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Nucleotide sequence, tissue expression patterns and phylogenetic analysis of estrogen receptor one mRNA in the Murray rainbowfish (*Melanotaenia fluviatilis*) (Atheriniformes, Actinopterygii)

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ABSTRACT

Estrogens are steroidal hormones that control many physiological processes in both female and male vertebrates. Like other vertebrates, fish have two distinct estrogen receptors (Esr) subtypes, Esr1 and Esr2a that have been isolated in a number of species, as well as a third subtype, Esr2b. The mRNA encoding the Esr1 was isolated from the female liver of an Australian freshwater fish, the Murray rainbowfish, *Melanotaenia fluviatilis*. The rainbowfish *esr1* cDNA was 2569 bp in length and with an open reading frame to encode a protein of 611 amino acids. Phylogenetic analysis and multiple amino acid sequence alignment indicated close relationship and high similarity with killifish (*Fundulus heteroclitus*) and gilthead sea bream (*Sparus aurata*). Expression of rainbowfish *esr1* mRNA was abundant in the liver, gonads and intestine of adult female and male rainbowfish. This is the first isolation of the full-length nucleotide sequence of an estrogen receptor from rainbowfish. This sequence provides a valuable molecular tool that can be used in future studies investigating estrogen mechanisms, actions and tissue-specific expression in juvenile and adult rainbowfish.

1. Introduction

Estrogen receptors (Esr) are members of a large superfamily of ligand-dependent nuclear receptors. These also include receptors for other steroid hormones, secosteroids, thyroid hormone, retinoids and a group of orphan receptors (Kumar and Thompson, 1999). In mammals, two distinct estrogen receptor genes exist, (Esr1 and Esr2) (Kuiper et al., 1997), whereas in teleost fish one Esr1 (formally known as ER) and two Esr2 subtypes, Esr2a and Esr2b (formally known as ER and ER, respectively) have been described (Hawkins et al., 2000). For two Esr subtypes (Esr1 and Esr2), one or multiple splice variants have been reported (Menuet et al., 2001; Caviola et al., 2007). The presence of these multiple Esr subtypes in fish has lead to an increase in the characterisation and tissue-specific expression of these subtypes in different fish species including channel catfish (*Ictalurus punctatus*) (Xia et al., 2000), largemouth bass (*Micropterus salmoides*) (Sabo-Attwood et al., 2004), European sea bass (*Dicentrarchus labrax*) (Halm et al., 2004), goldfish (*Carassius auratus*) (Choi and Habibi, 2003), killifish (*Fundulus heteroclitus*) (Greytak and Callard, 2007) and zebrafish (*Danio rerio*) (Legler et al., 2002). Although all three Esr subtypes are activated by estrogens, the tissue distribution and expression levels, the responses exhibited, and the ligand-binding affinity to other molecules may diverge (Hawkins and Thomas, 2004; Pinto et al., 2006; Greytak and Callard, 2007).

Differential sex-specific tissue expressions of the Esr have been shown in different fish species. For example, in largemouth bass (*M. salmoides*), females exhibited high levels of *esr1* in the liver tissue, while ovary tissue exhibited the highest levels of expression of both *esr2a* and *esr2b* (Sabo-Attwood et al., 2004). In goldfish (*C. auratus*), both male and female fish had the highest expression levels of *esr1* and *esr2a2* in the pituitary tissue, whereas for *esr2a1* the highest expression was noted in the gonadal tissue (Choi and Habibi, 2003). In yellow perch (*Perca flavescens*), the female liver and ovary had the highest expression of *esr1* and *esr2a*. In male liver tissue, moderate expression of both *esr* was reported, whereas no expression was observed for either *esr* subtype in the testis (Lynn et al., 2008). Comparative analysis of *esr* sex-specific tissue expression is important in identifying target tissues of estrogens in each species.

Although multiple forms of the Esr have been described, the cloning and characterisation of the Esr in an Australian freshwater fish species has not been previously reported. The Australian Murray rainbowfish (*Melanotaenia fluviatilis*) belongs to the class Actinopterygii (ray-finned fishes), order Atheriniformes (silversides) and

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family Melanotaeniidae, that includes a small group of fishes from northern and eastern Australia, and from New Guinea and some nearby islands. It residues in inland Murray-Darling Basin, spanning New South Wales, Victoria and South Australia (Allen, 1991). It is a small fish, with adult fish averaging 8 cm in length. The rainbowfish is oviparous and under controlled laboratory conditions, spawning can occur daily throughout the year. The Murray rainbowfish has been used successfully as a test species in previous laboratory studies and to monitor environmental contaminants in Australian water bodies. In 2002, a base-line reproductive study on this species was conducted by Pollino and Holdway (2002) and in 2007, Pollino et al. (2007) assessed the potential reproductive effects of laboratory exposures of 17β-estradiol (E2) to male and female fish. The authors reported changes in phosphoproteins and γ-glutamyltranspeptidase (GTP) in male fish exposed to E2 at concentrations ranging from 30 to 1000 ng/L within 14 days and changes in egg production in females were observed at 300 and 1000 ng/L (Pollino et al., 2007). A recent study by Woods et al. (2009) reported changes in the localisation and abundance of *esr1*, *esr2* and vitellogenin (*vtg*; female egg yolk protein) mRNA staining in the liver and testis of mature male rainbowfish exposed to E2 via the water for 7 days. This study revealed that in male rainbowfish *vtg* protein was detected in the testis and was up-regulated in response to E2 exposure. The function of this locally produced *vtg* is unknown and may suggest implication in the mediation of adverse effects of endocrine disrupting chemicals such as testicular growth inhibition, testis-ova and sex reversal. These studies indicate that the Murray rainbowfish may be a good candidate for investigating estrogen mechanisms, actions, and tissue-specific expression in juvenile and adult fish exposed to xenoestrogen contaminants in the Australian riverine environment.

The aim of the current study was to clone *esr1* in the Murray rainbowfish in order to determine the sex-specific tissue distribution of *esr1* in male and female rainbowfish. In addition, the deduced amino acid sequences were used to generate alignments and a phylogenetic tree with other *esr1* sequences. The results from this study form the basis for further investigations on the molecular regulation of *esr1* and estrogen actions in rainbowfish.

2. Materials and methods

2.1. Animals

Mature female and male *M. fluviatilis*, (approximately 1 year old weighing 2–3 g) were obtained from Ausyfish, Queensland. Upon arrival to the laboratory, fish were maintained under constant conditions in fish tanks containing an artificial freshwater recirculating system (at 24 °C, pH 7.6, 8.4 ppm oxygen and a hardness of 100 mg $CaCO_3$/L) with a flow through rate of 5 L/min and fish loading not exceeding 10 g fish/L. A 16 h light:8 h darkness photoperiod was maintained using cool white fluorescent lamps, with a 60 min dawn:dusk transition period. Fish were fed dried fish flakes (Nutrifin, Aggies Aquariums) at 2% body weight per day. Fish were killed in accordance with the guidelines and principles of the Institute of Medical and Veterinary Science Animal Ethics Committee and the target tissue (brain, gill, intestine, liver, muscle and gonads) was excised using sterile, RNase-free dissecting equipment. Tissues were flash-frozen in liquid nitrogen and stored at –80 °C until RNA isolation.

2.2. RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from the brain, gill, intestine, liver, muscle and gonads (ovary or testis) from adult male and female rainbowfish using a QIAGEN®RNeasy® mini total RNA isolation kit (QIAGEN® Pty. Ltd.) according to the manufacturer's protocol. RNA was treated with DNase 1 and the integrity was examined on a denaturing agarose gel. Total RNA yield was estimated using Quant-iT™ RiboGreen® RNA assay kit (Invitrogen). RNA was stored at −80 °C until use.

No published sequences exist for the rainbowfish. Therefore, consensus primers for *esr1* (*cesr11* and *cesr12*; Table 1) were designed on a conserved region of the LBD of the *esr1* of zebrafish (*D. rerio*; AF349412), gilthead sea bream (*Sparus aurata*; AF136979), Nile tilapia (*Tilapia nilotica*; U75604), channel catfish (*I. punctatus*; AF061275), goldfish (*C. auratus*; AY055725), medaka (*Oryzias* sp.; D28954) and rainbow trout (*Oncorhynchus mykiss*; AJ242740). These primers were designed in highly conserved areas between fish species, but poorly conserved areas between *esr1* and *esr2a*. Gene-specific primers were designed based on sequence analysis of cDNA fragments obtained from RT-PCR with the consensus primers. The gene-specific primer sets used for *esr1* (*esr11* and *esr12*) RT-PCR are listed in Table 1. The 18SrRNA1 and 18SrRNA2 primers (Table 1) were designed on a conserved region of the 18S rRNA of gilthead sea bream (*S. aurata*; AY993930), killifish (*F. heteroclitus*; M91180), Japanese medaka (*Oryzias latipes*; AB105163), European perch (*Perca fluviatilis*; AF518195) and Atlantic salmon (*Salmo salar*; AJ427629).

One step RT-PCR was used for amplification of fragments (which contained 300 ng of total RNA) following the manufacturer's protocol (QIAGEN® Pty. Ltd.). Negative controls (containing no RNA template) for each primer pair was run concurrently with each reaction. PCR amplifications were performed using an Eppendorf Master Cycler Gradient (ThermoFisher Scientific). Reverse transcription was conducted at 50 °C for 30 min followed by an initial PCR activation at 95 °C for 15 min. Amplification of *esr1* was performed by 35 successive PCR cycles (94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min). A final extension step was conducted at 72 °C for 10 min. Amplification of 18S rRNA was performed following the same conditions used for *esr1* except 0.3 μM of both forward and reverse primers were used and amplification was conducted using 22 successive PCR cycles. The number of PCR cycles was optimised to ensure that the reaction was in the log-linear phase of amplification in order for inter-tissue comparisons to be made. Values were normalised to the expression of the housekeeping gene, 18S rRNA. To estimate the molecular weights of the amplicons, 5 μL of amplified products were analysed on a 1% agarose gel stained with ethidium bromide. DNA was extracted and purified from the agarose gel using a QIAquick® gel extraction kit following the manufacturer's protocol (QIAGEN® Pty. Ltd.), cloned in pGEM®-T Easy Vector (Promega) and sequenced at the Molecular Pathology Sequencing Centre at the Institute of Medical and Veterinary Science, Adelaide, to confirm the identity of the products. Specific band and background densities were determined for each cDNA on a single gel by digital image analysis using the Bio-Rad Quantity One software (expressed as intensity/mm^2) in

Table 1
Primers for *esr1* and 18S rRNA used in RT-PCR and RACE PCR.

Oligo name	Sequence[a] (5′–3′)
cesr11[b]	TCACCATGATGACCCTGCTC
cesr12[b]	GGTGCDTTTTCDTTCTGCACT
esr11	GCTGAGCCGACCTTACACTG
esr12	CTGGTGCGTTTTTCTTTCTGC
18SrRNA1	CAGTTATGGTTCCTTTGATCGC
18SrRNA2	CTGCCCTATCAACTTTCGATG
GSP1esr1 (5')	CTGGAGGTGCTGATGATCGGGCT
GSP2esr1 (3')	TCACCATGATGACCCTGCTCACCA
NGSP1esr1	CCAAGTACTGCTGCTTGAGAGCTCATGG
5′esr1SEQ	GACGAGGCATGCGATGTG

[a] A = adenine, C = cytosine, G = guanine, T = thymine, degenerate base pairs D = A + T + G, R = G + A.
[b] Primers designed based on census with other fish species.