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Isolation and characterization of three estrogen receptor transcripts in *Oreochromis mossambicus* (Peters)

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ABSTRACT

Exposure of aquatic organisms to 17β-estradiol (E_2) induces a variety of estrogen-responsive genes, including vitellogenin (*vtg*)—the precursor protein of egg yolk in oviparous animals and to date the single most used gene product in screening for estrogenic endocrine disruption. Transcription regulation of *vtg* by E_2 is dependent on binding of the ligand (E_2) to a specific nuclear receptor (estrogen receptor, *ESR*) which in turn binds to an estrogen responsive element (ERE) in the promoter of *vtg*. Since a local tilapiine, *Oreochromis mossambicus* (Peters), is targeted as a model for estrogenic endocrine disruption in Southern Africa, a platform of knowledge is necessary for the ontogenic and tissue specific behavior of *ESR* in this species before *vtg* levels can be interpreted in relation to such endocrine disruption. Therefore, three *ESR* cDNA sequences (*ESR1*, *ESR2a* and *ESR2b*) in *O. mossambicus* were isolated and QPCR protocols were developed to ascertain their quantitative transcript levels in adult brain, gonadal and hepatic tissues. *ESR1* transcript levels were highest in female liver tissue compared to males and other tissues, whereas the levels for *ESR2a* and *b* were not statistically significantly different between male and female tissues. Quantitative gene levels during development demonstrated a sharp increase in *ESR1* during the stage of gonad differentiation (50–60 days post-fertilization) in this species. Finally, an induction experiment in adult male liver tissue confirms the upregulation of *ESR1* by E_2.

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1. Introduction

The steroid hormone 17β-estradiol (E_2) performs a multitude of functions in both the male and female physiological systems. Apart from its primary reproductive effects which include controlling reproductive processes in male and female vertebrates, gonadal function and many secondary reproductive functions, E_2 also plays an important role in liver and cardiovascular physiology, neural growth and differentiation, neuroprotection, cognition, and regulation of mood [1,2]. In addition, oviparous vertebrate species are known to be dependent on the presence of E_2 in order to produce large quantities of the egg yolk precursor protein, vitellogenin (VTG) in the liver, even in phylogenetically ancient fish [3,4].

This, along with the vast majority of endocrine disruption studies revealing estrogenic interference, provides a rationale for detailed studies on matters where estrogens are concerned, which in turn require detailed description of *ESR*s and its behavior.

Binding of estrogens to its specific nuclear receptors has been well documented for mammals, in which temporal and tissue specific actions of estrogens are mediated by either of two *ESR*s, denoted *ESR1* and *2* (formerly known as *ERα* or *ERI* and *ERβ* or *ERII* respectively) [5–9]. *ESR1* was the first *ESR* cloned and isolated from MCF-7 human breast cancer cells [6,7] with *ESR2* cloned about a decade later from rat prostate [9]. In fish, three *ESR*s have been identified [10–12]. These are transcribed from distinctive genes (*ESR1*, *ESR2a* and *ESR2b*, the latter also known as *ERγ* or *ERIIb*) to result in functionally different receptors that have distinct expression patterns *in vivo*, and which differentially recruit co-factors [13]. Substrate preference has been reported for the different *ESR*s in various species [12,14] and several different splice variants have been described for ERs in both mammals and teleosts [5,15–17].

In mammals, both *ESR1* and *2* are known to localize in the breast, brain, cardiovascular system, urogenital tract and bone [5]. In the liver *ESR1* is known to be the predominant paralogue, whereas *ESR2* is the main *ESR* in colon tissue [5]. A few studies demonstrated the expression of *ESR1* in liver tissue, whereas in ovarian tissue both *ESR2a* and *ESR2b* are the most transcribed *ESR*s in teleost fish [11,18]. This information for Mozambique tilapia (*Oreochromis mossambicus*) is not available, and can provide significant information with regards to the mechanism by which E_2 functions both during its developmental programme, and possibly in response

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Table 1
Oligonucleotide primer sequences used in this study shown in a 5′ to 3′ direction.

Target gene	Application	Primer	Sequence	Ta used	NCBI reference
ESR1	Cloning	onERI 3	ATGTACCCCGAAGAGAGCC	65 °C	U75604
		onERI 5	TCATGGGATGCGGGTGCAGTCG		U75604
		omERI 8	TCCAATCCTGTGCTCTCGTC		AM284390
		omERI 9	CACAGCGTCCCGCTTCC		AM284390
		omERI 12	GCACATGAGCAACAAAGGC		AM284390
		omERI 13	GCCTTTGTTGCTCATGTGC		AM284390
		omERI 14	AGGCACCAGAGTTTAGCA		AM284390
	QPCR	omERI 11	TGCTAAACTCTGGTGCCT	64 °C	AM284390
		onERI 5	TCATGGGATGCGGGTGCAGTCG		AM284390
ESR2a	Cloning	omERII 1	CAACATGTGCCTCAGTTC		U75605
		omERII 2	CTACTGGGATTCACCTCCG		U75605
		omERII 3	GTCATGTCAGTAACAAAGGC		AM284391
		omERII 4	GCCTTTGTTACTGACATGAC		AM284391
		omERII 5	GAAGCTGCGTCCAGGGC		AM284391
		omERII 6	CTGTTGGAGTGCTGCTGGC		AM284391
	QPCR	omERIIa7[a]	TAACTGGACCAGCTGAGGGT	66 °C	AM284391
		omERIIa8[a]	AGTTCCTCAGACGGCAGCGA		AM284391
ESR2b	Cloning	omERIIb3	ATGACCTCCTCCCCTGCCCTGG	65 °C	DQ462608
		omERIIb5	TCAAGCTGTTTCCGTGACAACTCTG		DQ462608
	QPCR	omERIIb1[a]	CAGTGCACTATTGACAAGAACCGAC	66.5 °C	EU140820
		omERIIb2[a]	CCAGCATGAGGATCTCCAACCAGC		EU140820

Ta = annealing temperature.
[a] Derived from Wang et al. [36].

to vitellogenic transcription inducers. The importance and development of *O. mossambicus* as a bioindicator species to study and monitor endocrine disruption activity in aquatic systems has been highlighted by Esterhuyse et al. [19]. The aim of the present study was to isolate and sequence *ESR* cDNA in *O. mossambicus* with the objective to describe the expression of *ESR* isoforms quantitatively in different tissues (spatial variation) and during the normal developmental programme (temporal changes) using quantitative real-time reverse transcription PCR (QPCR). To this end, three *ESR* transcripts in *O. mossambicus* were cloned, sequenced and subjected to phylogenetic analysis. Subsequent information was used to develop a QPCR protocol to describe the expression of these transcripts during developmental stages in *O. mossambicus* and confirm the E_2-responsiveness of *ESR1* in adult male liver tissue.

2. Methods

2.1. Animals and sampling procedure

Oreochromis mossambicus adult breeding stock was obtained from Aquastel (South Africa) and maintained in aquaria with water which was constantly aerated and filtered through activated charcoal. Water temperature was kept at 27 °C (±1 °C). The light regime followed a 14:10 light:dark cycle and fish were fed once daily with Tilapia pellets (AquaNutro, South Africa). Because of the mouth brooding characteristic of this species, offspring production was monitored daily—females carrying eggs in their mouths were removed from the breeding aquaria to culturing tanks. Each brooding female was kept individually in culturing tanks until the offspring reached the 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 (determined by age in 5-day intervals after fertilization) were collected, euthanized using 0.01% benzocaine (Heynes Mathew, Ltd., South Africa) and preserved in RNA*later* (Ambion Inc., USA) at 4 °C. Three or more different breeding pairs were used to generate offspring that were sampled at each developmental stage. For the adult tissue scan for determination of the various *ESR* amplicons presence, at least five ($n \geq 5$) males and five females were dissected and the RNA prepared as outlined below. RNA of different specimens was not pooled in this study.

For the estrogen exposure experiment, adult male *O. mossambicus* ($n = 10$ per group) were exposed to 60 ng/μl E_2 for 12 h as was previously described [19]. Livers of euthanized fish were dissected and preserved in RNA*later* for RNA isolation.

2.2. Total RNA isolation and cDNA preparation

Total RNA was prepared from specific tissues of adults or from whole body homogenates of juveniles using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Following resuspension of total RNA in diethyl pyrocarbonate (DEPC)-treated water, samples were treated with DNase I (Promega, USA) for 30 min at 37 °C and precipitated with 0.1 volumes of 3 M sodium acetate pH 5.6 and 2.5 volumes of 95% ethanol at −20 °C. The RNA pellets were washed with 70% ethanol and redissolved in 30–60 μl of DEPC-treated water. RNA yields were quantified spectrophotometrically at $\text{absorbance}_{260\,\text{nm}}$ 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^- M-MLV reverse transcriptase (Invitrogen, USA) as described by the manufacturer. Samples were diluted 40-fold prior to gene expression determination and stored at −20 °C or used as template for *ESR* cloning as described below.

2.3. Isolation of *O. mossambicus ESR* cDNA

PCR primer sequences for *ESR1*, *ESR2a* and *ESR2b* cDNA in *O. mossambicus* were initially designed from the closely related *Oreochromis niloticus* (GenBank accession nos. U75604, U75605 and DQ462608, respectively). cDNA from ovarian tissue of an adult female was used as template to perform long-range PCR for which a reaction consists of 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 1 μM of each primer (Table 1) and 2.5 Units of SuperTherm Gold Taq polymerase (JMR Holdings, UK) in a 25 μl reaction. To each reaction, 50 ng cDNA was added. PCR reaction volumes were denatured for

9 min at 95 °C, after which followed 30 cycles constituting of 30 s at 95 °C, 30 s at 64 °C and 3 min at 70 °C, with a final elongation step of 5 min at 70 °C. PCR products were checked for size on a 0.8% agarose gel. Amplified DNA fragments were cloned into pGEM-T Easy vectors (Promega, USA) and transformed into *E. coli* DH5α to sequence confirm the amplicons. Sequences were deposited into NCBI GenBank database (see Table 1).

2.4. DNA sequencing and sequence comparisons

Plasmid DNA of full length cDNA was isolated from positive clones detected by colony PCR, and insert DNA sequenced using SP6 and T7 primers on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, USA). The resulting *O. mossambicus ESR* sequence information was deposited in GenBank (accession nos. AM284390, AM284391 and EU140820 for *ESR1*, *ESR2a* and *ESR2b* respectively). Sequence analysis of the DNA and derived amino acid sequences was performed using ClustalW v2 software [20] according to Chenna et al. [21] and graphic illustrations were prepared with software available in Bioedit Sequence Alignment Editor v7 [22]. Sequence alignments were done for various vertebrates by using the BLAST program [23].

2.5. Phylogenetic tree analysis

A phylogenetic tree of *ESR1*, *ESR2a* and *ESR2b* was constructed for the derived amino acid sequences of the genes sequenced in this study as well as reported sequences for *ESR1*, *ESR2a* and *ESR2b* in other teleost species. The deduced sequences were aligned using ClustalW [24] implementing a Gonnet scoring matrix, and the phylogenetic tree was constructed with application of the Neighbor-Joining [25] method where bootstrap analysis calculated the probability of the presented branching of 1000 possible tree values recorded and are presented as percentage of times out of 1000 that a node was recovered [26]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA v4.0 [27].

2.6. Gene expression analysis by QPCR

Primers for analysis of gene expression by quantitative real-time PCR (QPCR) were designed for *ESR1*, *ESR2a* and *ESR2b* from the distinctive genes sequenced (Table 1). β-actin (GenBank accession no. AB037865) was used for normalization as has been described before [28] since transcript levels of β-actin were invariant. Gene expression was quantified using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, CA, USA). Each 15 μl QPCR reaction contained 2 μl of first strand cDNA (40-fold dilution), 7.5 μl SYBRgreen mix (Sigma, Germany), 0.08 μl reference dye (Sigma, Germany) and 0.27 μM of each primer. The thermocycle program included 95 °C (9 min), followed by 40 cycles of 95 °C (15 s), 31 s at the appropriate annealing temperature (Ta) for each amplicon (Table 1) and 72 °C (45 s). A dissociation curve was generated at the end of each programme as confirmation of amplicon size. Each DNA amplification run included control reactions containing no cDNA template and a standard concentration of each target DNA. Triplicate determinations were performed for each sample and the C_t values obtained across independent amplification runs for a given gene target which were used to transform the data into gene levels expressed as fold change compared to a standard (a 20 dpf sample) according to the $\Delta\Delta C_t$ [29–32] method. A dilution range was generated for each gene target in 5-fold dilution increments using the appropriate gene contained in the respective plasmid used for sequencing. QPCR analysis of these were used to determine PCR efficiency (PCR efficiency = $10^{(-1/\text{slope})} - 1$).

In each PCR run, standard curves generated using plasmids containing amplicons of interest showed a linear relationship between C_t values and plasmid concentration with the correlation coefficient (R^2) of 0.992, 0.998 and 0.995 for *ESR1*, *ESR2a* and *ESR2b* respectively. PCR efficiencies were calculated as 92.4, 94.1 and 91.8% for the respective genes, whereby the assumptions for the $\Delta\Delta C_t$ method [29–32] are met. QPCR primer sets as listed in Table 1 were validated for specificity using the authentic gene-containing plasmid. In each case, no product was amplified in plasmids with genes other than the specific gene that has been targeted, confirming appropriateness of primer sets.

Data published earlier on the yolk precursor protein, vitellogenin (*vtg*) [19] has been used to test for correlation with *ESR* data generated in this study (Table 2).

Table 2
Spearman Rank Order Correlation values for *O. mossambicus* liver *vtg*.

	vtg	*ESR1*	*ESR2a*	*ESR2b*
vtg	–			
ESR1	0.035961	–		
ESR2a	**−0.304725**	−0.073659	–	
ESR2b	**−0.311848**	0.009092	**0.323365**	–

Bold values are significant at $p < 0.05$. The levels of *vtg* transcripts have been described previously in Esterhuyse et al. [19] on the same samples used in this study.

2.7. Statistical analysis

Statistical analyses were performed using the software package STATISTICA version 8 (StatSoft, Inc., 2007). Data sets were first tested for normality. Quantitative data were log transformed before statistical analysis to achieve statistical homogeneity [33] when testing for correlations between gene effects. Correlations between genes (variables) were analyzed using linear regression on scatterplots and Pearson method [34] or in case of data not normally distributed, the Spearman Rank Order Correlations [35]. The significance level for each test was set at $p < 0.05$ throughout the study. Comparison between groups was calculated using the non-parametric Mann–Whitney *U*-test when all data in groups were not normally distributed. Percentage identities were calculated using DNAMAN v4.1 (Lynnon Biosoft).

3. Results

3.1. Amplification and cloning of ESR genes

We cloned and sequenced the full length sequences of *ESR1*, *ESR2a* and *ESR2b* (GenBank accession nos. AM284390, AM284391, and EU140820 respectively) in *O. mossambicus* coding sequence (CDS) for each gene. Alignment with previously described sequences in *O. niloticus* revealed cDNAs containing additional sequence within the coding region for *ESR1* and *ESR2a*. These sequences corresponded to two introns at the 3′ region of *ESR1* and one intron at the 3′ region of *ESR2a* in the genomic sequence of *O. niloticus* and thus represent putative alternative splice variants (Fig. 1). These result in the production of a prematurely terminated protein from the *ESR2a* protein which would likely produce a protein unable to bind to E_2. Hence primer pairs (ERI14/ERI5 and ERII7/ERII8, Fig. 1) were developed spanning intron/exon splice sites in *ESR1* and *ESR2a* respectively which allows to effectively amplify only transcripts encoding the functional ESR proteins from each of these genes. No putative splice variants were observed for the *ESR2b* transcript and consequently established primers were used from an earlier publication [36].

Comparison of the deduced amino acid sequences (Fig. 2) of isolated *O. mossambicus ESR*s to each other show that they are 53.7% identical after multiple alignment. The receptors cloned and analyzed in this study showed highly conserved areas for the DNA-binding domain (95, 97 and 98% respectively for *ESR1* and *ESR2a*,

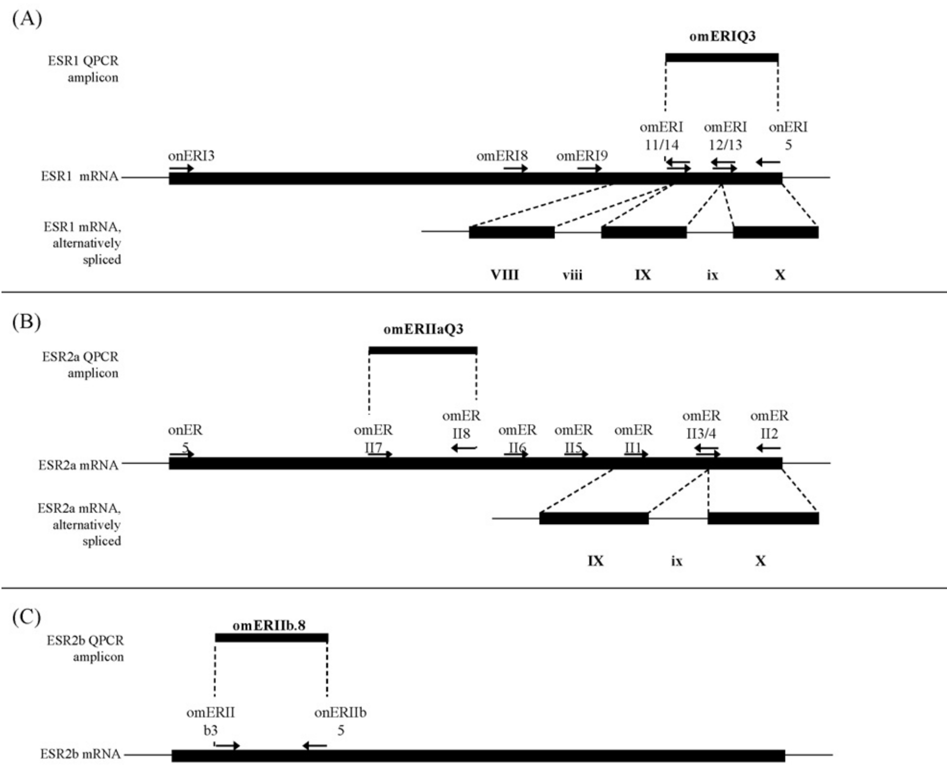

Fig. 1. Schematic overview of designed primers in *ESR1* (A), *ESR2a* (B) and *ESR2b* (C) which produced splice variant templates. No splice variants were amplified for *ESR2b* when using the primer pair OMER2b3/5 which are tested by Wang et al. [36]. Arrows indicate loci of primers used in this study.

ESR1 and *ESR2b*, and *ESR2a* and *ESR2b* respectively) and ligand binding domain (70, 67 and 83% respectively for *ESR1* and *ESR2a*, *ESR1* and *ESR2b*, and *ESR2a* and *ESR2b* respectively).

These sequences are 99, 99 and 98% identical to their respective sequences of *O. niloticus* (GenBank accession nos. U75604, U75605 and DQ462608 respectively).

3.2. Phylogenetic analysis

To further characterize *ESR* in *O. mossambicus*, deduced amino acid sequences of 33 entries found in GenBank for teleost *ESR1*, *ESR2a* and *ESR2b* in addition to *O. mossambicus ESR*s were subjected to phylogenetic analysis (Fig. 3). The resulting tree is well resolved

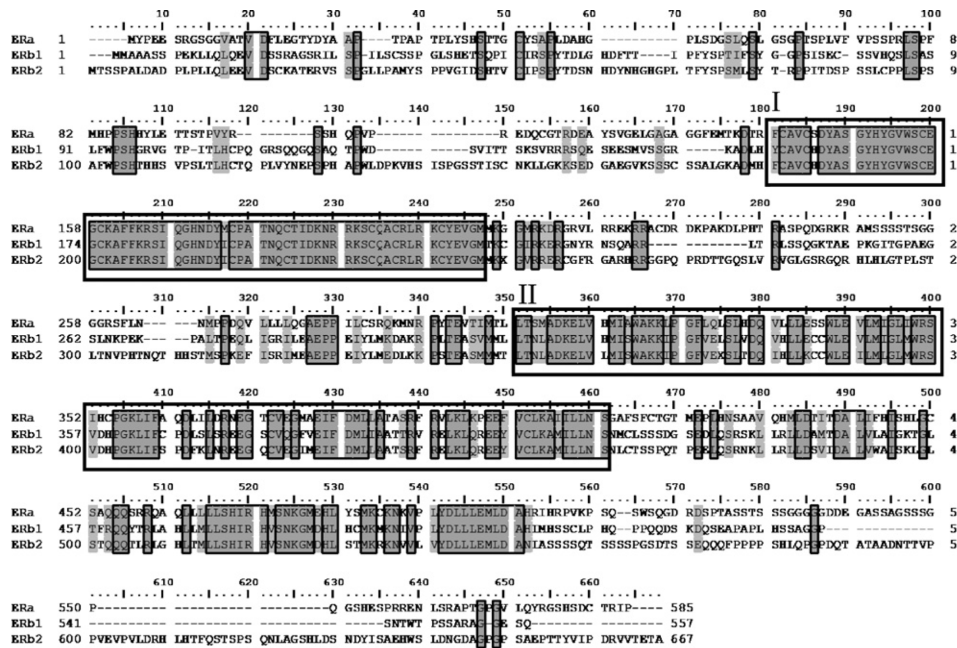

Fig. 2. Alignment of the amino acid sequences of *ESR1*, *ESR2a* and *ESR2b* of Mozambique tilapia. Darker shaded and outlined boxes point out identical amino acids, whereas lighter shaded fragments without outlines illustrate similar amino acids. Box I denotes the putative DNA-binding domain, and Box II the putative ligand binding domain. These sequences are 99, 99 and 98% identical to their respective sequences of *O. niloticus* (GenBank accession nos. U75604, U75605 and DQ462608 respectively for the *O. niloticus sequences*).

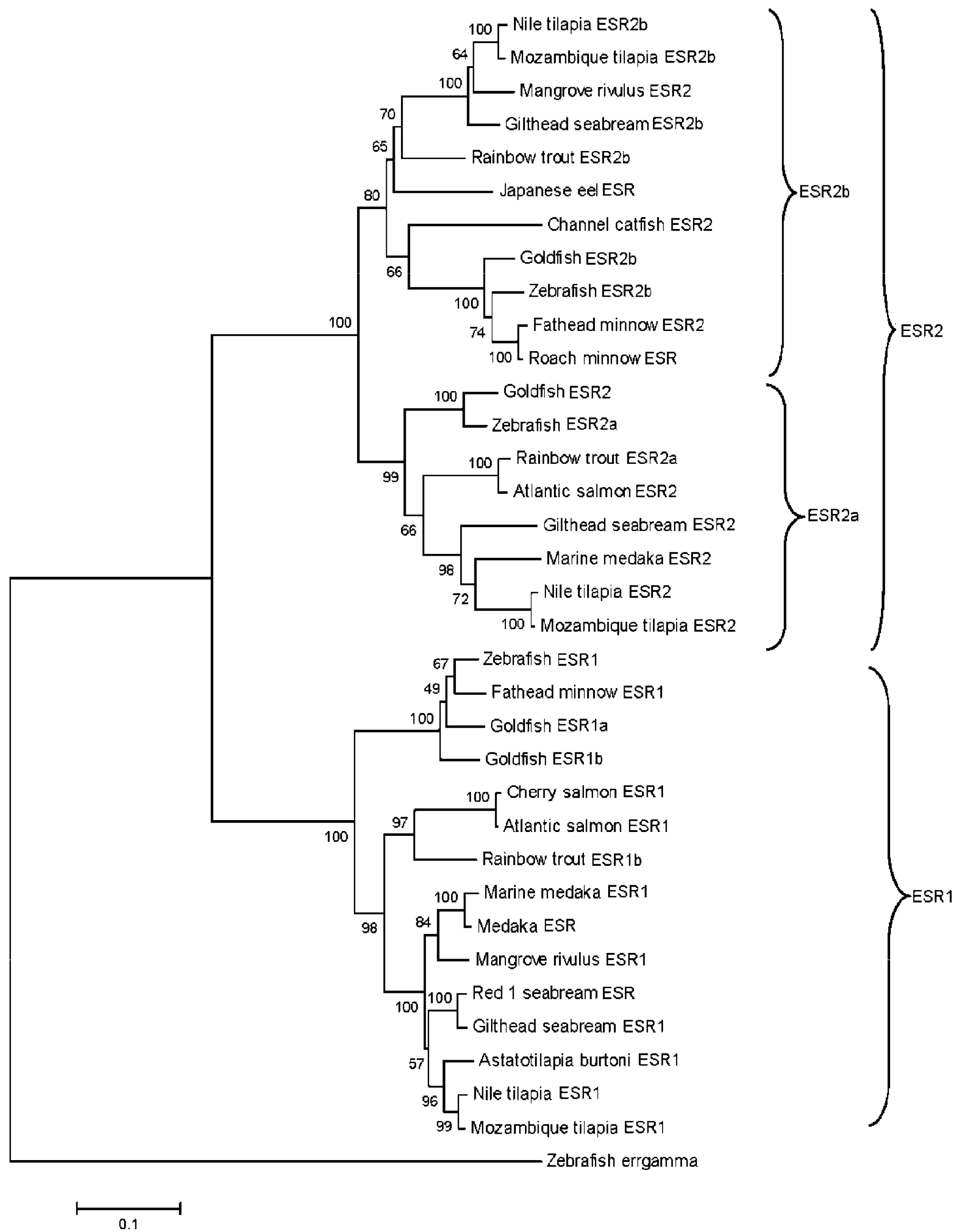

Fig. 3. Phylogenetic tree based upon the alignment of deduced amino acid sequences of Mozambique tilapia ESR sequences and reported *ESR* sequences in other teleostean species. Phylogenetic analysis was carried out by MEGAv4.1 using *p*-distance based on Neighbor-Joining method with 1000 bootstrap replicates. The tree was rooted by using Zebrafish estrogen related receptor as the outgroup. The number shown at each branch indicated the bootstrap values (%). GenBank accession nos. are as follows: Japanese eel (*Anguilla japonica*) ESR, AB003356.1; Astatotilapia burtoni (*Astatotilapia burtoni*) *ESR1*, AY422089.1; Goldfish (*Carassius auratus*) *ESR1*b, AY344444.1; Goldfish (*C. auratus*) *ESR1*a, AY055725.1; Goldfish (*C. auratus*) ESR2, AF061269.1; Goldfish (*C. auratus*) *ESR2b*, AF177465.1; Red seabream (*Chrysophrys major*) ESR, AB007453.1; Zebrafish (*Danio rerio*) *ESR1*, NM_152959.1; Zebrafish (*D. rerio*) *ESR2a*, NM_180966.2; Zebrafish (*D. rerio*) *ESR2b*, AJ414567.1; Channel catfish (*Ictalurus punctatus*) *ESR1*, AF185568.1; Mangrove rivulus (*Kryptolebias marmoratus*) ESR2, DQ339109.1; Mangrove rivulus (*K. marmoratus*) *ESR1*, DQ339108.1; Rainbow trout (*Oncorhynchus mykiss*) *ESR2b*, NM_001124570.1; Rainbow trout (*O. mykiss*) *ESR2a*, NM_001124753.1; Rainbow trout (*O. mykiss*) *ESR1b*, NM_001124558.1; Cherry salmon (*Oncorhynchus masou*) *ESR1*, AY520443.2; Nile tilapia (*Oreochromis niloticus*) ESR2, U75605.1; Nile tilapia (*O. niloticus*) *ESR1*, U75604.1; Nile tilapia (*O. niloticus*) *ESR2b*, DQ462608.1; Mozambique tilapia (*Oreochromis mossambicus*) *ESR2a*, AM284391.1; Mozambique tilapia (*O. mossambicus*) *ESR1*, AM284390.1; Marine medaka (*Oryzias javanicus*) ESR2, AY917148.1; Marine medaka (*O. javanicus*) *ESR1*, AY917147.1; Medaka (*Oryzias latipes*) ESR, D28954.1; Fathead minnow (*Pimephales promelas*) ESR2, AY566178.1; Fathead minnow (*P. promelas*) *ESR1*, AY775183.1; Roach minnow (*Rutilus rutilus*) ESR, AY770578.1; Atlantic salmon (*Salmo salar*) *ESR1*, NM_001123592.1; Atlantic salmon (*S. salar*) ESR2, NM_001123577.1; Gilthead seabream (*S. auratus*) *ESR1*, AF136979.2; Gilthead seabream (*S. auratus*) *ESR2b*, AJ580048.1; Gilthead seabream (*S. auratus*) ESR2, AF136980.1; Mozambique tilapia (*O. mossambicus*) *ESR2b*, EU140820.1; Zebrafish (*D. rerio*) estrogen related receptor gamma, NM_212954.1.

between the *ESR1* and *ESR2* groups. However, a further node with 100% bootstrapping was found between *ESR2a* and *ESR2b*. The tree revealed that *O. mossambicus ESR1*, *ESR2a* and *ESR2b* are related closest to the respective genes in *O. niloticus*. Within the teleost *ESR1* group, 100% bootstrap support distinguished a zebrafish lineage from the tilapia group. Within the *ESR2* group, *ESR2a* and *ESR2b* are clearly grouped separately with again 100% bootstrap support.

3.3. Tissue specific gene analysis

Expression of *ESR1*, *ESR2a* and *ESR2b* was further characterized through QPCR in adult male- and female-derived samples. *ESR1* transcript levels in females is significantly higher in liver tissue compared to brain ($p < 0.05$, Mann–Whitney *U*) but not ovaries (Fig. 4). *ESR1* transcripts were not differentially expressed in the

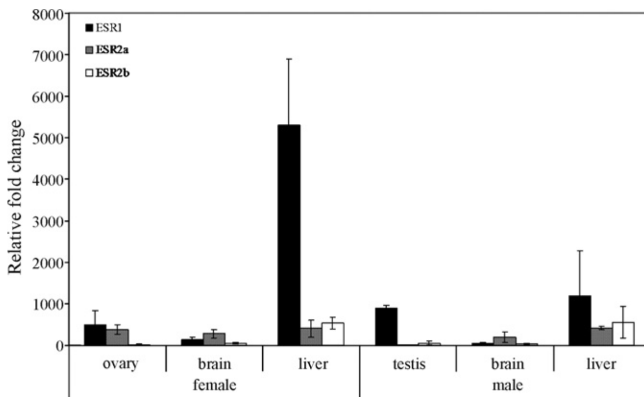

Fig. 4. Quantitative gene expression of *ESR1*, *ESR2a* and *ESR2b* amongst gonadal, brain and liver tissues in adult *O. mossambicus* ($n \geq 5$ for each data point). Relative fold change has been calculated against 20 dpf juvenile whole body homogenates ($n = 20$).

male tissues tested (liver, brain, testes). *ESR2a* was expressed at detectable levels in all tissue samples except testes. *ESR2b* was again mostly expressed in livers of the adult females ($p < 0.05$, Mann–Whiney *U*), however males showed no statistical significant tissue specificity for this form of *ESR*. *ESR2b* was found to be expressed in all tissues at very low levels, whereas both male and female livers showed elevated levels ($p < 0.05$, Mann–Whitney *U*) but no dimorphism between the sexes.

3.4. Gender specific gene analysis

Gender specific dimorphic expression patterns were found in *ESR1* for liver samples of adult specimens, but not in brain or gonad ($p < 0.05$, Mann–Whitney *U*). No significant difference between male and female samples for *ESR2a* and *ESR2b* was detected in any tissue examined.

3.5. Temporal expression during early development

We previously determined that whole body homogenates of juveniles may serve as an effective surrogate for the evaluation of exposure to estrogenic substances compared to adult males [19]. To further characterize this approach, we measured the levels of *ESR* transcripts during development.

Temporal up- and down-regulation of *ESR1* in *O. mossambicus* revealed a significant increase at 55 dpf and significant decrease again thereafter (Fig. 5). Expression of *ESR1* remains low again until 95 dpf when another significant increase occur which decrease again at 105 dpf. Hereafter basal levels were measured of *ESR1* until 110 dpf. Relative to the 5 dpf samples, *ESR2a* increased significantly

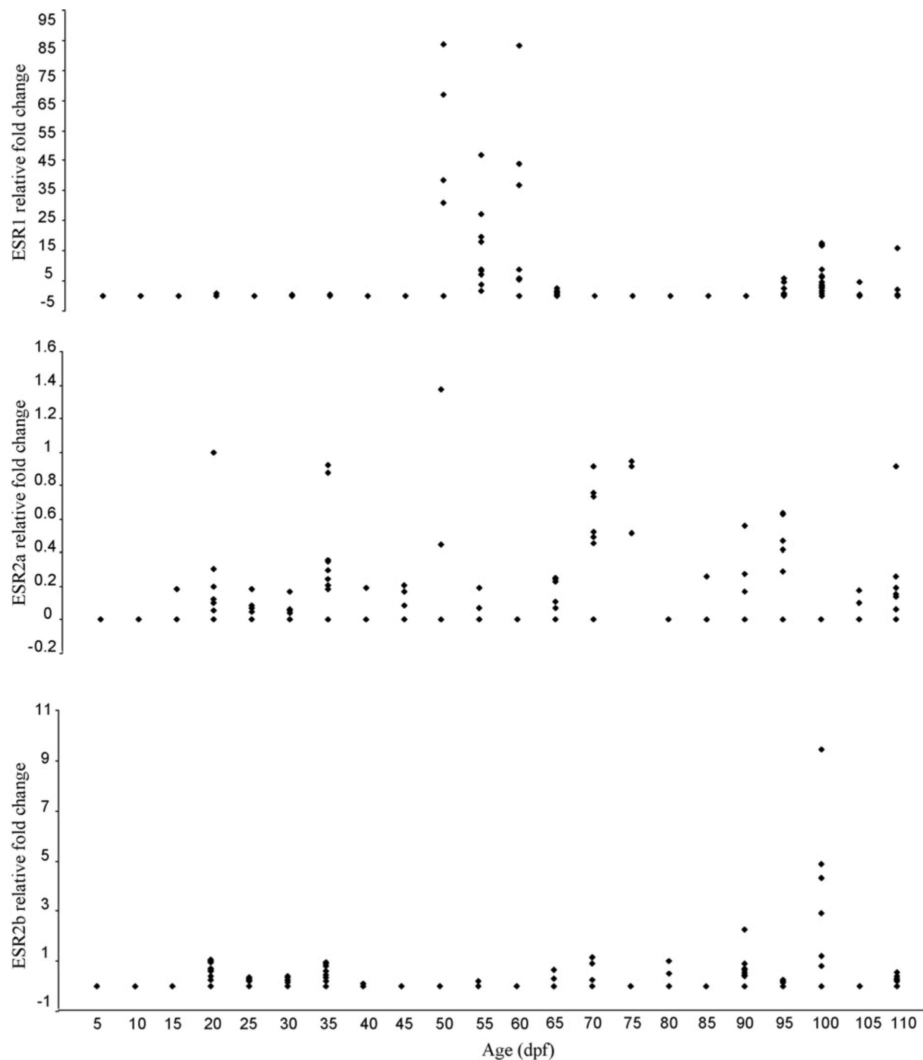

Fig. 5. Real-time PCR quantification of *ESR1*, *ESR2a* and *ESR2b* mRNA from whole body homogenates during the period 5–110 dpf.

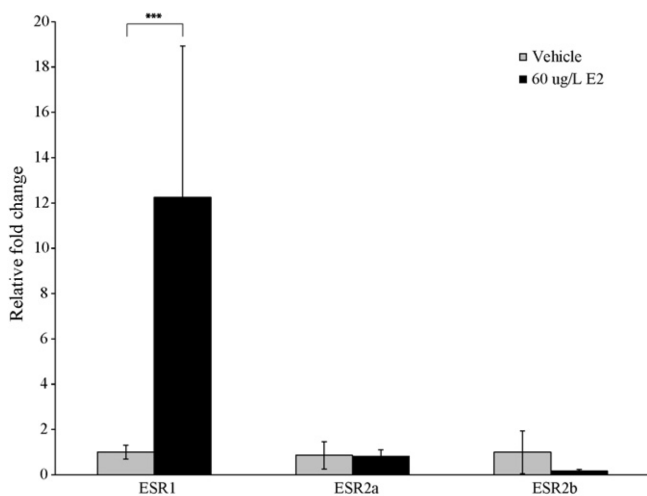

Fig. 6. Real-time PCR quantification of *ESR1*, *ESR2a* and *ESR2b* mRNA from liver of adult male *O. mossambicus* after exposure to 60 μg/L 17β-estradiol for 12 h. Light grey bars represent control animals exposed to vehicle only (EtOH) and black bars represent the fold change of the exposed samples relative to the controls. Error bars represent the standard error of the mean (SEM) while asterisks indicate significance at $p < 0.001$, Mann–Whitney *U*-test.

at 20 dpf ($p < 0.05$, Mann–Whitney *U*). Hereafter, the data revealed basal levels of *ESR2a* but include much variation. At 60 dpf significantly low levels were measured where after another increase occurred, peaking at 75 dpf. *ESR2b* mRNA increased significantly for the first time at 20 dpf, and continued to be present at an elevated level until 45 dpf significant decrease occur, remaining low with much variation and no significant increase or decrease.

When the juvenile data for *ESR1* is compared to adult females, juveniles at 60 dpf are maintaining similar levels of the gene than female ovaries, but ~10 times less than female liver tissue. As for the *ESR2*s, juveniles expressed *ESR2a* at ~50 times less than both ovarian and liver tissues, and *ESR2b* again at similar levels than female ovaries, but ~150 times less than female liver.

3.6. ESR expression correlating with vtg expression

Because of the effect of E_2 on *vtg* expression *ESR* transcript levels were correlated with *vtg* transcript levels [19] in the same samples in Table 2. Statistical significant correlation has been found between *vtg* expression and that of *ESR2a* and *ESR2b*.

3.7. Induction of ESR transcripts by estrogen

Relative induction of *ESR1* transcripts by E_2 in adult male liver tissue revealed a 12-fold increase ($p = 0.0006$) compared to that expressed in control fish (Fig. 6). The levels of *ESR2a* and *ESR2b* transcripts were not affected.

4. Discussion

Assessing the biologic consequences of chronic multigenerational exposures to endocrine disrupting compounds (EDCs) is a complex challenge. Consequently, some aspects of the endocrine system are studied in a piece-meal fashion [37–41]. EDCs known to have estrogenic effects are modeled and screened for by using *in vivo* and *in vitro* bioassays or a combination thereof [42–44] which are most often absolute or quantitative responses by estrogen-responsive genes. The latter are regulated by E_2 most often via the genomic pathway whereby the ligand binds to its specific nuclear receptor, ESR. To understand and monitor xenoestrogenic effects *in vivo*, it is thus imperative to fully characterize the normal expression of *ESR* in non-induced animals in order to make informed conclusions with regards to the expression levels of such estrogen responsive genes under exposure conditions.

Three *ESR* subtypes have previously been cloned from several fish species including the tilapiines, *O. niloticus* [36,66] and *O. aureus* [40,49]. The current study confirmed the presence of these three *ESR* paralogues in *O. mossambicus*. Typically vertebrate-like [12,36,45], *ESR* in *O. mossambicus* segregates firstly into two subclades (*ESR1* and *ESR2*, Fig. 3). Wang et al. [36] reported a third *ESR* subtype, *ESR2b* for *O. niloticus* that was consequently confirmed for *O. mossambicus* in this study.

We additionally report putative splice variants in at least *ESR1* and *ESR2a* as has been indicated in Fig. 1 when using primer pair omERI9/5 or omERII1/2 on their respective paralogues. Putative alternative splice variants have been reported for *O. mossambicus* before [19] which emphasizes the utmost importance of using the correct primer set when developing protocols for QPCR experiments.

The different *ESR* genes are believed to be the result of polyploidization or genome duplication amongst ancestral aquatic vertebrates [36]. After duplication of the ancestral *ESR1* gene, the coding sequences of *ESR2b* accumulated novel mutations at a greater rate than *ESR2a* as is indicated by patterns of amino acid divergence in other teleosts [13]. *In situ* hybridization of *ESR1*, *ESR2a* and *ESR2b* in Atlantic croaker hypothalamus illustrated different patterns of expression [13], which, in addition to differential expression in rats [46], suggests distinctive neuroendocrine roles. Quantified *ESR* expression levels in brain of the *O. mossambicus* (Fig. 4) for each of the *ESR*s reported in this study supports this hypothesis.

On the other hand, ESR forms part of the nuclear receptor superfamily [47] and has been studied extensively in mammalian models and to a lesser extent in other vertebrates, including teleosts [12,15,17,36]. The level of *ESR* transcription itself is known to be generally under control of E_2 and up- or down-regulation is tissue specific [48–50]. In oviparous species, the hepatic *ESR* concentration is markedly increased by E_2—not surprising since the liver is one of the main target organs for estrogens during the adult life of teleosts [51,52]. The current study supports this theory since within male and female adults, highest expression of *ESR1* was found in the liver, with the only sexual dimorphic pattern being in liver samples. The other two *ESR*s did not reveal such dimorphism (Fig. 4) and these data are in agreement of results found in zebrafish (*Danio rerio*) and trout (*Oncorhynchus mykiss*) confirming that the expression of *ESR1* is robustly stimulated by E_2 treatment *in vivo* [11,53]. More evidence in this regard was presented by the observation that E_2 induced the expression of *ESR1* mRNA transcripts in adult male livers (Fig. 6).

Expression of *ESR*s during ontogeny in fish has been reported to start soon after fertilization—in zebrafish as early as 48 h post-fertilization for all *ESR*s [54]. Such early expression of *ESR* is feasible in the light of maternally inherited E_2 in embryos which only diminishes after the onset of gonadal differentiation [55] in tilapia. Hereafter, E_2 remains low until after ovarian development [17] to be influenced partially by temperature as *cyp19b* and *ESR*s [56,57] are reported to be expressed increasingly at higher temperatures [58,59]. It therefore seems likely that differential expression of *ESR*s in response to environmental signals is an important factor contributing to gonadal and brain development [17]. E_2 function via this genomic pathway is therefore dependent on the availability of *ESR* which is subsequently implicated in estrogenic endocrine disruption.

In the present study all *ESR* expression is initiated at 20 dpf. *ESR* transcripts are shown to be maternally transferred but rapidly degraded post-fertilization in killifish, and *ESR1* transcripts are selectively expressed in preovulatory oocytes in contrast with

mRNA of neither of *ESR2a* nor *b* [12]. In the present study all three *ESR*s were poorly expressed during the 5–15 dpf developmental window. The relatively low expression of *ESR2b* in the ovary (which includes unfertilized eggs) therefore suggests that *ESR2b* found in juveniles is made *de novo*, and is not been maternally transferred, which agrees with the situation in killifish [12]. In comparison, *ESR1* is increased at stage 20 dpf in *O. mossambicus*. Gonads in tilapia are known to start to differentiate around 21 dpf [60–62] but histological data has shown this differentiation only to be somatic and no distinguished germ cells are present [28]. Germ cells were only identified in some cases at 40 dpf. Therefore, and in addition to Nakamura et al. [61], we postulate the liver of the different sexes may react differentially to sex specific stimuli (hormones and temperature) and therefore would be induced markedly in females and to a lesser degree in males.

The absence of a dimorphic expression profile in *ESR2b* during the complete period investigated in this study indicates that this gene may possibly not be under gender specific regulation which is supported by the absence of *ESR2a* or *ESR2b* induction by E_2 in livers of other teleosts [11,18].

On account of the expected functional relationship of *ESR1* in hepatic tissue of adult specimens during vitellogenin (VTG) production, correlation was tested for between the various *ESR*s and *vtg* quantified transcript levels in liver using *vtg* transcript expression data generated previously on the same sample set [19] (Table 2). In hepatic tissue of adult *O. mossambicus*, a significant ($p < 0.05$) correlation was found between *vtg* and *ESR2a* and *ESR2b* transcripts. VTG is known to have functions additional to egg yolk formation in teleosts [51,63,64]. Therefore it is not surprising that *vtg* expression in hepatic tissue correlates well with *ESR2b* expression. However, in the light of the previous discussion, not *ESR2*s, but *ESR1* is expected to correlate with *vtg* expression. Excluding males from this correlation did not change the results (data not shown). A possible explanation may be that females tested ($n = 9$) may be at various stages in the vitellogenic cycle, during which it is known for a female to have varying levels of VTG. We suggest that *ESR1* may be under direct regulation of E_2 which also induce *vtg* expression at the appropriate time in the vitellogenic cycle. In support of this is our observation that *ESR1* transcripts are induced along with *vtg* [19] in adult male livers following exposure to E_2 (Fig. 6). However, at stages of low *vtg* expression in the liver of females, *vtg* may be downregulated in turn by other transcription regulators as is discussed elsewhere [19]. Further, ligand binding to *ESR2b* might be the pathway whereby basal levels of *vtg* are maintained over and above the gender dependent regulation of *vtg* via *ESR1*, however not E_2 regulated. On the contrary, a study on largemouth bass (*Micropterus salmoides*) illustrated in adults that elevated *vtg* and E_2 levels were correlated with up-regulated *ESR1* transcription and to a lesser extent with *ER*γ (*ESR2b*) whereas *ESR2* (*ESR2a*) remained unchanged during the upregulation of *vtg* and E_2 [18]. Conversely, the present study and another on feral adult *O. mossambicus* found no correlation between *ESR1* and *vtg*, with the latter also not correlating to *ESR2* (*a* or *b* not distinguished) and *vtg* in an E_2 polluted river [65]. These results indeed question the current theory that *ESR* gene expression is induced in an isoform specific manner by xenoestrogens [12,15]. This challenges researchers in the toxicogenomics arena with the question of mode of action by E_2 with regards to which method is being used to assess E_2 activity in aquatic systems. Subsequent studies therefore on *O. mossambicus* may provide a tool at another level to monitor estrogenic exposure.

In conclusion, vitellogenesis as endocrine disruption endpoint in oviparous species has been used extensively for the past two decades to report E_2 activity in aquatic systems. But as we approach to understand the mechanisms of action estrogens incorporates, modern research on this matter is challenged with contradicting results between several species where this specific nuclear ligand receptor is concerned. Overall, the present study confirms the basal expression of *ESR2a* and *ESR2b* during temporal development, with a clear upregulation of *ESR1* during the time of gonadal differentiation, as well as induction following E_2 exposure. This study therefore underlines the necessity to firstly characterize the expression of *ESR*s at transcriptional and translational levels and secondly to investigate other pathways such as the suggested "non-genomic" pathway of estrogens proposed in recent literature for specific species under investigation before inferring any estrogenic endocrine disrupting effects.

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