. In the red-eared slider turtle (*Trachemys scripta*), a species that has temperature sensitive sex determination, the female-determining temperature shows a higher aromatase activity than the male-determining temperature [67]. Inhibition of aromatase at a temperature that normally would produce 80% females produces almost 100% males. In Japanese flounder (*Paralichthys olivaceus*) inhibition of aromatase leads to a repression of *cyp19* expression and masculinization of larvae results [59].

In summary, this study presents a possible mechanism by which estrogenic compounds can exert their feminizing potential. We have shown that extra-gonadal aromatase is also present in Japanese medaka. The presence of putative transcription factor binding elements in the promoter region of medaka *cyp19b*, including the ERE and several neurogenesis factors, suggests significance of *cyp19b* in brain development and responsiveness to estrogenic compounds. This study presents evidence of an increase in aromatase activity and *cyp19b* expression at feminizing treatment levels of a xenoestrogen. All this information suggests the importance of brain aromatase in male-to-female sex reversal in response to environmental estrogens.

Acknowledgements

The authors would like to thank Sue Barnes for assistance with medaka culture and exposure, Thea Brouwer and Ann Marie Flowers for help with cloning and PCR techniques, and Dr. Kristie Willitt and Monali Patel (University of Mississippi) for help with enzyme activity assay. This work was supported by NOAA/Sea Grant (Award NA16RG2258/CEH).

References

- [1] J.C. Semenza, P.E. Tolber, C.H. Rubin, L.J. Guillette, R.J. Jackson, Reproductive toxins and alligator abnormalities at Lake Apopka, Florida, *Environ. Health Perspect.* 105 (1997) 1030–1032.
- [2] R.S. White, S. Jobling, S.A. Hoare, J.P. Sumpter, M.G. Parker, Environmentally persistent alkylphenolic compounds are estrogenic, *Endocrinology* 135 (1994) 175–182.
- [3] L.C. Folmar, N.D. Denslow, K. Kroll, E.F. Orlando, J. Enblom, J. Marcino, C. Metcalfe, L.J. Guillette Jr., Altered serum sex steroids and vitellogenin induction in walleye (*Stizostedion vitreum*) collected near a metropolitan sewage treatment plant, *Arch. Environ. Contam. Toxicol.* 40 (2001) 392–398.
- [4] S. Gimeno, A. Gerristen, T. Bowmer, Feminization of male carp, *Nature* 384 (1996) 221–222.
- [5] S. Gronen, N. Denslow, S. Manning, S. Barnes, D. Barnes, M. Brouwer, Serum Vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-tert-octylphenol, *Environ. Health Perspect.* 107 (1999) 385–390.
- [6] A. Cheek, T.H. Brouwer, S. Carroll, S. Manning, M. Brouwer, J.A. McLachlan, Developmental exposure to anthracene and estradiol alters reproductive success in medaka (*Oryzias latipes*), *Environ. Sci.* 8 (2001) 31–45.
- [7] G.A. Hunter, E.M. Donaldson, Hormonal sex control and its application to fish culture, in: W.S. Hoar, D.J. Randall, E.M. Donaldson (Eds.), *Fish Physiology*, Academic Press, Orlando, FL, 1983, pp. 223–303.
- [8] Y. Guiguen, J.-F. Barioller, M.-J. Ricordel, K. Iseki, O.M. McMeel, S.A.M. Martin, A. Fostier, Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*), *Mol. Reprod. Dev.* 54 (1999) 154–162.
- [9] A.K. Shimada, K. Yoshida, N. Saito, Effects of aromatase inhibitor on sex differentiation and level of P45017 α and P450arom messenger ribonucleic acid of gonads in chicken embryos, *Gen. Comp. Endocrinol.* 102 (1996) 241–246.
- [10] F. Piferrer, S. Zanuy, M. Carrillo, I.I. Solar, R.H. Devlin, E.M. Donaldson, Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males, *J. Exp. Zool.* 270 (1994) 255S–262S.
- [11] J.Y. Kwon, B.J. McAndrew, D.J. Penman, Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile Tilapia, *Oreochromis niloticus*, *Mol. Reprod. Dev.* 59 (2001) 359–370.
- [12] J.M. Trant, S. Gavasso, J. Ackers, B.-C. Chung, A.R. Place, Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*), *J. Exp. Zool.* 290 (2001).
- [13] G.V. Callard, A. Tchoudakova, Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish, *J. Steroid Biochem. Mol. Biol.* 61 (1997) 387–392.
- [14] E.F.L. Chiang, Y.-L. Yan, Y. Guiguen, J. Postlethwait, B. Chung, Two *cyp19* (P450 Aromatase) genes on duplicated zebrafish chromosomes are expressed in ovary or brain, *Mol. Biol. Evol.* 18 (2001) 542–550.
- [15] S. Halm, M. Rand-Weaver, J.P. Sumpter, C.R. Tyler, Cloning and molecular characterization of an ovarian-derived (brain-like) P450 aromatase cDNA and development of a competitive RT-PCR assay to quantify its expression in the fathead minnow (*Pimephales promelas*), *Fish Physiol. Biochem.* 24 (2001) 49–62.
- [16] M. Kishida, G.V. Callard, Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development, *Endocrinology* 142 (2001) 740–750.
- [17] H.O. Scholz, Gutzeit, 17- α -ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*), *Aquat. Toxicol.* 50 (2000) 363–373.
- [18] A.C. Melo, J.S. Ramsdell, Sexual dimorphism of brain aromatase activity in medaka: induction of a female phenotype by estradiol, *Environ. Health Perspect.* 109 (2001) 257–264.
- [19] M. Tanaka, S. Fukada, M. Matsuyama, Y. Nagahama, Structure and promoter analysis of the cytochrome P-450 aromatase gene of the teleost fish, medaka (*Oryzias latipes*), *J. Biochem.* 117 (1995) 719–725.
- [20] M.B. Hawkins, J.W. Thornton, D. Crews, J.K. Skipper, A. Dotte, P. Thomas, Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts, *Proc. Natl. Acad. Sci. U.S.A.* 97 (2000) 10751–10756.
- [21] T. Madigou, P. Le Goff, G. Salbert, J.P. Cravedi, H. Senger, F. Pakdel, Y. Valotaire, Effects of nonylphenol on estrogen receptor conformation, transcriptional activity and sexual reversion in rainbow trout, *Aquat. Toxicol.* 53 (2001) 173–186.
- [22] K.W. Gaido, S.C. Maness, D.P. McDonnell, S.S. Dehal, D. Kupfer, S. Safe, Interaction of methoxychlor and related compounds with estrogen receptor alpha and beta, and androgen receptor: structure-activity studies, *Mol. Pharmacol.* 58 (2000) 852–858.
- [23] J. Min, S.-K. Lee, M.B. Gu, Effects of endocrine disrupting chemicals on distinct expression patterns of estrogen receptor, cytochrome P450 aromatase, and p53 genes in *Oryzias latipes* liver, *J. Biochem. Mol. Toxicol.* 17 (2003) 272–277.
- [24] T. Kawahara, H. Okada, I. Yamashita, Cloning and expression of genomic and complementary DNAs encoding an estrogen receptor in the medaka fish, *Oryzias latipes*, *Zool. Sci.* 17 (2000) 643–649.
- [25] M.J. Kennish, *Practical Handbook of Marine Science*, CRC Press, Boca Raton, FL, 1994.
- [26] A. Cheek, T.H. Brouwer, S. Carroll, S. Manning, J.A. McLachlan, M. Brouwer, Experimental evaluation of vitellogenin as a predictive biomarker for reproductive disruption, *Environ. Health Perspect.* 109 (2001) 681–690.
- [27] J.S.G. Edmunds, R.A. McCarthy, J.S. Ramsdell, Permanent and functional male-to-female sex reversal in d-rR strain medaka (*Oryzias latipes*) following egg microinjection of *o,p'*-DDT, *Environ. Health Perspect.* 108 (2000) 219–224.
- [28] G.M. Woodwell, C.F. Wurster, P.A. Isaacson, DDT residues in an east coast estuary: a case of biological concentration of a persistent insecticide, *Science* 156 (1967) 821–824.
- [29] W.W. Walker, C.S. Manning, R.M. Overstreet, W.E. Hawkins, Development of aquarium fish models for environmental carcinogenesis: an intermittent-flow exposure system for volatile, hydrophobic chemicals, *J. Appl. Toxicol.* 5 (1985) 255–260.
- [30] C.S. Manning, A.L. Schesny, W.E. Hawkins, D.H. Barnes, C.S. Barnes, Exposure methodologies and systems for long-term chemical carcinogenicity studies with small fish species, *Toxicol. Method* 9 (1999) 201–217.
- [31] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.* 22 (1994) 4673–4680.
- [32] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, *Evolution* 39 (1985) 783–791.
- [33] J. Felsenstein, PHYLIP: Phylogenic inference package (Computer program distributed by the author. Department of Genetic, University of Washington, Seattle), *Cladistics* 5 (1989) 164–166.
- [34] E.A. Thompson, P.K. Siiteri, Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione, *J. Biol. Chem.* 249 (1974) 5364–5372.
- [35] R.G. Contractor, C.M. Foran, S. Li, K.L. Willett, Evidence of gender and tissue-specific promoter methylation and the potential for ethinylestradiol-induced changes in Japanese medaka (*Oryzias latipes*) estrogen receptor and aromatase genes, *J. Toxicol. Environ. Heal. A* 67 (2004) 1–22.
- [36] L.D. Dikkeschei, B.G. Wolthers, I. Bos-Zuur, G. Brutel de la Riviere, G.T. Nagel, D.A. van der Kolk, P.H.B. Willemese, Optimization of a

- classical aromatase activity assay and application in normal, adenomatous and malignant breast parenchyma, *J. Steroid Biochem. Mol. Biol.* 59 (1996) 305–313.
- [37] A.E. Carey, K.W. Kutz, Trends in ambient concentrations of agrochemicals in humans and the environment of the United States, *Environ. Monit. Assess.* 5 (1985) 155–163.
- [38] H.L. Boul, M.L. Garnham, D. Hucker, D. Baird, J. Aislabie, Influence of agricultural practices on the levels of DDT and its residues in soil, *Environ. Sci. Technol.* 28 (1994) 1397–1492.
- [39] G. Ware, B. Estes, N. Buck, DDT moratorium in Arizona-agricultural residues after seven years, *Pestic. Monit. J.* 12 (1978) 1–3.
- [40] NOAA, PCB and chlorinated pesticide contamination in U.S. fish and shellfish: A historical assessment report. 1988. Rockville, MD, National Oceanic and Atmospheric Administration, Office of Oceanography and Marine Assessment.
- [41] E. Zeng, C. Yu, K. Tran, In situ measurements of chlorinated hydrocarbons in the water column off the Palos Verdes Peninsula, California, *Environ. Sci. Technol.* 33 (1999) 392–398.
- [42] G. Flouriot, F. Pakdel, B. Ducouret, Y. Valotaire, Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression, *J. Mol. Endocrinol.* 15 (1995) 143–151.
- [43] A. Arukwe, L. Forlin, A. Goksoyr, Xenobiotic and steroid biotransformation enzymes in Atlantic salmon (*Salmo salar*) liver treated with an estrogenic compound, 4-nonylphenol, *Environ. Toxicol. Chem.* 16 (1997) 2576–2583.
- [44] J.P. Sumpter, S. Jobling, Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment, *Environ. Health Perspect.* 103 (Suppl. 7) (1995) 173–178.
- [45] A.C. Nimrod, W.H. Benson, Reproduction and development of Japanese medaka following an early life stage exposure to xenobiotics, *Aquat. Toxicol.* 44 (1998) 141–156.
- [46] E.F. Orlando, W.P. Davis, L.J. Guillette, Aromatase activity in the ovary and brain of the eastern mosquitofish (*Gambusia holbrooki*) exposed to paper mill effluent, *Environ. Health Perspect.* 110 (2002) 429–433.
- [47] Y. Nagahama, Endocrine regulation of gametogenesis in fish, *Int. J. Dev. Biol.* 38 (1994) 217–229.
- [48] Y. Nagahama, Gonadal steroid hormones: major regulators of gonadal sex differentiation and gametogenesis in fish, *Sixth Int. Symp. Reprod. Physiol. Fish.* (1999).
- [49] R.H. Devlin, Y. Nagahama, Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences, *Aquaculture* 208 (2002) 191–364.
- [50] S.-K. Tong, E.F. Chiang, P.-H. Hsiao, B. Chung, Phylogeny, expression and enzyme activity of zebrafish *cyp19* (P450 aromatase) genes, *J. Steroid Biochem. Mol. Biol.* 79 (2001) 299–303.
- [51] S.M. Belcher, A. Zarnovszky, Estrogenic actions in the brain: estrogen, phytoestrogens, and rapid intracellular signaling mechanisms, *J. Pharmacol. Exp. Ther.* 299 (2001) 408–414.
- [52] D.T. Solum, R.J. Handa, Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus, *J. Neurosci.* 22 (2002) 2650–2659.
- [53] S. Graham-Lorence, B. Amarneh, R.E. White, J.A. Peterson, E.R. Simpson, A three-dimensional model of aromatase cytochrome P450, *Protein Sci.* 4 (1995) 1065–1080.
- [54] W.-H. Shen, C.C.D. Moore, Y. Ikeda, K.L. Parker, H.A. Ingraham, Nuclear receptor steroidogenic factor 1 regulates the Mullerian inhibiting substance gene: A link to the sex determination cascade, *Cell* 77 (1994) 651–661.
- [55] B. Meyer, Sex in the worm: counting and compensating X-chromosome dose, *Trends Genet.* 16 (2000) 247–253.
- [56] P. Koopman, M. Bullejos, J. Bowles, Regulation of male sexual development by *Sry* and *Sox9*, *J. Exp. Zool.* 290 (2001) 463–474.
- [57] S. Vriza, C. Joly, H. Boulekbache, H. Condamine, Zygotic expression of the zebrafish *Sox-19*, an HMG box-containing gene, suggests an involvement in central nervous system development, *Brain Res. Mol. Brain. Res.* 40 (1996) 221–228.
- [58] M. Ito, M. Ishikawa, S. Suzuki, N. Takamatsu, T. Shiba, A rainbow trout *SRY*-type gene expressed in pituitary glands, *FEBS Lett.* 377 (1995) 37–40.
- [59] T. Kitano, K. Takamune, Y. Nagahama, S.-I. Abe, Aromatase inhibitor and 17 α -methyltestosterone cause sex-reversal from genetic females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*), *Mol. Reprod. Dev.* 56 (2000) 1–5.
- [60] M.R. Gorrero, R.J. McEvilly, E. Turner, C.R. Lin, S. O'Connell, K.J. Jenne, M.V. Hobbs, M.G. Rosenfeld, Brn-3.0: A POU-domain protein expressed in the sensory, immune, and endocrine systems that functions on elements distinct from known octamer motifs, *Proc. Natl. Acad. Sci. U.S.A.* 90 (1993) 10841–10845.
- [61] V. Budhram-Mahadeo, M. Parker, D.S. Latchman, POU transcription factors Brn-3a and Brn-3b interact with the estrogen receptor and differentially regulate transcriptional activity via an estrogen response element, *Mol. Cell. Biol.* 18 (1998) 1029–1041.
- [62] S.-K. Choe, N. Vlachakis, C.G. Sagerstrom, Meis family protein are required for hindbrain development in the zebrafish, *Development* 129 (2002) 585–595.
- [63] E.J. Bellefroid, C. Bourguignon, T. Hollemann, Q. Ma, D.J. Anderson, C. Kintner, T. Pieler, X-MyT1, a *Xenopus* C2HC-type zinc finger protein with regulatory function in neuronal differentiation, *Cell* 87 (1996) 1191–1202.
- [64] E.P. Murphy, O.M. Conneely, Neuroendocrine regulation of the hypothalamic-pituitary-adrenal axis by the *nurr1/nur77* subfamily of nuclear receptors, *Mol. Endocrinol.* 16 (1997) 39–47.
- [65] J. Milbrandt, Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene, *Neuron* 1 (1988) 183–188.
- [66] M. Kishida, M. McLellan, J.A. Miranda, G.V. Callard, Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (*Danio rerio*), *Comp. Biochem. Phys. B* 129 (2001) 261–268.
- [67] D. Crews, J.M. Bergeron, Role of reductase and aromatase in sex determination in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination, *J. Endocrinol.* 143 (1994) 279–289.