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Estrogen receptor mRNA expression patterns in the liver and ovary of female rainbow trout over a complete reproductive cycle

James J. Nagler^{1,2}, Timothy D. Cavileer^{1,2}, Joseph S. Verducci³, Irvin R. Schultz⁴, Sharon E. Hook⁵, and William L. Hayton⁶

¹Department of Biological Sciences, University of Idaho, Moscow, ID, USA

²Center for Reproductive Biology, University of Idaho, Moscow, ID, USA

³Department of Statistics, The Ohio State University, Columbus OH, USA

⁴Battelle PNNL-Marine Science Laboratory, 1529 West Sequim Bay Road, Sequim, WA, USA

⁵CSIRO Centre for Environmental Contaminants Research, Kirrawee, NSW, Australia

⁶Division of Pharmaceutics, The Ohio State University, Columbus OH, USA

Abstract

Estrogens are critical hormones involved in reproduction and need to bind to estrogen receptors in target organs for biological activity. Fishes have two distinct estrogen receptor subtypes, alpha (α) and beta (β), with variable combinations of additional isoforms of each subtype dependent on the history of genome duplication within a taxon. The comparative expression patterns of estrogen receptor isoforms during the female reproductive cycle will provide important insights into the unique function and importance of each. The purpose of this study was to measure the mRNAs for the four estrogen receptor isoforms (*era1*, *era2*, *er β 1*, *er β 2*) in the liver and ovary of adult, female rainbow trout over the course of an annual reproductive cycle. The expression of estrogen receptor mRNA isoforms was measured by quantitative real-time RT-PCR. Several reproductive indices (gonadosomatic index, maximum oocyte diameter, plasma estradiol-17 β , plasma vitellogenin, and ovulation) were also quantified for comparison and used in a correlation analysis to examine any inter-relationships. Of the four isoforms, the expression of *era1* was highest in the liver, and had a significant positive correlation with liver *er β 1* expression. Liver expression of *era2* mRNA was the lowest, but showed a significant positive correlation with maximum oocyte diameter in the ovary. The pattern of the *er β* isoforms in liver was one of initially elevated mRNA expression followed by a gradual decrease as reproductive development proceeded. In the ovary the *er β 1* isoform had the highest mRNA expression of all estrogen receptor isoforms, at the beginning of the reproductive cycle, but then decreased afterward. Both ovarian *er β* isoforms had a significant positive correlation with one another. In contrast, *era2* mRNA expression showed a high maximum level in the ovary near the end of the cycle along with a significant positive correlation with plasma estradiol-17 β levels; the highest gonadosomatic indices, maximum oocyte diameter, and vitellogenin levels occurred then too.

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Corresponding Author: James J. Nagler, Department of Biological Sciences and Center for Reproductive Biology, P.O. Box 443051, University of Idaho, Moscow, ID, 83844, USA, Tel: 208-885-4382; Fax: 208-885-7905; jamesn@uidaho.edu.

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Keywords

isoform; estradiol-17 β ; vitellogenin; fish; oocyte

1.0 Introduction

Estrogens are responsible for the liver production of vitellogenins and zona radiata proteins, estrogen feedback at the level of the brain and pituitary, inhibition of oocyte maturation, and reproductive behavior in female fishes [20,31]. The signaling mechanism for estrogens involves binding to estrogen receptors either within the cell (intracellular) or on the cell plasma membrane [6,18,26,39]. Estrogen receptors have been shown to be widespread in the body of several fishes [25,29,38], but most of the attention has been on the liver due to the importance of estrogen for vitellogenesis [28,30,36]. Fewer studies have examined the fish gonad [1,5,41] and most of this effort has been on the ovary [25,27,39] to understand the role of estrogen within the tissues that synthesize it [12]. The presence and quantity of estrogen receptors in target organs such as the liver and ovary is important to interpret the biological role(s) of estrogens in fishes.

Estrogen receptor genes have undergone duplication over the course of vertebrate evolution, such that in mammals there are two genes (subtypes) termed estrogen receptor alpha (α) and estrogen receptor beta (β). Due to an additional genome duplication event in fishes [21] many studies have reported additional isoforms of either *era* or *er β* [7,13,14,22,25,36,43]. The rainbow trout (*Oncorhynchus mykiss*) has four isoforms (i.e., *era1*, *era2*, *er β 1*, *er β 2*; [25]) which are thought to comprise the complete estrogen receptor family for this species. In juvenile rainbow trout the presence of estrogen receptor isoform mRNA expression is found throughout the body, with all four isoforms detectable in the gonads and liver [25]. Comparable studies of mRNA expression in other fishes have shown similar results and expression patterns [36,38,43].

Of the four estrogen isoforms reported in fishes there is evidence that *era1* and *er β 2* are primarily involved with vitellogenin synthesis in the liver of the female [21,28,36,43]. The biological functions of the other estrogen receptor isoforms in fish have not been established. The ovarian expression of *era1* mRNA has been quantified previously in female rainbow trout sexually maturing for the first time and demonstrated high levels of expression coincident with large ovarian follicles toward the end of the reproductive cycle [27]. This is in contrast to the medaka where *era1* mRNA was higher in early-mid stages of ovarian development and lowest in pre-spawning fish [5]. The function of estrogen receptors in the ovary remain to be elucidated.

The goal of this study was to measure the expression pattern of the four estrogen receptor mRNA isoforms in the liver and ovary of adult, female rainbow trout over a complete annual cycle and compare it with established reproductive indices to determine the potential scope for individual isoform functions.

2.0 Materials and Methods

2.1 Study design and sample collection

This study used female rainbow trout (*Oncorhynchus mykiss*) an iteroparous, egg laying fish with an annual reproductive cycle. Seventy, two-year old, adult females from an autumn-spawning population [37] were obtained from a commercial rainbow trout supplier (Troutlodge, Inc., Sumner, WA) and transported to the Battelle Marine Sciences Facility (Sequim, WA) the day after they had spawned. All fish were spawned on the same day and

all were therefore synchronized reproductively entering their second, consecutive cycle. The fish were placed in a 1200 liter fiberglass tank with flow-through freshwater (12° C) and maintained under lighting to simulate the natural photoperiod regime at Sequim (48.079 N). Three fish were removed approximately every four weeks over the course of one year, anesthetized in 75 mg tricaine methanesulfonate/L, and killed. Blood was sampled by syringe from caudal vessels, and the body and ovary masses were recorded to the nearest 0.0g. The plasma was separated from the blood by centrifugation and frozen at -80° C. Small portions of the liver and ovary were excised and immediately placed in RNAlater™ solution (Ambion, Austin, TX), gradually chilled to 4° C, and eventually frozen at -80° C for storage. Other small pieces sampled from the middle of the ovary were fixed in Bouin's fluid and eventually stored in 70% ethanol for histology.

2.2 Reproductive Indices

The following reproductive indices were used to gauge the annual progression toward sexual maturation. Ovulation was assessed on all remaining fish (n=20) at the last two sampling times approaching the end of the reproductive cycle. These fish were anesthetized and checked for evidence of ovulation by applying gentle pressure to the abdomen and looking for the extrusion of eggs from the genital opening. The gonadosomatic index was calculated from fish sampled monthly using the formula (ovary mass/body mass-ovary mass) × 100. The mean maximum oocyte diameter was calculated based on the ten largest ovarian follicles observed on randomly selected areas (n=3) from a histological section of ovary from all fish sampled monthly. In addition, the histology of the ovarian follicles was assessed according to the scheme proposed by van den Hurk and Peute [40]. Fixed ovarian tissue pieces were dehydrated through a graded series of ethanol, cleared in 100% xylene, and embedded in paraffin. Tissue sections were cut in cross-section at 5µm thickness, stained for histological analysis with hematoxylin and eosin, and ovarian follicles selected for measurement that were cut through the middle of the nucleus [4]. Plasma samples were analyzed by radioimmunoassay for estradiol-17β using a commercially available kit (Coat-A-Count Estradiol, Diagnostic Products, Los Angeles, CA) at the Center for Reproductive Biology Assay Core Laboratory (Department of Animal Sciences, Washington State University, Pullman, WA). Plasma samples were analyzed for vitellogenin using a rainbow trout vitellogenin ELISA kit (Biosense, Cayman Chemical, Ann Arbor, MI). Statistical analysis for gonadosomatic index, oocyte diameter, estradiol-17β, and vitellogenin utilized an analysis of variance (p<0.05) followed by a Dunnet's Test for multiple comparisons.

2.3 Quantitative Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), DNase treated with Ambion Turbo DNase-free (Invitrogen), and quantified using the Quant-iT RiboGreen RNA Reagent kit (Invitrogen). Ribosomal RNA standards (*E. coli* 16S and 23S rRNA) included in the Quant-iT RiboGreen RNA Reagent kit was used to develop a standard curve. The standard curve samples and total RNA unknowns were measured on a fluorometer (Turner BioSystems Modulus luminometer, Model 9200-003). Four hundred nanograms of total RNA was reverse transcribed using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). The resulting cDNA was diluted and stored at -20° C until used in quantitative reverse transcription-polymerase chain reactions (QRT-PCRs).

The mRNAs for the four estrogen receptor isoforms (*era1*, *era2*, *erβ1*, *erβ2*) were quantified using primers and protocols for QRT-PCR originally described in Nagler *et al.* [25]. Enhanced green fluorescent protein (eGFP) cRNA generated by *in vitro* transcription was used as an internal standard to account for amplification differences between samples because an endogenous gene could not be found that consistently amplified in samples of both organs. Generation of the eGFP plasmid and application of the *in vitro* transcription

protocol were as described in Gilsbach et al. [10] and Nagler *et al.* [25]. Messenger RNA levels for all target genes and the eGFP internal standard were determined using absolute quantities calculated from standard curves [25]. Each estrogen receptor isoform was measured in three independent QRT-PCRs (i.e., three technical replicates) and an average of these values was used to represent the level of that isoform for each individual. Statistical analysis utilized an analysis of variance ($p < 0.05$) followed by a Tukey's Test for multiple comparisons.

2.4 Correlation Analysis

Correlation tests were conducted in *R* [34] using Pearson's rho [8] and Kendall's tau [15] correlations as well as a modification of the tau-path test [42] to detect association over restricted ranges. The tau-path test was modified to accommodate multiple independent samples at each time point by constructing all possible ($3 \times 3 = 9$) pairings of observations between each pair of time points.

3.0 Results

3.1 Reproductive Indices

The autumn-spawning population of female rainbow trout used in this study completed a second, consecutive reproductive cycle by ovulating in early November almost 1 year after they were spawned for the first time. Mean maximum oocyte diameter first showed a significant increase in May, and gradually increased after May to reach a maximum of almost 6 mm in late October shortly before ovulation occurred in early November (Figure 1A). The histology of the largest ovarian follicles present in the ovaries would be classed as vitellogenic from shortly after spawning until October (of the following year), after which they were mature [40]. There was considerable heterogeneity in size and histological appearance (a mixture of small pre-vitellogenic and larger vitellogenic ovarian follicles) early in the cycle (November to February) but afterward much more consistency (uniformly vitellogenic) until all ovarian follicles were mature in October. Similar to mean maximum oocyte diameter, the gonadosomatic index began to increase significantly in June and continued steadily to a maximum in late October (Figure 1B). The plasma estradiol-17 β levels were at their lowest levels at the beginning of the cycle (November) and remained low until May when they increased dramatically to peak levels in September after which they began to decline. Plasma vitellogenin levels were high after spawning in November and then significantly decreased to their lowest levels by February, before beginning a gradual increase to high levels again by the end of the cycle (October; Figure 1D).

3.2 Liver Estrogen Receptor Isoform Expression

The pattern of liver expression was quite different both between *era* and *er β* subtypes and amongst subtype isoforms (Figure 2). Of the four estrogen receptor isoforms in the liver *era1* had the highest absolute mRNA quantities, while *era2* had the lowest. The pattern of the *era1* isoform started with a low level at the beginning of the cycle, which remained fairly static until August, when a significant increase to a peak level in September occurred before falling back toward the baseline at the end of the cycle (Figure 2). The *era2* isoform was low and essentially unchanging throughout the cycle (Figure 2). The absolute quantity of *era1* was always ~ten-fold higher than *era2*. With respect to the *er β* subtype, the *er β 2* isoform was more dynamic than *er β 1* (Figure 2). Although the *er β 1* isoform showed a significant peak in expression in February it was otherwise relatively unchanging. In contrast, the *er β 2* isoform showed a sharp decrease in February before a rebound, followed by a gradual decline toward the end of the cycle. In general, the absolute quantity of *er β 2* was always higher than *er β 1* by ~two-fold.

3.3 Ovary Estrogen Receptor Isoform Expression

The patterns of ovary expression for each estrogen receptor isoform were all uniquely different from each other over the reproductive cycle (Figure 3). The *era1* isoform expression in the ovaries was low and relatively unchanging (Figure 3). The *era2* isoform had two significant peaks of mRNA expression the first in August and then again in September, with the last one the greatest in amplitude (Figure 3). The pattern of both the *erβ* isoforms began with the highest level of expression at the outset of the reproductive cycle, with *erβ1* much higher than *erβ2*, and then both gradually decreased until May (Figure 3). At this point in the cycle both *erβ* isoforms showed an oscillating pattern of expression that continued until cycle completion. The amplitude of these significant increases and decreases was more obvious for *erβ1* than *erβ2*.

3.4 Correlation Analysis

Each endpoint (estrogen receptors and reproductive index variables) was first summarized by its mean value over all fish sampled at each time point, and then tested using both the Pearson and the more robust Kendall correlations between endpoints, as well as a modification of the tau-path test to detect association over restricted ranges. This procedure identified five strongly positive correlations and four weakly negative correlations (Table 1; Supplementary Data). The only positive correlation that did not involve an estrogen receptor occurred between maximum oocyte diameter and the gonadosomatic index (which was not unexpected). The remaining positive correlations resulted between ovary *era2* and plasma E2, maximum oocyte diameter and liver *era2*, liver *era1* and liver *erβ1*, and between the two ovary *erβ* isoforms. The weakly negative correlations occurred between plasma vitellogenin and liver *era1*, maximum oocyte diameter and both liver *erβ2*/ovary *erβ2*, and liver *era2* and liver *erβ2*. These correlations are depicted in Figure 4, highlighting those pairs that were individually significant, both within and between the liver and ovary.

4.0 Discussion

The expression of estrogen receptor mRNAs in the liver and ovary of female rainbow trout in this study represents the first time all estrogen receptor isoforms have been measured in a seasonally spawning female fish over the complete reproductive cycle. Estrogen receptors are necessary to transduce the estrogen signal in target organs and therefore the magnitude and pattern of their mRNA expression provides insight into potential function and regulation. In general, *era* mRNAs exhibited higher absolute transcript levels compared to the *erβ*s, with the exception of *erβ1* in the ovary early in the reproductive cycle. Of the *eras*, *era1* expression was more prominent in the liver than *era2* expression, but this was reversed in the ovary. The high levels of ovarian *era2* expression during the last 25% of the cycle suggest the potential for a significant role of this estrogen receptor in the latter phase of development in the rainbow trout ovary.

The only estrogen receptor isoform that has been linked to a biological function in fishes is *era1* and vitellogenin synthesis in the liver. This has been most convincingly established by gene knockdown experiments in the goldfish [28]. Other studies show correlative associations of *era1* with vitellogenesis [5,7,30,36], which were mostly corroborated by the results of this study on rainbow trout. On one hand, the pattern of *era1* liver expression in this study was distinctly different and quantitatively expression was higher than the other three isoforms throughout the reproductive cycle. This is presumably because, of the four estrogen receptor isoforms in the liver, *era1* is the only one regulated by estradiol-17β. Several studies have shown that estradiol-17β increases the transcription of *era1* mRNA via estrogen response elements in the gene promoter region [2,9,10] and contributes significantly to *era1* mRNA stability and prevents degradation of the transcript [3]. Given

this degree of isoform-specific estradiol-17 β regulation it can be reasonably concluded that estrogen signaling from the ovary coordinates the transcription of *era 1* in the liver where it is needed for the control of genes important for reproduction (e.g., vitellogenin, zona radiata genes; [20]). However, there was a weak, negative correlation between liver *era 1* expression and plasma vitellogenin concentrations. This relationship is likely significantly influenced by the drop in plasma vitellogenin for several months after spawning at a time when liver *era 1* expression is beginning to increase due to estradiol-17 β stimulation.

Previously, *era 2* mRNA was demonstrated in juvenile (immature) rainbow trout ovaries to have the lowest absolute mRNA expression of the four isoforms measured [25]. At the beginning of the reproductive cycle in the adult female *era 2* mRNA levels are low too, but a significant increase in ovarian expression is observed late in the cycle (unlike ovary *era 1*) that correlates positively with plasma estradiol-17 β levels. This implies that *era 2* could play one or more physiological roles associated with the final phase of ovarian growth occurring at this time. The timing of the peak in *era 2* expression is coincident with when plasma luteinizing hormone levels [33,37] are the highest in the blood of female rainbow trout, shortly before ovulation. A plausible scenario is that *era 2* is involved in the estrogenic induction of gonadotropin receptors in granulosa cells that make up the follicle surrounding the developing oocyte, as has been reported in mammals [16,35] and more recently a fish [19]. Another possibility is that *era 2*, coupled with high estradiol-17 β levels, is responsible for shutting down vitellogenin uptake through a reduction in the cycling of vitellogenin receptors to the oocyte plasma membrane that occurs at this time [32].

The pattern of the *er β* isoforms in both liver and ovary, in general, is one of initially high mRNA expression followed by a gradual decrease over the reproductive cycle. The pattern in the liver is not suggestive of a prominent role of *er β* s during the mid to late phases of the reproductive cycle, in which this organ is steadily increasing the production of vitellogenin, for example, and the ovary is undertaking considerable growth. However, during the early part of the cycle in February there is a significant increase in liver *er $\beta 1$* (and decrease in *er $\beta 2$*) when vitellogenin is at its lowest plasma concentration before increasing, and overall a significant positive correlation between liver expression of *er $\beta 1$* and *era 1* is apparent. It is possible that these changes in *er β* liver levels are biologically important at this time and this fits with evidence in the goldfish that *er β* s affect estrogen-induced *era* mRNA expression [28]. In addition, it has been reported that using an *er β* selective agonist (diarylpropionitrile) *in vitro*, rainbow trout hepatocytes were stimulated to produce vitellogenin [17]. In the ovary *er β* s were positively correlated with one another and highest at the beginning of the cycle. They could play a role at the initiation of a new reproductive cycle by stimulating oogonal proliferation, an effect ascribed to estrogen in the ovaries of Japanese huchen and common carp [24]. This explanation however, does not address the oscillating pattern of *er β* expression beginning after June and continuing to the end of the cycle. One caveat of the experimental design of this study is that each time point represents a “transcriptional snapshot” that could change from day to day and any assignment of biological relevance to *er β* s will require more detailed mRNA measurements over the latter phase of ovarian development.

5.0 Conclusion

The expression of estrogen receptor mRNAs in the liver and ovary of adult female rainbow trout, a seasonally spawning fish, has been measured over a complete reproductive cycle in conjunction with indices of reproductive development. The pattern of the liver expression of *era 1* fits with its known regulation by estradiol and involvement with vitellogenin synthesis. The *era 2* isoform showed a significant increase of expression during the final phase of ovarian growth and could be involved with estradiol-gonadotropin receptor regulation in the

ovary. The pattern of the *erβ* isoforms in both liver and ovary is one of higher expression at the beginning of the reproductive cycle, particularly for *erβ1*. These receptors may be important for estrogen signaling that initiates the next cycle of germ cell development in the ovary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- estrogen receptor gene expression quantified in female rainbow trout
- estrogen receptor alpha 1 was highest in the liver
- ovary estrogen receptor alpha 2 peaked toward the end of the reproductive cycle
- estrogen receptor beta levels were high at the onset of a new cycle then decreased

Figure 1.

Monthly reproductive index measurements from adult female rainbow trout over the course of a complete annual cycle. A. maximum oocyte diameter of the ten largest ovarian follicles on histological sections from the ovaries; B. gonadosomatic index; C. plasma estradiol-17 β concentrations; D. plasma vitellogenin concentrations (note: logarithmic y-axis scale). Each point represents a mean ($\pm$ standard deviation) for three individual fish. The asterisk (*) indicates a statistically significant ($p < 0.05$) difference from the first time point in November. The x-axis labels denote the first letter of each calendar month.

Figure 2.

Mean (± standard deviation) levels of estrogen receptor isoform mRNA expression in the liver of female rainbow trout (n=3) over a complete annual reproductive cycle. The asterisk (*) indicates a statistically significant (p<0.05) difference from the first time point in November. The x-axis labels as described in Figure 1.

Figure 3.

Mean (± standard deviation) levels of estrogen receptor isoform mRNA expression in the ovary of female rainbow trout (n=3) over a complete annual reproductive cycle. The asterisk (*) indicates a statistically significant (p<0.05) difference from the first time point in November. The x-axis labels as described in Figure 1.

Figure 4. Correlation connection diagram depicting the strongly positive correlations (solid lines; $0.00001 < p < 0.002$) and weakly negative correlations (dashed lines; $0.02 < p < 0.04$) between experimental variables (E₂- plasma estradiol-17β, VG- plasma vitellogenin, GSI- gonadosomatic index, MOD- maximum oocyte diameter, ERa1- *era* 1, ERa2- *era* 2, ERb1- *erβ* 1, ERb2- *erβ* 2,) and the two organs examined, ovary and liver, in the adult female rainbow trout.