# Estrogen receptor-related receptors in the killifish *Fundulus heteroclitus*: diversity, expression, and estrogen responsiveness

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## Abstract

The estrogen receptor-related receptors (ERRs) are a group of nuclear receptors that were originally identified on the basis of sequence similarity to the estrogen receptors. The three mammalian ERR genes have been implicated in diverse physiological processes ranging from placental development to maintenance of bone density, but the diversity, function, and regulation of ERRs in non-mammalian species are not well understood. In this study, we report the cloning of four ERR cDNAs from the Atlantic killifish, *Fundulus heteroclitus,* along with adult tissue expression and estrogen responsiveness. Phylogenetic analysis indicates that *F. heteroclitus* (Fh)ERRα is an ortholog of the single ERRα identified in mammals, pufferfish, and zebrafish. FhERRβa and FhERRβb are co-orthologs of the mammalian ERRβ. Phylogenetic placement of the fourth killifish ERR gene, tentatively identified as FhERRγb, is less clear. The four ERRs showed distinct, partially overlapping mRNA expression patterns in adult tissues. FhERRα was broadly expressed. FhERRβa was expressed at apparently low levels in eye, brain, and ovary. FhERRβb was expressed more broadly in liver, gonad, eye, brain, and kidney. FhERRγb was expressed in multiple tissues including gill, heart, kidney, and eye. Distinct expression patterns of FhERRβa and FhERRβb are consistent with subfunctionalization of the ERRβ paralogs. Induction of ERRα mRNA by exogenous estrogen exposure has been reported in some mammalian tissues. In adult male killifish, ERR expression did not significantly change following estradiol injection, but showed a trend toward a slight induction (three- to five-fold) of ERRα expression in heart. In a second, more targeted experiment, expression of ERRα in adult female killifish was downregulated 2·5-fold in the heart following estradiol injection. In summary, our results indicate that killifish contain additional ERR genes relative to mammals, including ERRβ paralogs. In addition, regulation of ERRα expression in killifish apparently differs from regulation in mammals. Together, these features may facilitate determination of both conserved and specialized ERR gene functions.

*Journal of Molecular Endocrinology* (2006) **37,** 105–120

## Introduction

The estrogen receptor-related receptors (ERRs) are members of the nuclear receptor superfamily of transcription factors (Giguere *et al.* 1988). Three ERR genes have been identified in mammals, ERRα (NR3B1), ERRβ (NR3B2), and ERRγ (NR3B3) (Giguere *et al.* 1988, Chen *et al.* 1999, Hong *et al.* 1999, Heard *et al.* 2000). ERRs and estrogen receptors (ERs) have overlapping affinities for co-activators and DNA-binding sites, but differ markedly in ligand binding and activation (Vanacker *et al.* 1999*a*,*b*, Giguere 2002). Unlike ERs, ERRs do not bind estradiol, and have been reported to be either constitutively active (Hong *et al.* 1999, Xie *et al.* 1999, Greschik *et al.* 2002) or activated by an unidentified ligand (Vanacker *et al.* 1999*b*). While high-affinity ERR agonists have not been identified, some ER ligands, including 4-hydroxytamoxifen and diethylstilbestrol, can antagonize ERR activity (Coward *et al.* 2001, Tremblay *et al.* 2001*a*,*b*).

The functions and target genes of ERRs are not yet well understood. ERRα helps to regulate bone growth and maintenance by binding to the osteopontin promoter, a gene target that is shared with ERα (Bonnelye *et al.* 1997*b*, 2001, Vanacker *et al.* 1999*a*). ERRα has also been shown to repress activity of PPARγ co-activator 1α (PGC-1α), a co-activator that interacts with PPARs (peroxisome proliferator-activated receptors) to regulate gluconeogenesis and adaptive thermogenesis (Ichida *et al.* 2002). ERRα-null mutant mice are essentially normal with reduced body weight and peripheral fat deposits, which supports the hypothesis that ERRα helps to regulate energetic metabolism and fat storage (Luo *et al.* 2003). In contrast to the mild ERRα knockout phenotype, ERRβ-null mutants die during development due to defects in placental formation (Luo *et al.* 1997).


0952–5041/06/037–105 
DOI: 10.1677/jme.1.01976
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ERRβ-null mutants rescued from embryonic lethality exhibit behavioral abnormalities and reduced numbers of germline cells (Mitsunaga *et al.* 2004). The role of ERRγ has not been elucidated through knockout experiments, but high expression has been noted in differentiating neural tissues (Hermans-Borgmeyer *et al.* 2000). Other proposed target genes for ERRs include lactoferrin, aromatase, small heterodimer partner, endothelial nitric oxide synthase, SULT2A1, and thyroid hormone receptor-α (Yang *et al.* 1996, Vanacker *et al.* 1998, Zhang & Teng 2000, Sanyal *et al.* 2002, Sumi & Ignarro 2003, Seely *et al.* 2005).

In mice, ERRα and ERRγ are broadly expressed in adult and embryonic tissues (Bonnelye *et al.* 1997*a*, Shigeta *et al.* 1997, Heard *et al.* 2000, Hermans-Borgmeyer *et al.* 2000). In contrast, ERRβ has more limited expression, most notably in a subset of placental cells during early embryonic development and in developing germ cells (Pettersson *et al.* 1996, Luo *et al.* 1997, Mitsunaga *et al.* 2004). In the adult, ERRβ is expressed at low levels in a few tissues including kidney, heart, testis, hypothalamus, hippocampus, cerebellum, and prostate (Giguere *et al.* 1988, Pettersson *et al.* 1996). ERRα expression can be upregulated by estrogen exposure in some mammalian tissues (Shi *et al.* 1997, Shigeta *et al.* 1997, Liu *et al.* 2003), but regulation of ERR expression is not well understood.

Examination of teleost genomic databases has revealed that fishes contain additional diversity of ERR genes as compared with mammals: six ERR genes have been identified in the Japanese pufferfish *Takifugu rubripes* ('fugu'), five in the spotted green pufferfish *Tetraodon nigroviridis* ('tetraodon'), and five in the zebrafish *Danio rerio* (Bardet *et al.* 2002, Bertrand *et al.* 2004). A genome duplication within the teleost lineage (Amores *et al.* 1998, Taylor *et al.* 2001, Christoffels *et al.* 2004, Jaillon *et al.* 2004, Postlethwait *et al.* 2004) may account for some of the additional ERR diversity, but it has also been suggested that the ERRδ identified in zebrafish and fugu has been secondarily lost from mammals and tetraodon (Bardet *et al.* 2002). Additional diversity of ERR genes within the teleost lineage is of evolutionary interest, but also provides an opportunity to gain mechanistic insight into mammalian ERR genes. In particular, the duplication, degeneration, complementation hypothesis predicts that the multiple functions of a gene (e.g. a mammalian ERR) may be partitioned between duplicated co-orthologs (Force *et al.* 1999, Lynch & Force 2000).

The few published studies of ERR function in fishes and invertebrates have provided insight into evolutionary biology and novel aspects of ERR function. For example, *in situ* hybridization showed that ERRs are developmentally expressed in a segmented pattern in both the amphioxus (single ERR) and the zebrafish (ERRα, ERRβb, and ERRγa) hindbrain, which indicates that a structure similar to a segmented hindbrain predated the divergence of invertebrates and vertebrates (Bardet *et al.* 2005*b*). Knockdown of ERRα expression using morpholino antisense oligonucleotides in zebrafish indicated a novel role for ERR in regulating morphogenic movement during gastrulation (Bardet *et al.* 2005*a*). ERRs of zebrafish and human are similar with respect to ligand binding and transactivation (Bardet *et al.* 2004); however, further investigation is needed to understand ERR signaling in teleosts. For example, ERR expression patterns have not been described in adults of any teleost species and regulation of teleost ERR expression (e.g. in response to estradiol exposure) has not been described.

In this study, we report the cloning, adult tissue-expression patterns and estrogen responsiveness of ERR cDNAs in the Atlantic killifish, *Fundulus heteroclitus*. *F. heteroclitus* has been used as a model species for several recent studies of endocrine disruption. In particular, laboratory and natural populations exposed to environmental contaminants show altered levels of sex steroids (Dube & MacLatchy 2001, Hewitt *et al.* 2002, MacLatchy *et al.* 2003, Boudreau *et al.* 2004, Greytak *et al.* 2005), thyroid hormones (Zhou *et al.* 2000, Carletta *et al.* 2002), and aromatase mRNA (Greytak *et al.* 2005). Given the cross-talk between mammalian ERs and ERRs (Vanacker *et al.* 1999*a*, Giguere 2002), and the hypothesized regulatory role of ERRα in aromatase and thyroid receptor expression (Vanacker *et al.* 1998, Yang *et al.* 1998), elucidation of ERR signaling may provide insight into endocrine regulation and disruption in this model species. Specific objectives of this study were: (1) to determine whether *F. heteroclitus* contained duplicated co-orthologs of any mammalian ERR gene, which could provide insight into gene function, (2) to compare adult tissue expression patterns of killifish ERR cDNAs with expression patterns reported for mammalian ERRs, and (3) to determine whether estradiol exposure affects killifish ERR cDNA expression, especially the expression of ERRα.

## Materials and methods

### Animals and RNA isolation

For cloning of ERR and determination of tissue-specific expression patterns, *F. heteroclitus* (Atlantic killifish or mummichog) were trapped in salt marshes surrounding Scorton Creek on Cape Cod, MA, USA in May and June 2003. Fish were reproductively active, with mature eggs visible in the ovaries. Three adult male fish and three adult female fish were anesthetized with MS-222 and killed *via* cervical transection. Liver, gonad, brain, eye, kidney, gill, gut, heart, and spleen were dissected and pooled for the three fish of a given sex. Total RNA

was isolated from tissues using RNA STAT-60 (Tel-Test, Inc.). A negative control consisted of a sham RNA extraction with no tissue added.

Two experiments were conducted to determine the effects of estradiol exposure on ERR mRNA transcript expression. In both the experiments, reproductively regressed adult *F. heteroclitus* were collected from Scorton Creek and injected intra-peritoneally with estradiol (5 μg/g body weight, as a 1 μg/μl solution in sesame oil) or with a vehicle control (sesame oil). Both concentration and method of exposure are predicted to produce a high spike in plasma estradiol concentration that is cleared rapidly (Pankhurst *et al.* 1985) and a robust induction of vitellogenesis in male fish (Pait & Nelson 2003).

In the first experiment, male *F. heteroclitus* were injected in November 2003 and sacrificed after 2 or 5 days. The organs were immediately flash-frozen, and the total RNA was extracted subsequently from organs of individual fish using Tri-reagent (Sigma). Thirteen fish were injected in total (6 with estradiol and 7 with vehicle), giving a sample size of 3–4 fish per time point within a treatment.

In the second experiment, adult female *F. heteroclitus* were injected in January 2005 and sacrificed after 2 days. The organs were flash-frozen as in the previous experiment and total RNA was extracted from organs of individual fish with STAT-60. Twenty five fish were injected in total ($n=12$, estradiol treatment; $n=13$, vehicle control).

### Reverse transcriptase (RT)-PCR

cDNA was synthesized from 3 μg total RNA using random hexamers and the Omniscript cDNA Synthesis Kit (Qiagen). Degenerate oligonucleotide primers, ERRf1 and ERRr1, were designed based on highly conserved regions of the fugu-predicted ERR genes (Table 1). PCRs with these degenerate primers resulted in two different cDNAs (441 bp *F. heteroclitus* (Fh)ERRα and 432 bp FhERRβa) when used with Advantage2 Polymerase (BD Biosciences Clontech) with the following cycling conditions in a Perkin-Elmer GeneAmp 2400 thermocycler: 95 °C/60 s (95 °C/30 s, 65 °C/45 s, 68 °C/45 s) for 35 cycles, 68 °C/60 s. Additional degenerate primers were targeted toward other ERR genes predicted from the fugu genome. These reactions were conducted using AmpliTaq Gold Polymerase (Applied Biosystems, Framingham, MA, USA). A 398 bp cDNA fragment (FhERRβb) was amplified using ERRf2 and ERRr1 under the following conditions: 94 °C/5 min (94 °C/15 s, 62·5 °C/15 s, 72 °C/30 s), 72 °C/5 min, followed by (94 °C/15 s, 62 °C/15 s, 72 °C/30 s) for 10 cycles, 72 °C/5 min. ERRf3 and ERRr1 amplified a 324 bp fragment (FhERRγb) at 95 °C/10 min (94 °C/15 s, 63·5 °C/15 s, 72 °C/30 s) for 35 cycles, 72 °C/10 min.

### Rapid amplification of cDNA ends (RACE) and amplification of full-length PCR products

5′-/3′-RACE reactions were performed using a SMART RACE cDNA amplification kit (BD Biosciences Clontech). Briefly, adapter-ligated, oligo(dT)-primed cDNA was produced from brain or liver total RNA. Gene-specific primers were used with adapter primers in PCR. To most RACE PCR, 5% dimethylsulfoxide (DMSO) was added. Touchdown PCRs cycling conditions were used according to the manufacturer's instructions with primers shown in Table 1. For FhERRα 5′-RACE, primer ERRAr1 and nested primer ERRAr2 were used to generate a partial 5′-RACE product. To obtain the 5′ end of FhERRα, primer ERRAr3 was used as a nested primer. For FhERRα 3′-RACE, primer ERRAf1 was used. For FhERRβa 5′-RACE, a partial 5′-RACE product was obtained using primer ERRB1r1. To obtain a complete 5′ sequence, additional fragments were amplified using nested primers ERRB1r2 and ERRB1r3. For FhERRβa 3′-RACE and FhERRβb 5′-RACE, primers ERRB1f1 and ERRB2r1 were used, respectively, in two rounds of PCR with nested adapter primers. For FhERRβb 3′-RACE, primer ERRB2f1 and nested primer ERRB2f2 were used. For FhERRγb 5′-RACE, primer ERRGr1 and nested primer ERRGr2 were used. For FhERRγb 3′-RACE, primer ERRGf1 and nested primer ERRGf2 were used.

Once full-length RACE products were obtained, additional gene-specific primers (Table 1) were designed within the untranslated regions to amplify full-length PCR products for each gene. All full-length products were amplified using Advantage2 Polymerase with 5% DMSO added to the reactions. FhERRα, FhERRβb, and FhERRγb were amplified from cDNA made from brain total RNA using the following cycling conditions: 94 °C/1 min, 35–37 cycles of (94 °C/s, 65 °C/10 s, 68 °C/2 min), 72 °C/7 min. FhERRβa was amplified from cDNA made from brain poly-A+ RNA using the following cycling conditions: 94 °C/1 min, 40 cycles of (94 °C/s, 64 °C/10 s, 69 °C/2 min), 72 °C/7 min. During analysis of 5′-RACE products and full-length cDNA clones for FhERRα, an apparent frame shift was noted in the sequence. When these sequences were aligned with the other fish ERRα sequences, it appeared that the FhERRα clones might be missing a section coding for 16 amino acid residues. Using specific primers, we amplified a 300 bp product that overlapped previous sequences and contained an additional 56 bp in the frame shift region. The 300 bp product and the 56 bp insert had 68 and 80% GC content respectively. The high GC content of this region is likely to have resulted in the secondary structure leading to errors in RT-PCR. The complete predicted cDNA sequence is thus a composite of full-length clones with the 56 bp region inserted; the

**Table 1** Oligonucleotide sequences of primers. In degenerate primers S=C or G, W=A or T, R=A or G

| Application | Name | Oligonucleotide sequence |
|---|---|---|
| Amplification of partial cDNA | ERRf1 | 5′-TCCTCTGAGGCCTGCAARGC-3′ |
| | ERRf2 | 5′-TCCATGCCSAAGAGRCTG-3′ |
| | ERRf3 | 5′-GTCAAGTGCCTGGCTGTGG-3′ |
| | ERRr1 | 5′-WGGRATGTGYTTGGCCCAGC-3′ |
| 5′ RACE of FhERRα | ERRAr1 | 5′-CCAGCTCGCGGTCGGCAAGGTCAC-3′ |
| | ERRAr2 | 5′-GCACTCGTTGGACGCAGGGCAGC-3′ |
| | ERRAr3 | 5′-CACACCAGACACAGCCTCTTGGG-3′ |
| 3′ RACE of FhERRα | ERRAf1 | 5′-CACCACCCTGTGTGACCTTGCCG-3′ |
| 5′ RACE of FhERRβa | ERRB1r1 | 5′-GCTTTTTTGCTGGTGGAGGAAGAGTG-3′ |
| | ERRB1r2 | 5′-GGGTTGAACATCGGAGGTGAGTC-3′ |
| | ERRB1r3 | 5′-TCACAAGAGGCGACCCCGTAGTG-3′ |
| 3′ RACE of FhERRβa | ERRB1f1 | 5′-GATGCTGAGAATGGGTCTTACCTG-3′ |
| 5′ RACE of FhERRβb | ERRB2r1 | 5′-GGGTTGAACATCGGAGGTGAGTC-3′ |
| 3′ RACE of FhERRβb | ERRB2f1 | 5′-TGCTCAAAGAAGGGGTTCGTCTGG-3′ |
| | ERRB2f2 | 5′-GGAGGCTGGACACAGAAAACAACC-3′ |
| 5′ RACE of FhERRγb | ERRGr1 | 5′-ACCACCTTGTTTTCCACTGCGTC-3′ |
| | ERRGr2 | 5′-CCAGACGGACGCCTTCTCTCAAC-3′ |
| 3′ RACE of FhERRγb | ERRGf1 | 5′-TGTTGAGAGAAGGCGTCCGTCTG-3′ |
| | ERRGf2 | 5′-CGACATCAAGGCTCTGACCACGC-3′ |
| Cloning of FhERRα ORF | ERRAFLf | 5′-CAGGCACACCTGACCTTTGAGTG-3′ |
| | ERRAFLr | 5′-CCCCAATCCCATCTTTATGTCCT-3′ |
| Cloning of FhERRβa ORF | ERRB1FLf | 5′-AACAGAAAGCCTGCACGAGT-3′ |
| | ERRB1FLr | 5′-CGTCTCTGACTGATGATG C-3′ |
| Cloning of FhERRβb ORF | ERRB2FLf | 5′-CCAGGCTTTGTTGTCCAAAT-3′ |
| | ERRB2FLr | 5′-GGGATCCACACAATGAGGAG-3′ |
| Cloning of FhERRγb ORF | ERRGFLf | 5′-TCCTTGAGACTGACTGACTGC-3′ |
| | ERRGFLr | 5′-CTAAAATGGACGAATACACCG-3′ |
| QPCR | ERRAQf | 5′-GAGGGAGTACGTCTCGACAGAG-3′ |
| | ERRAQr | 5′-CACGATGATGTTGGAAGAACC-3′ |
| | ERRBaQf | 5′-GCATATCCCAGGTTTTTCCAC-3′ |
| | ERRBaQr | 5′-AAACACAATGCTCAGCACCAG-3′ |
| | ERRBbQf | 5′-TGGTGCGCAAGTACAAGAAG-3′ |
| | ERRBbQr | 5′-CCTCTATGTGCATGGAGTCTG-3′ |
| | ERRGbQf | 5′-CAGGGTAACATCGAATACAGC-3′ |
| | ERRGbQr | 5′-GACGCCTTCTCTCAACATGC-3′ |
| | FhACrt-F | 5′-TGGAGAAGAGCTACGAGCTCC-3′ |
| | FhACrt-R | 5′-CCGCAGGACTCCATTCCGAG-3′ |
| | FhEF1-F | 5′-GGGAAAGGGCTCCTTCAAGT-3′ |
| | FhEF1-R | 5′-ACGCTCGGCCTTCAGCTT-3′ |
| | FhVtg-F | 5′-GAGGATCTGTGCTGATGCAGTTGTG-3′ |
| | FhVtg-R | 5′-GGGTAGAAGGCAGTCTTTCCCAGG-3′ |

ORF, open reading frame; QPCR, quantitative real-time PCR

location of the insert is marked in Fig. 1. The insert is well conserved among fishes (not shown) but not between fishes and mammals.

## Cloning and sequencing

All PCR products were cloned into pGEM-T Easy (Promega). PCR products were sequenced by the University of Maine DNA Sequencing Facility (Orono, ME, USA) or at the Bay Paul Center Sequencing Facility (Marine Biological Laboratory, Woods Hole, MA, USA). Both strands from multiple clones were sequenced to ensure accuracy. DNA sequences were analyzed, assembled, and translated using the Wisconsin Package (GCG, Accelrys, Burlington, MA, USA) and Bioedit Sequence Alignment Editor software (Hall 1999).

## Phylogenetic analysis

*F. heteroclitus* ERR-deduced amino acid sequences were aligned with previously reported ERR sequences from fishes, mammals, and *Drosophila melanogaster* using Clustal X 1·81 with default parameters (for accession numbers see Table 2). Gaps and the highly variable A/B domain were excluded from phylogenetic analysis. The aligned amino acid sequences were used to create phylogenetic trees using maximum parsimony and distance (minimum evolution) criteria with

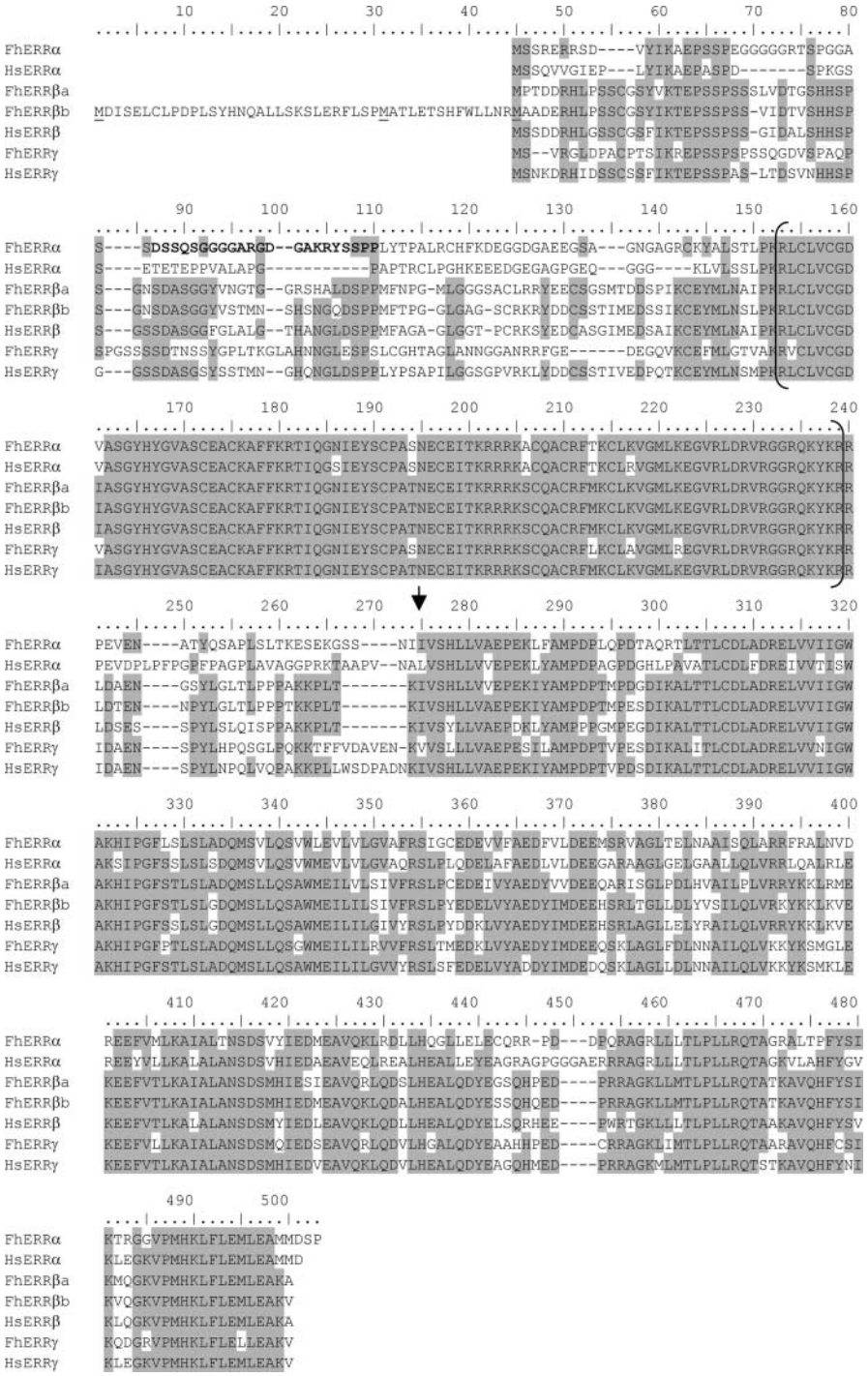


**Figure 1** Alignment of *F. heteroclitus* ERR amino acid sequences. Deduced amino acid sequences of four ERR genes cloned from *F. heteroclitus* were aligned with three human ERR genes using ClustalW within Bioedit. Identical residues are shaded. Sixteen amino acid residues shown in boldface indicate a portion of the FhERR$\alpha$ sequence that was obtained from a separate PCR product. See Results for further information. The start codon of FhERR$\beta$b is not known; three potential translation initiation sites are underlined. The DBD (C domain) is enclosed in brackets, and the start of the LBD (E/F domain) is indicated by an arrow. All GenBank accession numbers are given in Table 2.

**Table 2** ERR gene names, synonyms, and accession numbers. Ensembl predicted proteins from the following databases: human 31·35d, Takifugu build 2c, release 31·2f, Tetraodon version 7, Danio release 31·4d. Other Ensembl predicted proteins are abbreviated names and correspond to older assemblies for comparison with previously published literature (i.e. Bertrand *et al.* 2004)

| | **Official name** | **Trivial name**(s) | **Pubmed accession number** (protein) | **Ensembl predicted protein** | **'Other' Ensembl predicted protein** |
|---|---|---|---|---|---|
| **Species** | | | | | |
| Human | NR3B1 | ERRα, ESRRA | NP_004442 | ENSP00000000442 | |
| | NR3B2 | ERRβ, ESRRB, ERR2, ESRL2 | NP_004443 | ENSP00000261532 | |
| | NR3B3 | ERRγ, ERR3 | NP_001429 | ENSP00000354584 | |
| Takifugu | | ERRα | N/A | SINFRUP 00000138848 | SIN67383 |
| | | ERRβa | N/A | SINFRUP 00000140232 | SIN72315 |
| | | ERRβb | N/A | SINFRUP 00000141263 | FRUP141263/62880 |
| | | ERRγa | N/A | SINFRUP 00000162788 | SIN57192 |
| | | ERRγb (β/γ) | N/A | SINFRUP 00000130211 | FRUP 130211(51057) |
| | | ERRδ | N/A | SINFRUP 00000134462 | FRUP134462 |
| Tetraodon | | ERRα | N/A | GSTENP 00020389001 | GST20389 |
| | | ERRβa | N/A | GSTENP 00032328001 | GST32328 |
| | | ERRγa | N/A | GSTENP 00032849001 | GST32848 |
| | | ERRγb (β/γ) | N/A | GSTENP 00030242001 | FS563 |
| Danio | | ERRα, *esrral* (zfin) | AAS66634 | ENSDARP 00000050433 | |
| | | ERRβb *esrrb* (zfin) | AAS66635 | ENSDARP 00000040417 | |
| | | ERRγa *esrrg* (zfin) | AAS66636 | ENSDARP 00000005364 | |
| | | ERRγb, ERRβ/γ *esrrgl* (zfin) | AAS66638 | ENSDARP 00000002838 | |
| | | ERRδ *esrrd* (zfin) | AAS66637 | ENSDARP 00000013201 | |
| Fundulus | | ERRα | DQ241376 | N/A | |
| | | ERRβa | DQ241377 | N/A | |
| | | ERRβb | DQ241378 | N/A | |
| | | ERRγb (β/γ) | DQ241379 | N/A | |
| Drosophila | | ERR | NP_729340 | CG7404 (flybase) | |

PAUP*4.0b10 software (Swofford 2003). The *D. melanogaster* ERR was used as the outgroup. Trees were constructed with a heuristic search strategy, and branch swapping and tree-bisection reconnection were repeated to obtain bootstrapping values from 1000 replicates.

In an attempt to further resolve the ERRβ and ERRγ clades, a second alignment was created using fish and mammalian ERRα, ERRβ, and ERRγ sequences (i.e., no ERRδ or invertebrate ERR sequences). Phylogenetic trees were created using parsimony and minimum evolution criteria, as previously, using ERRα genes as the outgroup. In addition, a maximum likelihood tree was constructed using Phylip 3.64 (Felsenstein 2004). Weights corresponding to 1000 bootstrap replicates were generated by SEQBOOT (within Phylip), and a γ-law parameter, $\alpha$, was estimated by PHYML (Guindon *et al.* 2005). Maximum likelihood trees were constructed using the Jones, Taylor and Thornton (JTT) substitution model (Jones *et al.* 1992) as implemented in ProML (within Phylip) with a γ distribution of rates between sites (four categories). Consensus trees were created by CONSENSE and rooted with the ERRα sequences. Alternative tree topologies were compared with the maximum likelihood consensus tree using the -Shimodaira–Hasegawa test (SH-test; Shimodaira & Hasegawa 1999), as implemented in ProML.

### Nomenclature

We have named teleost co-orthologs (e.g. FhERRβa and FhERRβb) to be consistent with zebrafish nomenclature rules (Sprague *et al.* 2001). Similar nomenclature has been applied to duplicated teleost ER genes (Hawkins & Thomas 2004).

### Quantitative real-time RT-PCR (qPCR)

*F. heteroclitus* ERR splice sites were predicted by comparing genomic sequences for human, fugu, and zebrafish ERRs with cDNA sequences (human, zebrafish) or gene predictions (fugu). Splice sites were generally well conserved among species and among various ERR genes within a species (data not shown). Primers for ERRα, ERRβa, ERRβb, ERRγb, and β-actin (Table 1) were designed with one primer spanning a predicted exon–exon junction to avoid amplification of genomic DNA. Primers for EF-1 (Bears *et al.* 2006) and vitellogenin (Garcio-Reyero *et al.* 2004) were taken from published studies. cDNA was synthesized from 2 μg total RNA using random hexamers and the Omniscript cDNA Synthesis Kit (Qiagen). In the tissue-distribution study, cDNA was diluted in a ratio of 1:3 in ERRβb and EF-1 assays. qPCR was performed using the iQ SYBR Green Supermix (Bio-Rad) and reactions were run in an iCycler iQ Real-Time PCR Detection System (BioRad). The PCR mixture consisted of the following: 11 μl molecular biology grade distilled water, 12·5 μl iQ SYBR Green Supermix, 0·25 μl 5′-primer (10 μM), 0·25 μl 5′-primer (10 μM), and 1 μl cDNA.

In the analysis of tissue distribution of ERRs, the PCR conditions for FhERRα and FhERRβb were: 95 °C/3 min, 95 °C/15 s, 66 °C/1 min, 40 cycles. PCR conditions of other genes were identical except that annealing/extension temperatures were adjusted to maximize the amplification of the specific product: FhERRβa (64 °C), FhERRγb (67·9 °C), EF-1 (60 °C). At the end of each PCR cycle, the PCR products were subjected to melt-curve analysis to ensure that only a single product was amplified. For both males and females, each of the nine tissues was represented by a single cDNA derived from pooled total RNA from three fish. There were three technical replicates (qPCR well) per sample per gene. Expression data were quantified based on threshold cycle ($C_t$) values and the $-2^{\Delta\Delta C_t}$ method (Livak & Schittgen, 2001). β-Actin expression was highly variable among tissues (data not shown), so for each ERR gene, $C_t$ values were normalized to EF-1 (Bears *et al.* 2006). Relative mRNA expression for each gene was calculated as the fold change compared with the tissue with the lowest $C_t$ (i.e. data were normalized such that the tissue with the highest expression was set equal to one).

For analysis of qPCR data from dosing experiments, a standard curve for each ERR gene was generated by serially diluting plasmids containing a full-length copy of each gene from $10^3$ to $10^8$ molecules/μl. The PCR conditions for FhERRα, FhERRγb, and Fh-β-actin were: 95 °C/3 min, 95 °C/15 s, 66 °C/1 min, 40 cycles. PCR conditions for FhERRβb were nearly identical: 95 °C/3 min, 95 °C/15 s, 64 °C/1 min, 40 cycles. At the end of each PCR cycle, the PCR products were subjected to melt-curve analysis to ensure that only a single product was amplified. The number of molecules/μl for each gene of interest in each RNA sample was calculated from the standard curve. ERR expression was presented as unnormalized, and the β-actin expression is shown for comparison. The data were transformed via the natural logarithm to obtain a normal distribution (Shapiro–Wilk test and visual inspection of normal probability plots) and equality of variance (visual inspection of residuals) for each gene. Gene expression in estradiol-treated and control tissues were compared using two-tailed *t*-tests.

## Results

### Cloning and phylogenetic analysis of killifish ERRs

Using RT-PCR and 5′/3′ RACE, full-length cDNA and deduced amino acid sequences were determined for four ERR genes in *F. heteroclitus* (Fig. 1).

Identical 441 bp fragments of one ERR cDNA (FhERRα) were cloned initially from total RNA derived from killifish liver, brain, kidney, and heart. The full-length ERRα cDNA sequence is 1617 bp in length, including 131 bp 5′ untranslated sequence, an open reading frame of 1302 bp, and 185 bp 3′ untranslated sequence including a poly-A+ tail. The predicted amino acid sequence encodes a polypeptide 415 amino acid residues in length with a predicted molecular mass of 45·8 kDa. Phylogenetic analysis using distance (minimum evolution, Fig. 2) or parsimony (not shown) criteria clearly indicate that FhERRα is closely related to ERRα genes found in mammals and other species of fish.

Each of the mammalian and teleost species included in this study contains a single ERRα gene. In contrast, the teleost ERRβ genes form two clades, which together are a sister group to the mammalian ERRβ genes (Bertrand *et al.* 2004, Fig. 2). We have identified two ERRβ genes in *F. heteroclitus* (FhERRβa and FhERRβb); these genes are apparent co-orthologs of the mammalian ERRβ genes. Fragments (429 bp) of FhERRβa cDNA were cloned from killifish brain and heart total RNA, and the full-length cDNA was cloned from brain poly-A+ RNA. The sequence is 1656 bp long, including 185 bp of 5′ untranslated sequence, an open reading frame of 1305 bp, and 166 bp 3′ untranslated sequence. Available sequence for the 3′ UTR lacks a poly-A+ tail

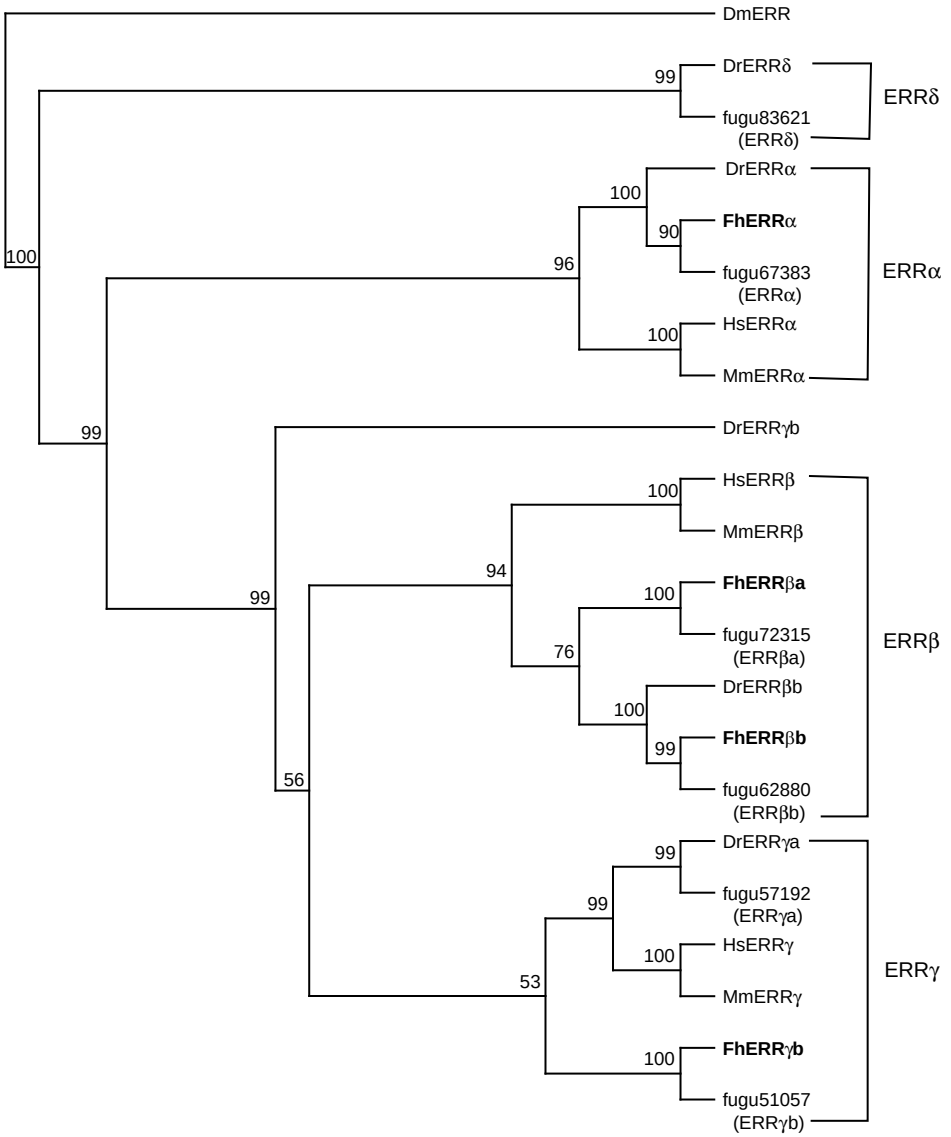


**Figure 2** Phylogenetic analysis (minimum evolution) of ERR-predicted proteins. Deduced amino acid sequences of *F. heteroclitus* ERR genes (bold) were aligned with ERRs from fugu, zebrafish, human, mouse, and fruitfly using ClustalX 1.81. The A/B domains and gaps were excluded from analysis, and the distance criterion (minimum evolution) was used to produce a consensus tree with the *D. melanogaster* ERR as the designated outgroup. Bootstrapping values from 1000 replicates are shown. GenBank accession numbers are given in Table 2.

and may be incomplete. The predicted amino acid sequence encodes a polypeptide, 435 amino acid residues in length, with a predicted molecular mass of 48·2 kDa. FhERRβa is most closely related to a predicted protein in fugu (fugu72315, Fig. 2).

A 499 bp fragment of FhERRβb cDNA was cloned from killifish eye total RNA. The putative complete cDNA sequence (1634 bp) was obtained using brain total RNA. The translated sequence contains three methionine residues near the 5′ end. The predicted proteins corresponding to these potential start codons are 443, 447, and 477 amino acid residues in length, with 176 bp of 3′ UTR. Thus, the predicted molecular mass ranges from 48·5 to 53·6 kDa. Both distance and parsimony analyses indicate that FhERRβb is most closely related to an ERRβ cDNA cloned from zebrafish (Bardet *et al.* 2004) and to a predicted protein in fugu (fugu62880, Fig. 2).

A 324 bp fragment of a fourth ERR cDNA, FhERRγb was cloned from killifish heart cDNA. The cDNA sequence corresponding to the complete coding region was obtained by RACE and PCR with gene-specific primers using brain total RNA. The sequence includes 132 bp of 5′ UTR, 1320 bp coding region, and a partial 3′ UTR of 88 bp. The predicted protein contains 439 amino acid residues and the predicted molecular mass is 48·3 kDa. Phylogenetic analysis using distance criterion (minimum evolution) indicates that FhERRγb is a form of ERRγ and an ortholog of a predicted protein in fugu (fugu51057, Fig. 2), but inclusion of FhERRγb and fugu51057 in the ERRγ clade has a relatively low bootstrap support (Fig. 2). Parsimony-based analysis of the same alignment indicates that these two fish ERRs form a sister group to the ERRβ and ERRγ clades (not shown), as described for zebrafish (Bardet *et al.* 2004).

To further investigate the evolutionary relationships, we aligned the full-length vertebrate ERRα, ERRβ, and ERRγ sequences and constructed phylogenetic trees with distance, parsimony, and maximum likelihood criteria (Fig. 3). As in the previous analysis, placement of FhERRγb, fugu51057, and DrERRγb were equivocal. The parsimony-based analysis placed the three genes within the ERRγ clade (with bootstrap support of 54%), but there were polytomies within the clade. The distance tree placed all the three genes as sister to the ERRβ and ERRγ clades, in contrast to the previous distance analysis (which included ERRδs and the ERR from *D. melanogaster* Fig. 2), which placed the fish genes within the ERRγ clade. The maximum likelihood consensus tree was consistent with a fish-specific duplication of ERRγ, with low bootstrap support (Fig. 3), which is similar to the results from a previous study (Bertrand *et al.* 2004). However, an SH-test showed that the topology indicated by the maximum likelihood tree was not significantly better than two alternative topologies: (1) FhERRγb, fugu51057, and DrERRγb grouping outside the clade formed by mammalian ERRs and teleost ERRγa genes (SH-test, $P=0{\cdot}45$) and (2) DrERRγb grouping outside the other fish and mammalian ERRγs ($P=0{\cdot}155$). The maximum likelihood tree was also unable to resolve the branching patterns within the ERRβ clade.

Possible explanations for the 'outgroup topology' (i.e. fish ERR genes grouping outside the ERRβ and ERRγ clades) include an ancient duplication, or artifacts due to differences in evolutionary rate or saturation (Van de Peer *et al.* 2003). We used the program AsaturA (Van de Peer *et al.* 2002) with a range of cut-off values, substitution matrices, and distance correction methods to explore the effects of amino acid saturation on the topology of trees made with distance and parsimony criteria. In no case did the tree topology provide clear evidence for two groups of fish ERRγ co-orthologs of mammalian ERRγ genes, as would be expected if fish ERRγ diversity resulted from a duplication in the teleost lineage. The results from these parsimony- and distance-based analyses contradict both the predictions from a teleost genome duplication and the weakly supported results from maximum likelihood analysis. Thus, the evolutionary history of FhERRγb and related genes in fugu and zebrafish remains unresolved. We have provisionally named FhERRγb based on the hypothesis that this gene resulted from a teleost-specific duplication of an ancestral ERRγ, as suggested by Bertrand *et al.* (2004).

### Tissue-specific expression

FhERRα, FhERRβa, FhERRβb, and FhERRγb transcripts were measured by qPCR in tissues from male and female fish. *F. heteroclitus* ERR genes showed distinct, partially overlapping expression patterns (Fig. 4). FhERRα was widely expressed and detectable in all tissues studied. FhERRβa was expressed at low levels in brain, female eye, and ovary. FhERRβb was detected primarily in gonad, eye, brain, and male liver, whereas FhERRγb was detected primarily in kidney, eye, heart, and gill. Males and females showed some differences in ERR expression including the ovarian, but not testicular, expression of ERRβa. Since each tissue was represented by a single pooled sample, a more detailed study is needed to determine the sex-specific expression patterns.

### Effects of estradiol exposure on ERR expression

In the first experiment, male fish were injected with estradiol, or a vehicle control, and transcript expression of ERRα, ERRβb, and ERRγb was quantified in several tissues using qPCR (Fig. 5). Two days after exposure, expression of ERRα, ERRβb, and ERRγb was not significantly affected by estradiol dosage for any tissue ($P>0{\cdot}05$), although there was a high degree of variability among the samples. In particular, 2 days after injection, a single fish displayed relatively high levels of ERRα expression in heart, testis, and gill. Because the same fish showed elevated β-actin expression in some tissues, statistical analysis was repeated on unnormalized data, but this did not result in any significant differences. The greatest trend toward induction was observed for ERRα expression in heart tissue. In comparing the mean transcript levels, FhERRα expression was 2·6-fold higher in $E_2$-treated normalized heart tissues relative to the control. The fish with the highest ERRα expression had transcript levels 5·3-fold greater than the control mean transcript level. A power

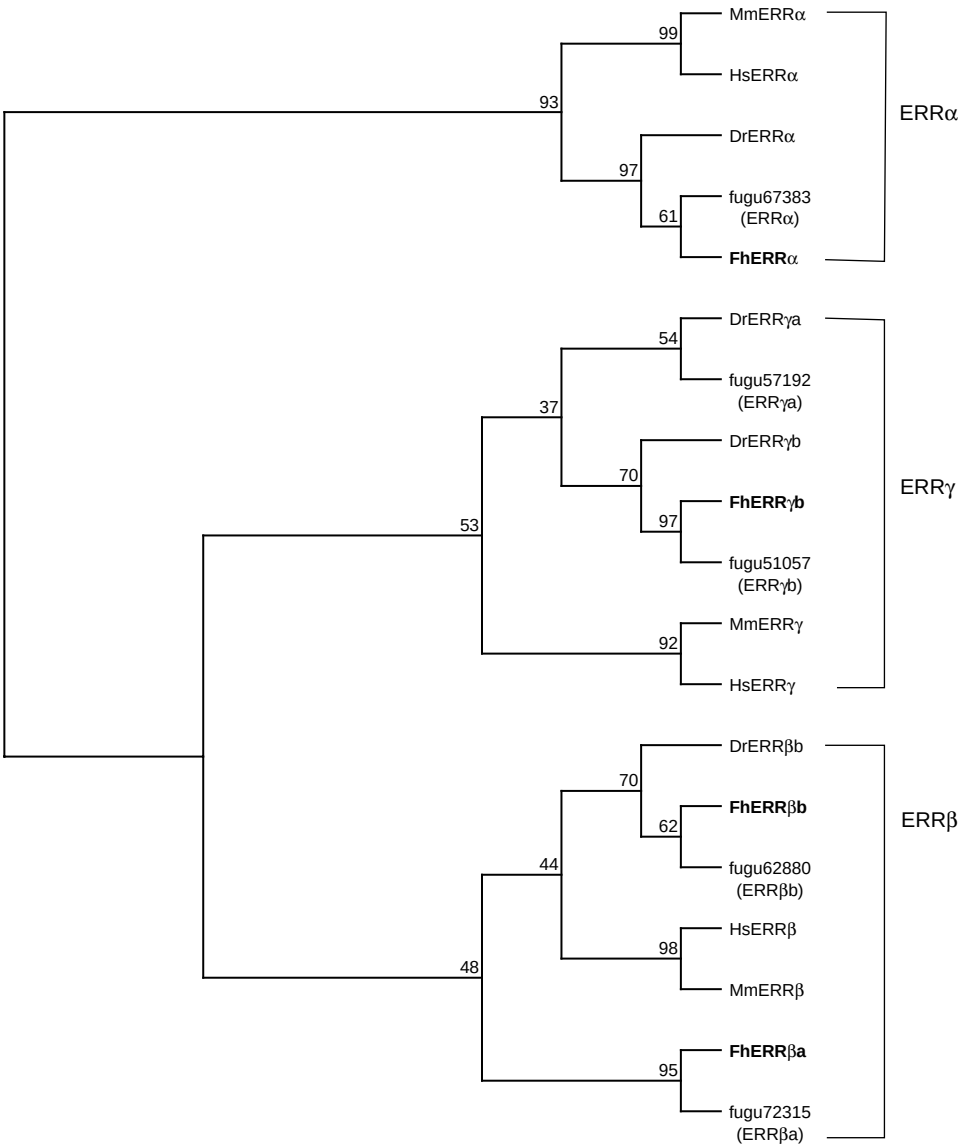


**Figure 3** Maximum likelihood analysis of ERRα-, ERRβ-, and ERRγ-predicted proteins. Deduced amino acid sequences of *F. heteroclitus* ERR genes (bold) were aligned with ERRs from fugu, zebrafish, human, and mouse using ClustalX 1.81. The A/B domains and gaps were excluded from analysis, and maximum likelihood criterion was used to produce a consensus tree that was rooted with the ERRα sequences. Bootstrapping values from 1000 replicates are shown. GenBank accession numbers are given in Table 2.

analysis demonstrated that to detect a threefold induction in FhERRα expression in heart ($\alpha=0{\cdot}05$, $1-\beta=0{\cdot}80$), a sample size of 12 fish per treatment would have been needed. In the experiment conducted in this study ($n=3$), a 15-fold induction would have been detected with a power of 0·874 ($\alpha=0{\cdot}05$). Thus, the high inter-individual variability of ERR expression precludes the detection of modest differences in expression. We also measured FhERRα and β-actin expression in fish 5 days after exposure to $E_2$ or a vehicle control. We detected no effect of $E_2$ exposure on FhERRα expression in these fish (data not shown). In a related study using qPCR with the same tissues, we have detected a twofold induction of cytochrome P450 aromatase B (AroB) expression in brain and greater than 100-fold induction of vitellogenin in the liver (SR Greytak, AM Tarrant, ME Hahn & GV Gallard, unpublished observations). Induction of AroB and vitellogenin by estradiol demonstrates that the fish were effectively exposed and normally responsive to estradiol.

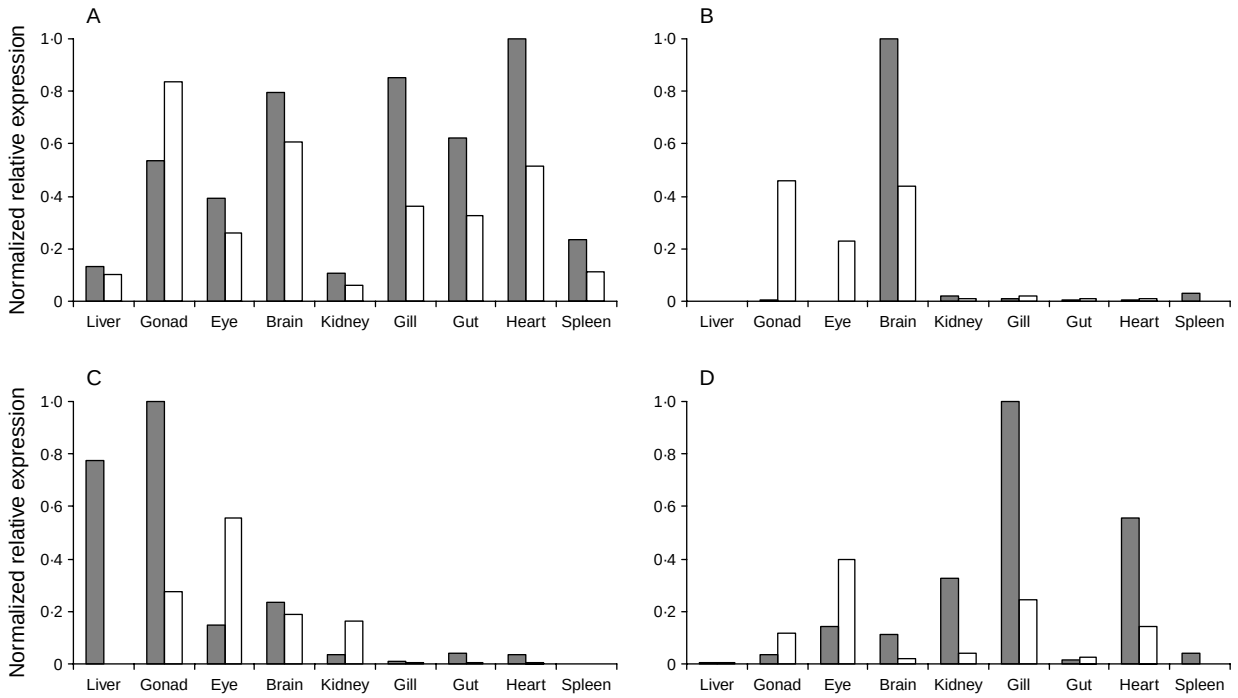


**Figure 4** Tissue-specific expression of *F. heteroclitus* ERRs. Relative expression of four *F. heteroclitus* ERRs ((A) ERRα, (B) ERRβa, (C) ERRβb, (D) ERRγb) was measured in cDNAs from adult male (shaded bars) and female fish (open bars) by qPCR, as described in Materials and methods. Expression of each ERR gene was normalized to EF-1. Relative mRNA expression for each tissue is represented as the fold change relative to the expression in the highest-expressing tissue (thus setting maximum relative expression equal to one). For each sex, relative expression was calculated from the mean of three technical replicates from a single cDNA pool derived from three fish.

In a second more targeted experiment, ERRα transcript expression was quantified in the hearts from female fish injected with estradiol or a vehicle control (Fig. 6). Two days after exposure, expression of ERRα was downregulated 2·5-fold in heart relative to the control ($P=0·001$). Liver from a subset of these fish showed a greater than 100-fold induction of vitellogenin (data not shown), demonstrating the effectiveness of the estrogen exposure.

## Discussion

### ERR diversity in *F. heteroclitus*

Examination of genomic databases and cloning efforts have demonstrated that teleost fish have additional diversity of ERR genes relative to mammals (Maglich *et al.* 2003, Bardet *et al.* 2004, Bertrand *et al.* 2004, this study). Increased diversity of nuclear receptors and other genes has been attributed to frequent gene duplication or to a genome duplication within the teleost lineage (Robinson-Rechavi *et al.* 2001*a*,*b*, Taylor *et al.* 2003). In zebrafish, the five ERR genes are each on separate chromosomes, as predicted from version 4 of the genome assembly. (In version 5 of the assembly, the zebrafish ERRβ is on a scaffold that has not been mapped to a chromosome.) In tetraodon, two genes (GSTENG00030324001, an ortholog of FhERRβa, and GSTENG00030242001, an ortholog of FhERRγb) are both on chromosome 14, but they are separated by approximately 800 kb. Thus, the additional ERR diversity observed in teleosts cannot be explained by recent tandem duplication events.

In the present study, we have identified four ERR genes from *F. heteroclitus*. These genes are predicted to be orthologs of four of the six ERR genes predicted from the fugu genome. Without a fully sequenced genome, it is not possible to know whether we have identified the full complement of ERR genes in *F. heteroclitus*. In our cloning efforts, we did not identify orthologs of ERRγa or ERRδ genes, which are present in pufferfish genomes and expressed in zebrafish embryos (Bardet *et al.* 2004, Bertrand *et al.* 2004). These genes may have been lost from *F. heteroclitus* or may have been difficult to detect, possibly due to the low expression in the adult tissues examined. Among the four killifish ERRs we identified, FhERRα is an ortholog of the single ERRα identified in mammals, pufferfish, and zebrafish. FhERRβa and

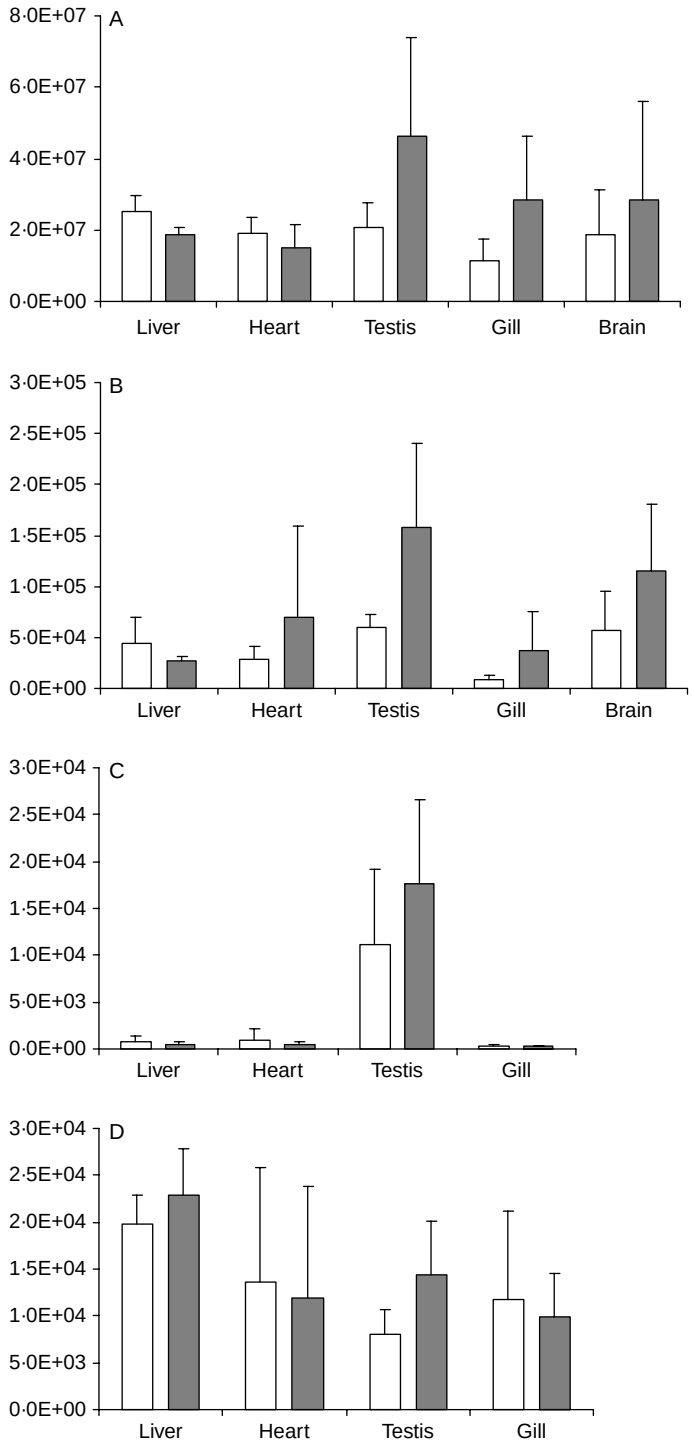


**Figure 5** Effects of estradiol treatment on ERR expression in *F. heteroclitus* males. Adult male *F. heteroclitus* were injected intra-peritoneally with a vehicle control (open bars) or with 5 μg/g body weight $E_2$ (shaded bars) and sacrificed after 2 days. Tissue-specific expression of: β-actin (A), ERRα (B), ERRβb (C), and ERRγb (D) was measured by qPCR. Error bars represent S.D. ($n=3$ fish). No significant effects ($\alpha=0{\cdot}05$) of $E_2$ treatment were detected (see the text for further details).

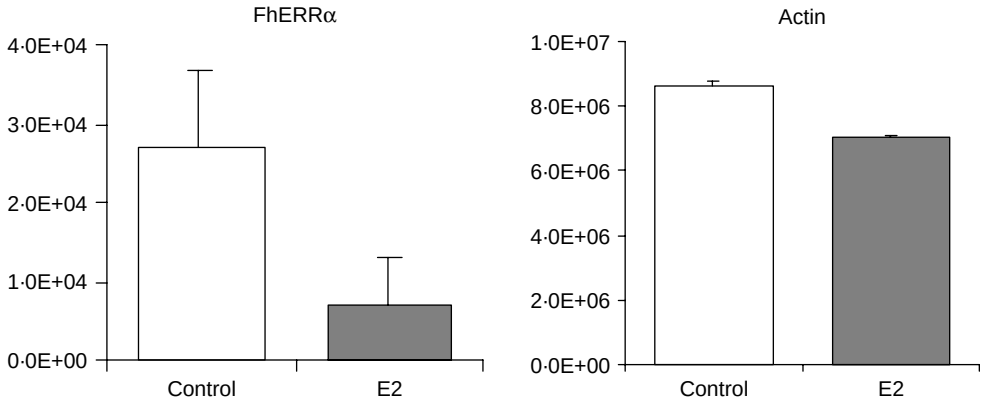


**Figure 6** Effects of estradiol treatment on ERRα expression in *F. heteroclitus* female heart. Adult female *F. heteroclitus* were injected intra-peritoneally with a vehicle control (open bars) or with 5 μg/g body weight $E_2$ (shaded bars) and sacrificed after 2 days. Expression of ERRα and β-actin was measured by qPCR. Error bars represent S.D. ($n=12$ estradiol treatment, $n=13$ vehicle control). ERRα expression was 2·5-fold lower in estradiol-treated fish relative to fish injected with a vehicle control ($P=0·001$; see the text for further details).

FhERRβb are co-orthologs of the mammalian ERRβ, as indicated by parsimony and distance analysis. Similarly, duplicated ERRβ genes have been identified in the fugu, tetraodon, and medaka genomes (Bertrand *et al.* 2004, AM Tarrant, unpublished data). In contrast, only a single ERRβ, similar to FhERRβb, is present in the zebrafish (Bardet *et al.* 2004); however, the tree topology is consistent with the loss of an ERRβa-like gene from the zebrafish lineage.

The phylogenetic placement of FhERRγb is less clear. Fugu and zebrafish each have one gene that groups clearly as an ERRγ and a second gene that groups as an ERRγ or within a sister group to the ERRβ and ERRγ clades, depending on the analysis. Our likelihood analysis and a previously published likelihood analysis (Bertrand *et al.* 2004) are consistent with the hypothesis that there has been a duplication of ERRγ within the teleost lineage. However, bootstrap support for the grouping of two fish ERRγ genes is low in both analyses. We have tentatively identified these groups of fish genes as ERRγa and ERRγb. FhERRγb is an apparent ortholog of this second group of fish genes.

Translation of the FhERRβb cDNA sequence revealed three methionine residues near the N-terminus of the predicted protein, and it is not clear which of these residues represents the translation initiation site(s). The third methionine aligns with the predicted start site of most other ERRs, including HsERRβ and FhERRβa, and this methionine has an adenine at the –3 position, the most conserved position in the Kozak consensus sequence (Kozak 1987). We did not detect multiple potential start codons of FhERRβa, and similarly did not detect multiple potential start codons in genomic sequences corresponding to other fish ERRβ genes. The predicted ERRβ sequence for the chimpanzee (GenBank accession number XP510082) similarly has an additional methionine upstream of the predicted start site for most ERRβ genes, and the sequence near the N terminus is highly similar to FhERRβa. It is possible that multiple initiation sites are used for some ERRβ genes. Indeed, apparent isoforms of HsERRγ that use different initiation sites have been described based on cDNA sequences obtained from different tissues (Heard *et al.* 2000). Further, application of several specific antibodies revealed that mammalian glucocorticoid-receptor mRNAs produce multiple functional isoforms that differ primarily in the N-termini and have distinct expression patterns and functional properties (Lu & Cidlowski 2005). The biological significance of multiple predicted start sites within the FhERRβb sequence is currently unknown.

### Tissue-specific expression

This study contains the first description of spatial patterns of ERR-transcript expression in adult fish. Like its mammalian ortholog, FhERRα was broadly expressed and detectable in all tissues. FhERRβa was apparently expressed at low levels in the eye, brain, and ovary. FhERRβb was detected primarily in the liver, gonad, eye, brain, and kidney, and FhERRγb in the eye, kidney, gill, and heart. While expression patterns were similar between males and females, some differences are apparent, such as expression of ERRβa in female eye and ovary. In the initial tissue comparison, the tissues from male and female fish are each represented by a single cDNA, and therefore, a more detailed analysis with multiple independent samples throughout the reproductive cycle will be needed to robustly compare expression patterns.

The ERR expression patterns indicate the utility of *F. heteroclitus* and other fish models for the characterization of ERR function in future. For example, FhERRβa and FhERRβb have distinct spatial expression patterns, which may indicate subfunctionalization of the co-orthologs. Thus, *F. heteroclitus* may serve as a particularly useful model for dissecting the ERRβ function. Mammalian ERRβ helps regulate placental development and primordial germ cell proliferation. Expression of FhERRβa in ovary is particularly interesting in this respect and is consistent with some role of ERRβ in killifish reproduction. The role of ERRβ in teleost development is unknown and would also be interesting to study, particularly given the differences in extraembryonic tissues between fishes and mammals. To give a second example, ERRγ function remains poorly characterized in any organism, and mouse knockout phenotypes have not been described. The additional diversity of ERRγ-like genes in teleosts, such as zebrafish, may facilitate characterization of function through knockdown experiments. In addition, FhERRγb expression in gill might indicate unique function relative to mammalian ERRs.

### Effects of estradiol dosage on ERR expression

In the first experiment, we detected no significant effects of exposure to $E_2$ (5 μg/g body weight) on ERR gene expression in any tissue. We did observe substantial variability among individual fishes and a trend towards a slight induction (three- to five-fold) of ERRα expression in heart. In a more targeted experiment with female fish, we observed a highly significant 2·5-fold downregulation of ERRα expression in heart following estrogen exposure. This downregulation contrasts with reports of ERRα induction by estrogens in some mammalian tissues (Shigeta *et al.* 1997, Liu *et al.* 2003).

The effect of estrogen exposure on mammalian ERRα expression is primarily mediated through multiple steroid hormone-response element half-sites that are conserved between the human and mouse ERRα gene promoters (Liu *et al.* 2003). While fish ERR gene promoters have not yet been characterized fully, in preliminary searches of teleost genomic databases, we have not identified any of the predicted ER response elements upstream of fish ERR genes (data not shown). Downregulation of ERRα in female heart following exposure to estradiol may indicate an important difference in regulation of expression between teleosts and mammals. This difference warrants further investigation and may provide an opportunity to identify estrogen-independent pathways of ERR expression. For example, ERRα expression in some mouse tissues displays circadian rhythmicity (Horard *et al.* 2004).

In conclusion, we have identified four ERR genes in *F. heteroclitus*. Phylogenetic analysis of our sequences and other teleost ERRs indicates that fishes possess additional diversity of ERRs relative to mammals and specifically that *F. heteroclitus* contains co-orthologs of ERRβ. Further, characterization of the duplicated genes may provide insight into conserved or teleost-specific functions of ERRβ. Downregulation of ERRα in the female heart by estradiol in our study also suggests that ERR expression is regulated differently in fishes and mammals.

## Acknowledgements

We thank Ann Michelle Morrison for *F. heteroclitus* RNA samples used to measure tissue-specific expression. Sibel Karchner, Diana Franks, and Brad Evans assisted with the development of qPCR assays. Jed Goldstone, Rob Jennings, and Ken Halanych provided advice on maximum likelihood analysis.

## Funding

This research was supported by the National Institutes of Health under Ruth L Kirschstein National Research Service Award (F32 ES013092-01) from the National Institute of Environmental Health Sciences, the Seward Johnson Foundation and the Superfund Basic Research Program (P42ES007381). The authors declare that there is no conflict of interest that would prejudice the impartiality of this work.

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Received in final form 1 May 2006
Accepted 8 May 2006
Made available online as an Accepted Preprint 9 May 2006