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Temporal expression of two Cytochrome P450 Aromatase isoforms during development in *Oreochromis mossambicus*, in association with histological development

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ABSTRACT

Oreochromis mossambicus is targeted as a sentinel species for assay development to detect disruption of estrogen-dependent pathways in Southern Africa. Aromatase — an enzyme encoded by either of two cytochrome P450 19 (*cyp19*) genes in *O. mossambicus* is the only enzyme able to catalyze the aromatization of androgens to estrogens. As part of background studies relating to evaluating the use of early life stages of *O. mossambicus* as an endocrine disruptor screen, we investigated the tissue specificity and temporal expression of *cyp19* transcripts, *cyp19a* (ovarian *cyp19*) and *cyp19b* (brain *cyp19*) during development and estrogen exposure. The *cyp19a* transcript was detected only in the ovaries of adults, and expression during development reflected this result as induction of *cyp19a* coincides with histological development of putative ovaries. Using primers that only identify the ovarian transcript, *cyp19a* transcript levels demonstrated a typical reflection of puberty — after an initial surge in *cyp19a*, juveniles expressed very low levels, which increased again at the time histologically discernable vitellogenic ovaries were detected. Moreover, we found evidence of putative alternate transcript of *cyp19a* whose function is currently unknown. *cyp19b* transcripts were expressed in brain and muscle tissue of both male and female adults, in addition to ovaries in females. During development, *cyp19b* transcript levels were increased coincidental with *cyp19a* at 20 days post fertilization but the expression pattern was distinct from that observed for *cyp19a*. These studies set the foundation for utilizing this native species as a possible indicator of endocrine disruption and accentuates the importance of understanding "normal" basal levels of transcript levels and the nature of amplification of QPCR targets.
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1. Introduction

17β-Estradiol (E_2) is responsible for a multitude of functions in the vertebrate physiology including primary and secondary sexual characteristics, maintenance of bone mass, and cardio-protective effects (Grumbach, 2000; Vasudevan et al., 2002). In addition, E_2 permanently affects central nervous system organization during development and general neurotrophic factors in several brain regions and life stages (Maclusky and Naftolin, 1981; Tchoudakova et al., 2001).

Estrogen biosynthesis in mammals involves a series of enzymatic steps, converting cholesterol into the steroid hormone, E_2. This process requires various forms of cytochrome P450 gene (*cyp*) products, one of which encodes for the enzyme cytochrome P450 19 (*cyp19*), or "aromatase". This enzyme catalyzes the synthesis of estrogens from androgens (Conley and Walters, 1999; Tsuchiya et al., 2005).

The cytochrome genes consist of a superfamily encoding heme-containing monoxygenases. These are responsible for the oxidative metabolism of many drugs and environmental chemicals as well as endogenous substances including steroids (Nelson et al., 1996). *cyp19* is present as a single copy in the genome of most mammals (Simpson et al., 1994; Choi et al., 1997) whereas in several other vertebrates, including fish, two distinctive genes denoted *cyp19a* and *cyp19b* are expressed predominantly in ovarian and brain tissues, respectively (Callard et al., 2001; Greytak et al., 2005; Chang et al., 2005). These genes give rise to functionally different enzymes, with the heme- and steroid-binding sites highly conserved between these homologues and among different species (Cheshenko et al., 2008).

Vertebrates with only one *cyp19* gene achieve tissue-specific expression by alternative splicing and/or different promoter usage in these species, whereas in species with two homologues of the gene, differential transcript levels are obtained on account of the difference in the respective promoters (Tsuchiya et al., 2005; Cornil et al., 2006).

Cheshenko et al. (2008) and Forlano et al. (2006) reviewed the potential regulatory factors for *cyp19* in teleosts which include responsive elements for cAMP responsive element binding protein (CREB), Steroidogenic factor 1/adrenal 4 binding protein (SF1/Ad4BP), estrogen receptor (ESR), aryl hydrocarbon receptor (AhR), and nuclear hormone receptors (including PPAR, RXR and RAR). Some of these elements are exclusively found flanking either the ovary or brain isoforms of the gene. In tilapia (*Oreochromis niloticus*), Chang et al. (2005) identified SRY, WT1-KTS, GATA-4, CRE and SF-1/Ad4 as binding

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sites for transcription factors in *cyp19a*. From these only GATA-4 and CRE was found in *cyp19b*, in addition to ESR- and RAR-response elements (Chang et al., 2005).

Therefore, in the brain of tilapia and some other teleosts (Callard et al., 2001), *cyp19* expression can potentially be regulated directly by E_2 and its receptor via the conventional genomic pathway (Falkenstein et al., 2000). E_2 induces the transcription of *cyp19b* (Kishida et al., 2001; Kazeto et al., 2004). In zebrafish, estrogenic exposure resulted in increased *cyp19* expression in the brain of female fish, whereas no change was reported for the male brain (Andersen et al., 2003). However, the opposite effect, was reported for fathead minnows (Halm et al., 2002). In tilapia, the enzyme activity decreased upon E_2 induction in the post-natal fish, whereas it increased significantly at the time of gonadal differentiation (Tsai et al., 2000). This and other studies clearly illustrate the importance of stage of development, tissue type and gender when measuring *cyp19* expression and activity (reviewed by Cheshenko et al., 2008). Alteration in the *cyp19* expression and/or activity following exposure to estrogenic EDCs, may adversely affect sex determination/differentiation pathways.

O. mossambicus, a gonochoristic teleost fish targeted as sentinel species for endocrine disruption in Southern Africa, and indigenous to the area, is known to follow a sex determination mechanism that is largely gene-dependent, but can be altered environmentally by temperature (Wang and Tsai, 2000) and hormonal imbalance (Eckstein and Spira, 1965; Nakamura et al., 1998). Assays for aromatase activity and vitellogenin mRNA transcript levels have been used in the past. However, additional markers of EDC effects are needed. As a first step for this species, we set forward to clone and sequence partial fragments of *cyp19* genes in order to develop quantitative PCR (QPCR) protocols. Subsequently tissue specific expression was quantified in adult specimens, and expression levels determined of *cyp19a* and *cyp19b* during the juvenile development stages of this species in comparison with gonadal development histologically to evaluate baseline expression at early developmental stages.

2. Materials and methods

2.1. Animals

Adult breeding stock of *Oreochromis mossambicus* (Cichlidae, Perciformes) (Mozambique tilapia) was obtained from Aquastel (South Africa) and kept in aquaria with water, constantly aerated and recycled through an activated charcoal filter. Water temperature was kept at 27 °C (±1 °C). Fish were fed once daily with tilapia pellets (AquaNutro, South Africa). Light regime followed 14:10 L:D cycle. On a daily basis aquaria were monitored for females carrying eggs in their mouths. These females were removed from the breeding aquaria to culturing tanks. In the culturing tanks each brooding female was kept alone until the offspring reached swim-up fry stage, at which time the adult female was removed and re-introduced into the breeding tank. Each batch of offspring was reared separately in the same water conditions as for breeding stock. Animals at the appropriate developmental stage were collected, euthanized using 0.01% Benzocaine (Heynes Mathew, Ltd., South Africa) and preserved in RNA *later* (Ambion Inc., USA) at 4 °C or fixed in buffered formaldehyde (Bancroft and Stevensal., 1977) for RNA isolation or histological purposes, respectively. At least three groups from different breeding pairs ($n \geq 5$ per group) were sampled at 5 day intervals starting at 5 days post fertilization (dpf). At no point was any tissue or sample pooled between individuals.

2.2. Histological procedures

Five specimens per age class were processed for histology. Standard histological procedures (Bancroft and Stevens, 1977) were used to determine the relationship between age and the formation of gonadal structures. Whole bodies were fixed in buffered formaldehyde (Bancroft et al., 1977) and embedded in paraffin wax (Merck, South Africa). Histological sections (5 μm) were stained with Harris hematoxylin and eosin (Humason, 1967) within each age group.

2.3. RNA isolation and cDNA preparation

For the initial cloning of either of the expressed *cyp19* sequences, RNA was prepared from brain, ovary and liver tissue of an adult female using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Following resuspension of total RNA in DEPC-treated water, samples were treated with DNase I (Promega, USA) for 30 min at 37 °C and precipitated with 2.5 volume (vol) 95% ethanol and 1/10 vol 3 M sodium acetate pH 5.6 at -20 °C. The RNA pellets were washed with 70% ethanol and redissolved in 30 to 60 μL of DEPC-treated water.

For analysis of *cyp19* gene expression during development, total RNA was isolated from whole body homogenates (WBH), three groups per age class ($n=5$ to 10 per age group). RNA yields were quantified spectrophotometrically at $Absorbance_{260nm}$ and stored at −70 °C.

First strand cDNA was prepared from 2 μg of total RNA using oligo d $(T)_{15}$ primers and SuperScript III RNase H^- MMLV reverse transcriptase as described by the manufacturer (Invitrogen, USA). Samples were stored at −20 °C and diluted 40-fold prior to gene expression determination.

2.4. Primer development

To obtain partial *cyp19* gene sequences, PCR was performed using cDNA derived from *O. mossambicus* brain and ovary tissue as template and primers designed against the related *O. niloticus cyp19a* and *cyp19b* sequences (GenBank accession no. AF472620 and AF472621, respectively). Expected product sizes based on the *O. niloticus* sequences, are 251 and 241 bp respectively. PCR conditions include 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 1 μM of each primer (Table 1) and 2.5 Units of in-house Taq polymerase in a 25 μL reaction. In each reaction, 50 ng cDNA from liver, ovary or brain was added. PCR reaction volumes were denatured for 2 min at 94 °C, after which followed 30 cycles constituting of 1 min at 94 °C, 30 s at 55 °C and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. PCR products were checked for size on a 2% agarose gel. Amplified DNA fragments of interest were cloned into pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5α. Plasmid DNA was isolated from positive clones detected by colony PCR and insert DNA sequenced using SP6 and T7 primers. The resulting *O. mossambicus cyp19* sequence information was compared to all sequences in the NCBI database using Blastn (Altschul et al., 1990) and were found to be 100% identical to *O. niloticus*.

2.5. QPCR assay development and verification

Primers and clones for analysis of gene expression by quantitative PCR (QPCR) are listed in Table 1. For housekeeping gene (internal invariant standard) purposes, β-actin was used. Primers for analysis of β-actin were designed using Primer Premier Version 5.00 software

Table 1
Primer sequences used for cloning or QPCR

Target Gene	Application	Primer	Sequence
cyp19a	Cloning	OMAO6	AATTAAACCCCAGAAAGCCAGG
		OMAO4*	CTGTGAACTAAATATGTATGACATGC
		OMAO1	CACAAAACCACGGTGAGCTGTCTGCT
		AromaOfwd	CAATCGCATGGGATATCAATGG
		AromaOrev*	GAAGATCTGCTTAGTATGAGCGTC
	QPCR	OMAO3	CACAAGACAGCAACCCAGGAGTTA
		OMAO2*	CTGTCTCTCACCCACAACAGCG
cyp19b	Cloning and QPCR	AromaBfwd	GAGCGTCAGAAGTCACTGC
		AromaBrev*	GCTCAAAATCAGGGTCTCCTC
β-actin	QPCR	OMBA1	TGTGATGGTGGGTATGGG
		OMBA2*	CTGTGGTGGTGAAGGAGTAG

Asterisk (*) indicate anti sense primers.

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