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Quantification of rainbow trout (*Oncorhynchus mykiss*) zona radiata and vitellogenin mRNA levels using real-time PCR after in vivo treatment with estradiol-17β or α-zearalenol

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Abstract

Estrogen receptor-mediated induction of zona radiata (ZR) and vitellogenin (VTG) mRNA and protein in rainbow trout (*Oncorhynchus mykiss*) was compared to assess their utility as biomarkers for exposure to estrogenic compounds. Partial sequences of rainbow trout ZR and β-actin were cloned by reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate primers based on conserved regions across a number of species. A 549 bp fragment of the rainbow trout ZR-gene showed a high degree of amino acid sequence identity to that of salmon (77%), winter flounder (64%), carp ZP2 (63%) and medaka (61%) ZR-proteins. The 1020 bp β-actin fragment was approximately 100% identical to sequences from several species. Real-time PCR was used to quantify the induction of ZR-gene and VTG in rainbow trout liver after in vivo exposure to estradiol-17β (E_2) (0.01, 0.1, 1.0 or 10 mg/kg body weight (bw) fish) or α-zearalenol (α-ZEA) (0.1, 1.0 or 10 mg/kg bw). Real-time PCR and indirect enzyme-linked immunosorbent assay (ELISA) showed that ZR and VTG were induced in both the liver and the plasma after a single injection of E_2 or α-ZEA. ZR was more responsive to low levels of E_2 and α-ZEA than VTG, and real-time PCR was shown to be more sensitive than the ELISA. Rainbow trout ZR-gene and proteins provide a sensitive biomarker for assessing estrogenic activity. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Real-time PCR; Zona radiata; *Oncorhynchus mykiss*

1. Introduction

Teleostean eggs are enclosed by an extracellular envelope that protect the developing embryo from the environment. Commonly used terms to describe this eggshell structure are zona radiata (ZR), zona pellucida (ZP), chorion, choriogenin and the vitelline envelope. In mammals, the eggshell is denoted ZP and its major components are ZP1, ZP2, and ZP3. The eggshell in teleosts consists of minor outer ZP and a major inner ZR. ZP is composed of complex proteoglycans [1], while ZR in fish consists mainly of filamentous proteins [2]. In this paper we focused on the ZR, and we refer to the proteins and gene as ZR-proteins and ZR-gene, respectively.

The development of the eggshell (oogenesis) in teleost fish can be divided into two main parts: oogenetic growth and final maturation of the ovum. The growth phase consists of two different processes, zonagenesis, the synthesis of eggshell and vitellogenesis, the synthesis of yolk material (vitellogenin, (VTG)). Both proteins are physiologically induced by estrogens, synthesized in the liver and transported to the ovary during oogenesis in several fish species [3–7]. The ZR of a number of fish species consists of 2 to 4 protein monomers [4,5,8]. ZR-proteins in Atlantic salmon (*Salmo salar* L.), cod (*Cadus morhua*), and rainbow trout (*Oncorhynchus mykiss*) exist as 3 monomers known as α, β, and γ

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[4,9,10]. The ZR-gene from several fish species has been identified [11–17]. Among these, two different ZR-genes corresponding to the mammalian ZP2 and ZP3 genes have been identified in carp [14,15], and medaka [12,13].

Several naturally occurring and synthetic compounds are suspected to adversely affect human health and wildlife [18]. Recently, concern has increased regarding exposure to synthetic chemicals and natural products that mimic or alter the effect of physiological estrogen, E_2. Chemicals referred to as estrogenic endocrine disruptors (EEDs) include a wide range of compounds such as environmental chemicals, industrial products and natural compounds. The negative effects of estrogenic substances on human health were seen after the use of diethylstilbestrol (DES), a synthetic hormone used by women to avoid abortion in early pregnancy [19]. Studies have shown that several structurally diverse chemicals have the ability to compete with E_2 for the binding to the estrogen receptor (ER) [20], as well as alter the expression of a number of estrogen responsive genes [21–23]. It has been suggested that direct-acting estrogen mimics are responsible for adverse effects in wildlife including fish [24]. However, other mechanisms of action that are independent of the ER can not be excluded [25–27].

Zearalenone (ZEA) is a mycotoxin produced by several strains of the fungus *Fusarium*, and is a common contaminant of cereals and other plant product [28]. ZEA has previously been reported to reduce the number and quality of spermatozoa in carp [29] as well as causing reproductive failure in swine and cattle fed contaminated grains [30]. ZEA undergoes metabolic reduction to the more estrogenic zearalenols, and the estrogenicity greatly depends on the stereochemical (α, β) configuration of the hydroxyl group in the non-aromatic moiety of their structure. α-Zearalenol (α-ZEA) is 2–3 times more estrogenic that β-zearalenol (β-ZEA) based on molar comparison and binding to ER [20,22].

Because of the potential of estrogenic contaminants to cause adverse effects in wildlife and humans, a number of methods have been developed to screen for estrogenic effects both in vitro and in vivo [25,31]. Also, a number of biomarkers and end points have been suggested for use in monitoring the exposure of fish to estrogen agonists in the environment [31]. Increase in mRNA and protein levels of VTG are well known, and frequently used markers for screening of EEDs [23,32]. However it has recently been shown that the induction of ZR-proteins in fish was a useful and more sensitive marker for EEDs exposure than VTG [21,33,22]. In this study, a real-time PCR assay, also know as the TaqMan assay, was developed to compare the induction profiles of VTG and ZR mRNAs in rainbow trout following treatment with increasing concentrations of α-ZEA or E_2. For comparison, indirect ELISA using heterologous antiserum to salmon ZR-proteins and heterologous antibody to salmon VTG were used to analyze the levels of ZR-proteins and VTG in blood plasma from exposed fish.

2. Material and methods

2.1. Chemicals

The RNA later was purchased from Ambion (Austin, TX). Trizol and Superscript reverse transcriptase II were from GibcoBRL (Life Technologies, MD, USA). Monoclonal antibodies to salmon VTG and polyclonal antiserum to salmon ZR-proteins were obtained from Biosense Laboratories (Bergen, Norway). Secondary antibodies, goat anti-rabbit IgG-HRP and anti-mouse IgG-HRP, were purchased from Bio-Rad (Richmond, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. E_2 and α-ZEA were from Sigma (St. Louis, MO). DNA *Taq* polymerase, T4 ligase and restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Optical PCR tubes and caps, and TaqMan Universal Master Mix were ordered from PE Applied Biosystems (Foster City CA, USA). All primers were made by Applied Biosystems (Macromolecular Facility, Michigan State University, East Lansing, MI, USA). Fluorescence labeled probes were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). All other reagents were of highest quality available from commercial sources.

2.2. Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) (30–50 g) were obtained from the Stoney Creek Fish Farm, (Grant, MI, USA). Fish were maintained in a 370 l tank with continuously running fresh water (4 l/min) at 16 ± 1°C, and a dark/light cycle of 8/16 h. Fish were allowed to acclimatize for 7 days prior to treatment.

2.3. Experimental design, dosing and sampling

A total of 80 rainbow trout were randomly assigned to each of eight treatment groups. All of the fish in a single treatment group were held together in a single tank. Fish received single intraperitoneal injections of corn oil (control), E_2 (0.01, 0.1, 1.0, and 10 mg/kg body weight (bw) fish, corresponding to 3.7×10^{-8}–3.7×10^{-5} mol/kg bw) or α-ZEA (0.1, 1.0, and 10 mg/kg bw, corresponding to 3.1×10^{-7}–3.1×10^{-5} mol/kg bw) on day 1 of the experiment. Fish were euthanized on day 10 of the experiment and liver and body weights were recorded. Blood samples were taken from the caudal vein using heparinized syringes, and the liver

stored in RNA Later at −80°C for later RNA isolation. Heparinized blood was centrifuged at 5000 rpm at 4°C for 10 min. The clear plasma was stored at −20°C until analysis.

2.4. RNA isolation

Liver samples (≈50 mg) were homogenized in the presence of Trizol reagent using a Brinkman Polytron homogenizer. Total RNA was purified according to the manufacturer's instructions (Life Technologies, MD, USA). This purification strategy is a modification of the method developed by Chomczynski and Sacchi [34].

2.5. Cloning of rainbow trout zona radiata and β-actin partial gene sequences

RT-PCR was preformed using an oligo dT primer. Total RNA (1 μg) and 100 ng oligo dT primer (Pr1r) were incubated for 10 min at 70°C followed by a 5 min incubation on ice. PCR buffer (20 mM), $MgCl_2$ (3.75 mM), dNTP (500 μM), dithiothreitol (DTT) (10 mM) and Superscript II reverse transcriptase (200 U) were added to bring the final reaction volume to 20 μl and then incubated at 42°C for 50 min. The reaction was terminated with a 15 min incubation at 70°C.

For the cloning of ZR-genes rapid amplification of cDNA 3′ ends (RACE) PCR was preformed using a uniform amplification primer (Pr2r) and two degenerate primers, rainbow trout ZR-gene forward (Pr3f) and rainbow trout ZR-gene reverse (Pr4r). The degenerate primers were designed from sequence alignment of available sequences from four different fish species, including Atlantic salmon (AJ000665), carp (Z72495, Z48974, L41636), winter flounder (U03674) and medaka (D89609, D38630). The first PCR reaction (PCR1) contained PCR buffer (20 mM), $MgCl_2$ (1 mM), dNTP (0.2 mM), Pr2r (100 ng), Pr3f (100 ng) and Taq DNA polymerase (2.5 U) in a total of 50 μl. Following the addition of template (10% of the RT-PCR reaction) the reaction mixtures were incubated at 95°C for 2 min and amplified for 35 cycles. Each cycle consisted of 45 s of denaturation at 95°C, 45 s annealing at 54°C and 1 min elongation at 72°C. Nested PCR was performed using 10% of the PCR1 reaction as a template and 100 ng of each degenerate primer (Pr3f and Pr4r). The reaction produced approximately a 700 bp fragment, which was digested with *Xho*I and *Eco*RI and cloned into a pSG5 based eukaryotic expression vector.

Cloning of β-actin was performed using a similar strategy. Two degenerate primers (Pr5f and Pr6r) designed from a conserved region obtained from sequence alignment of β-actin from human (X00351), mouse (number X03672), sheep (number U39357), horse (AF035774), chicken (L08165), sea bream (X89920), goose (M26111), salmon (AF012125), xenopus (AF079161) and zebrafish (AF057040). RACE PCR was performed using 10% of the RT-reaction and Pr5f and Pr6r, which resulted in a 1100 bp long fragment that was digested with *Bam*HI and *Xho*I restriction enzymes and cloned into a pSG5 vector. The ZR-gene and β-actin products were sequenced using ABI/Prism automated DNA sequencing (PE Applied Biosystem, Foster City, CA). The verified sequences were aligned to known sequences using Mac Vector 6.5 and the GCG Wisconsin Package (Oxford Molecular, Beaverton OR). All primers are listed in Table 1.

2.6. Real-time PCR primers and probes

Primers and probes for rainbow trout ZR-gene, VTG, and β-actin were selected using the ABS Primer Express program (PE Applied Biosystems, Foster City, CA, USA). This program was used to select probe and primer sets with optimized melting temperatures, secondary structure, base composition and amplicon lengths, for use in the real-time PCR product detection system and the ABI Prism model 7700 sequence detector. Fluorescent probes labeled with 6-carboxyfluorescein (FAM) in the 5′-end and 6-carboxy-tetramethylrhodamine (TAMRA) in the 3′-end were purchased from Integrated DNA Technologies (Coralville, Iowa).

To verify that each primer pair only hybridized to one part of the target sequence, RT-PCR reactions were performed under the conditions described above. The PCR products were then analyzed on a 10% polyacrylamide gel. When a single band was visualized on the gel the primers were considered specific. In addition, RNA gels of selected RNA samples was performed to verify the quality of the RNA preparation and to ensure that equal amount of RNA was used for the RT-PCR reaction. Prior to quantification the optimal concentrations of primers and probes for all targets were determined. Optimal primer concentrations were found by amplifying a known amount of each target sequence at increasing amounts of primers (50–1000 nM). The amounts of forward and reverse primers giving the greatest increase in fluorescence (ΔR) above base line were selected. Furthermore, optimal probe concentration was found by amplifying each target sequence at optimal primer concentrations and increasing amounts of probe (25–225 nM). Optimal probe concentration were found to be the amount of probe giving the lowest PCR cycle (C_T) were significant increase in flourescense above baseline was observed. See Table 2 for optimal primer and probe concentrations.

Table 1
PCR primers used in the cloning of rainbow trout zona radiata gene and β-actin and primers and probes used in the TaqMan assay

Primer	Description	Sequence
Pr1r[a]	Oligo dT	5′-GGCCACGCGTCGACTAGTACT$_{17}$-3′
Pr2r	Universal amplification primer	5′-CU[d]ACUACUACUAGGCCACGCGTCGACTAGTAC-3′
Pr3f[b,c]	Degenerate primer for ZR-gene (correspond to salmon ZR-gene cDNA from bp 409–431)	5′-CAAAGAATTCGGATCCGA[c]NGGACAGCAGTGCTTCTA/TC/T/GG-3′
Pr4r[d,e]	Degenerate primer for Zr-gene (correspond to salmon ZR-gene cDNA from bp 1082–1057)	5′-CAAA[e]AGATCTCTCGAGCACA[f]/CCA/GTCAA/TN[g]YAGA/TA/TNGTCCCACTG-3′
Pr5f	Degenerate primer for β-actin (correspond to human β-actin gene from bp 34–57	5′-CAAAGAGCTCGGATCCAAC/T/GGCTCCGGC/T/ATGTGCAA[h]RGCC-3′
Pr6r	Degenerate primer for β-actin (correspond to human β-actin gene from bp 1059–1083	5′-CAAAAAGCTTCTCGAGCTCCTGCTTGCTGATCCACATCTGC-3′
TaqMan	ZR-gene probe	5′[i]FAM-TGATGTGAAGCCGGTTCCTCCTCC-[j]TAMRA3′
TaqMan f	ZR-gene	5′-CAGTACCATTGTGGCTGTGGTT-3′
TaqMan r	ZR-gene	5′-GGCCCAGGAGCTATATCAGGAT-3′
TaqMan	Vitellogenin probe	5′FAM-CCTGCAAAATTTGCAGCACAGCTTGAC-TAMRA3′
TaqMan f	Vitellogenin	5′-GAGCTAAGGTCCGCACAATTG-3′
TaqMan r	Vitellogenin	5′-GGGAAACAGGGAAAGCTTCAA-3’
TaqMan	β-Actin probe	5′FAM-TCCGGTGACGGCGTGACCC-TAMRA3′
TaqMan f	β-Actin	5′-CCACCGGTATCATGGA-3′
TaqMan r	β-Actin	5′-CGTAGTCCTCGTAGATGGGTACTGT-3′

[a] r Denotes reverse.
[b] f Denotes forward.
[c] N denotes any of the four bases A (adenine), T (thymine), C (cytosine) and G (guanine).
[d] U denotes uracil.
[e] Restriction enzyme sites are underlined.
[f] / Denotes or.
[g] Y denotes C or T.
[h] R denotes A or G.
[i] 6-Carboxyfluorescein (FAM).
[j] 6-Carboxy-tetramethylrhodamine (TAMRA).

2.7. Quantification of PCR products using the real-time (RT)-PCR

Quantification of VTG and ZR-genes by the TaqMan assay was preformed using 5% of the RT-PCR reactions. An oligo dT primer was used in the RT-PCR reaction (as described in the cloning section), hence all targets are analyzed from one reaction. The level of β-actin in each RT-reaction was used to normalize for the variability in RNA quality, RNA quantity or differences in the efficiency of the RT-reaction, among samples. Results are reported as the ratio of ZR-genes/β-actin and VTG/β-actin. All samples were analyzed in duplicate.

Each reaction (30 μl) contained 15 μl of TaqMan Universal PCR Master Mix (consisting of AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTP with dUTP, passive reference, and optimized buffer components), and the optimized concentrations for each primer set and probe.

Quantities of mRNA in samples were quantified by comparison with a reference standard curve derived from known amounts of the target genes. The target sequences were sub-cloned into a pSG5 based vector, and the concentrations of the standards were calculated taking into consideration the combined size of the vector and the different target sequences. The standards ranged between 100 and 500 000 copies of target for all three genes. Amplification and detection of samples and standards were performed with the ABI 7700 system using the following thermal cycling conditions: 50°C for 2 min, 95°C for 10 min, 40 or 50 cycles (dependent on the target) at 95°C for 15 s, and 60°C for 1 min.

Table 2
Optimal primer and probe concentration for real-time PCR

Target	Description	Concentration (μM)
ZR	Probe	0.025
ZR	Forward primer	0.300
ZR	Reverse primer	0.300
VTG	Probe	0.025
VTG	Forward primer	1.000
VTG	Reverse primer	1.000
β-Actin	Probe	0.025
β-Actin	Forward primer	0.900
β-Actin	Reverse primer	0.900

```
  1 GATGCCACTCTGCCCAGCCTGGAACTGGACTCCATCAGCCTGCTGGGGACAAACGGAGCC
    D  A  T  L  P  S  L  E  L  D  S  I  S  L  L  G  T  N  G  A   20

 61 CACTGCCACCCTATTGGCACAACTTCTGTCTTTGCCATCTACCAGTTTAAAGTCACTGAA
    H  C  H  P  I  G  T  T  S  V  F  A  I  Y  Q  F  K  V  T  E   40

121 TGTGGAACTGTCATGACGGAGGAAACGGATACTATTATCTATGAGAATAGGATGTCCTCT
    C  G  T  V  M  T  E  E  T  D  T  I  I  Y  E  N  R  M  S  S   60

181 TCATATCAAGTGGGTGTTGGCCCCTTTGGCTCCATCACCAGGGACAGCCAATATGATCTA
    S  Y  Q  V  G  V  G  P  F  G  S  I  T  R  D  S  Q  Y  D  L   80

241 ACATTCCAGTGCAGATATAAGGGCAGTACCATTGTGGCTGTGGTTATTGATGTGAAGCCG
    T  F  Q  C  R  Y  K  G  S  T  I  V  A  V  V  I  D  V  K  P  100

301 GTTCCTCCTCCAAATCCTGATATAGCTCCTGGGCCCCTCATAGTTGAGCTCAGACTGGGC
    V  P  P  P  N  P  D  I  A  P  G  P  L  I  V  E  L  R  L  G  120

361 AGCGGAGGATGCCTTACCAAGGGATGTAATGAAGAGGAAGTGGCCTACACCTCTTACTAC
    S  G  G  C  L  T  K  G  C  N  E  E  E  V  A  Y  T  S  Y  Y  140

421 ACAGAGGCAGACTACCCTGTCACCAAGGTCCTCAGGGATCCTGTCTACACTGAGGTTCGC
    T  E  A  D  Y  P  V  T  K  V  L  R  D  P  V  Y  T  E  V  R  160

481 ATCCTGGCGAGGACAGATCCCAACATTGTGCTGACCCTGGGTCGCTGCTGGGCTACCACA
    I  L  A  R  T  D  P  N  I  V  L  T  L  G  R  C  W  A  T  T  180

541 AACCCAAAC
    N  P  N
```

Fig. 1. Partial nucleotide and protein sequence of rainbow trout ZR-gene. The numbers on the left indicate the nucleotide position, and the values on the right correspond to the amino acid residues (GeneBank Accession Number AF185274).

2.8. Indirect enzyme-linked immunosorbent assay (ELISA)

Concentrations of VTG and ZR-proteins in plasma were determined by ELISA according to Celius and Walther [33]. Samples of plasma were diluted 1:3000, anti-salmon VTG antibody were diluted 1:500, and anti-salmon ZR-protein antiserum were diluted 1:1000. Secondary antibodies, goat anti-rabbit IgG (H + L)-HRP and anti-mouse IgG-HRP were diluted 1:3000. ELISA absorbance was measured at 490 nm.

3. Results

3.1. Cloning of rainbow trout zona radiata and β-actin partial sequences

The partial nucleotide sequence of a rainbow trout ZR-gene and the corresponding protein sequence are shown in Fig. 1. Sequence alignment of a conserved region of the sequences of ZR-genes from four different fish species (salmon bp 409–1082, carp ZP2 bp 778–1448, carp ZP3 bp 394–1075, medaka bp 811–1478 and winter flounder bp 640–1310), were used to design primers for cloning of ZR-gene from rainbow trout. A 549 bp fragment was cloned that corresponded to a 183 amino acid sequence. The ZR-protein amino acid sequence exhibited 77% sequence identity the Atlantic salmon ZR-protein (AJ000665), 64% identity to winter flounder ZR-protein (U03674), 63% identity to carp ZP-protein (Z72495), and 61% sequence identity to medaka ZR-protein (D89609) (Fig. 2).

Cloning of rainbow trout β-actin resulted in a 1020 bp fragment corresponding to a 340 amino acid sequence (Fig. 3) that exhibited approximately 100% sequence identity to the β-actin cDNAs from human (X00351), mouse (X03672), sheep (U39357), horse (AF035774), chicken (L08165), sea beam (X89920),

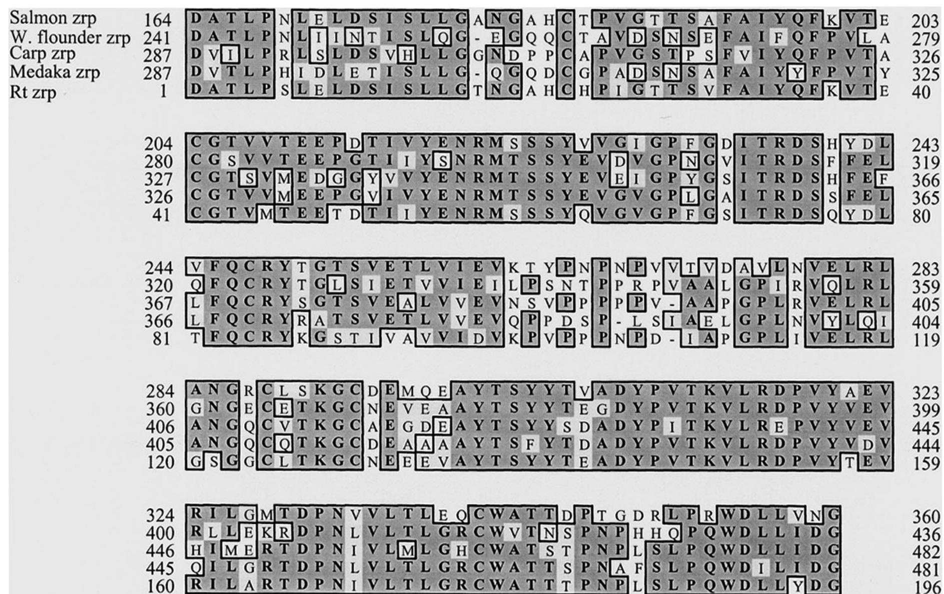

Fig. 2. Comparison of the partial amino acid sequence of known ZR-proteins with the cloned rainbow trout sequence. Identical residues are shaded in dark gray, similar amino acids in terms of residue type are lightly shaded and non-conservative changes are not shaded. The numbers flanking each sequence refer to the amino acid residues. The alignment was generated using the ClustalW alignment in MacVector 6.5 (Oxford Molecular).

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  1  GCCGGAGATGACGCGCCTCGGGCTGTCTTCCCCTCCATCGTCGGGCGTCCCAGGCATCAG
     A  G  D  D  A  P  R  A  V  F  P  S  I  V  G  R  P  R  H  Q  20

 61  GGAGTGATGGTTGGGATGGGCCAGAAAGACAGCTACGTGGGAGACGAGGCTCAGAGCAAG
     G  V  M  V  G  M  G  Q  K  D  S  Y  V  G  D  E  A  Q  S  K  40

121  AGGGGCATCCTGACCCTGAAGTACCCCATTGAGCATGGCATCGTCACCAACTGGGACGAC
     R  G  I  L  T  L  K  Y  P  I  E  H  G  I  V  T  N  W  D  D  60

181  ATGGAGAAGATCTGGCATCACACCTTCTACAACGAGCTGAGGGTGGCTCCAGAGGAGCAC
     M  E  K  I  W  H  H  T  F  Y  N  E  L  R  V  A  P  E  E  H  80

241  CCCGTCCTGCTCACAGAGGCCCCCCTCAACCCCAAAGCCAACAGGGAGAAGATGACCCAG
     P  V  L  L  T  E  A  P  L  N  P  K  A  N  R  E  K  M  T  Q  100

301  ATCATGTTTGAGACCTTCAACACCCCTGCCATGTACGCGGCCATCCAGGCCGTGTTGTCC
     I  M  F  E  T  F  N  T  P  A  M  Y  A  A  I  Q  A  V  L  S  120

361  CTGTACGCCTCTGGCCGTACCACCGGTATCGTCATGGACTCCGGTGACGGCGTGACCCAC
     L  Y  A  S  G  R  T  T  G  I  V  M  D  S  G  D  G  V  T  H  140

421  ACAGTACCCATCTACGAGGACTACGCTCTGCCCCACGCCATCCTGCGTCTGGATCTTGCC
     T  V  P  I  Y  E  D  Y  A  L  P  H  A  I  L  R  L  D  L  A  160

481  GGCCGCGACCTCACAGACTACCTGATGAAGATCCTGACGGAGCGCGGTTACAGCTTCACC
     G  R  D  L  T  D  Y  L  M  K  I  L  T  E  R  G  Y  S  F  T  180

541  ACCACGGCCGAGAGGGAAATCGTACGAGACATCAAGGAGAAGCTGTGCTACGTGGCGCTG
     T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  C  Y  V  A  L  200

601  GACTTTGAGCAGGAGATGGGCACCGCTGCCTCCTCTTCCTCTCTGGAGAAGAGCTACGAG
     D  F  E  Q  E  M  G  T  A  A  S  S  S  S  L  E  K  S  Y  E  220

661  CTGCCTGACGGACAGGTCATCACCATCGGCAACGAGAGGTTCCGCTGCCCAGAGGCCCTC
     L  P  D  G  Q  V  I  T  I  G  N  E  R  F  R  C  P  E  A  L  240

721  TTCCAGCCCTCCTTCCTCGGTATGGAGTCTTGCGGTATCCACGAGACCACCTACAACTCC
     F  Q  P  S  F  L  G  M  E  S  C  G  I  H  E  T  T  Y  N  S  260

781  ATCATGAAGTGTGACGTGGACATCCGTAAGGACCTGTACGCCAACACAGTGCTGTCCGGA
     I  M  K  C  D  V  D  I  R  K  D  L  Y  A  N  T  V  L  S  G  280

841  GGAACCACCATGTACCCCGGCATCGCTGACAGGATGCAGAAGGAGATCACCTCTCTGGCC
     G  T  T  M  Y  P  G  I  A  D  R  M  Q  K  E  I  T  S  L  A  300

901  CCCTCCACCATGAAGATCAAGATCATCGCCCCCCCAGAGCGTAAATACTCCGTCTGGATC
     P  S  T  M  K  I  K  I  I  A  P  P  E  R  K  Y  S  V  W  I  320

961  GGAGGCTCCATCTTGGCTTCTCTCTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAG
     G  G  S  I  L  A  S  L  S  T  F  Q  Q  M  W  I  S  K  Q  E
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Fig. 3. Partial sequence of rainbow trout β-actin. The numbers on the left refer to the nucleotide position, and the values on the right correspond to the amino acid residues (GeneBank Accession Number AF254414).

goose (M26111), salmon (AF012125), *Xenopus* (AF079161) and zebrafish (AF057040) (data not shown).

3.2. Exposure of rainbow trout to E_2 and α-ZEA

Treatment of rainbow trout with a single injection of E_2 or α-ZEA did not elicit overt toxicity. No mortality was observed after injection with the appropriate doses of E_2 and α-ZEA or throughout the duration of the experiment. However, four fish died during acclimatization, probably due to stress after transportation. Fish were not fed during the experiment, due to the possibility of interference with phytoestrogens in the food. There was no significant increase in liver somatic index (LSI) for any of the treatment groups compared to the control group (data not shown).

3.3. Measure of the relative amounts of VTG and ZR-proteins by ELISA

All concentrations (0.01, 0.1, 1.0, and 10 mg/kg) of E_2 significantly induced ZR-proteins ($P < 0.05$ or $P < 0.001$) resulting in 2.0-, 1.6-, 4.6-, and 7.6-fold induction in ZR-proteins, respectively (Fig. 4A). Injection of 0.01, 1.0 and 10 mg/kg E_2 induced VTG 0.2-, 0.9-, and 17-fold induction, respectively (Fig. 4B). In addition, treatment with α-ZEA (1.0 and 10 mg/kg) significantly induced ZR-proteins ($P < 0.05$ or $P < 0.001$, respectively), 1.4- and 4.8-fold, respectively, and 0.1 mg/kg ($P < 0.1$) 0.75 fold induction (Fig. 4A), while VTG was only significant induced at the greatest dose (10 mg/kg bw) ($P < 0.001$) giving a 0.8-fold induction (Fig. 4B). A maximal response was not obtained for ether treatments and therefore EC_{50} values were not calculated.

3.4. Quantitation of ZR-gene and VTG by real-time PCR

All standard curves for quantification of VTG, ZR-gene and β-actin mRNA were linear over six orders of magnitude with the linear correlation (r), between C_T and the number of copies of target, being ≥ 0.99 in each case. A representative amplification plot of standards and the corresponding standard curve are shown in Fig. 5A and B. Results from real-time PCR show that both α-ZEA and E_2 induced expression of both ZR-gene and VTG mRNAs (Fig. 6A and B). The ZR-gene was significantly induced at all α-ZEA concentrations, (0.1, 1.0 and 10.0 mg/kg) ($P < 0.05$ or 0.001) with increases in mRNA of 2.0-, 1.2-, and 7.0-fold, respectively, while VTG mRNA was induced by 1.0 and 10.0 mg/kg (1.4-, 3.4-fold, respectively) ($P < 0.1$ and $P < 0.001$, respectively) (Fig. 6B). E_2 induced ZR-gene ($P < 0.05$ or 0.001) and VTG-gene ($P < 0.1$ and $P < 0.05$) expression at all doses except for the 0.01 mg/kg dose. Again it is not known whether maximal response was obtained for both E_2 and α-ZEA, hence EC_{50} values could not be calculated. The amount (absolute and relative) of ZR-gene mRNA and ZR-proteins were greater than the amounts of VTG mRNA and VTG for all doses except 10 mg/kg E_2.

As observed with the ELISA, E_2 was significantly more potent and efficacious in inducing both responses when compared to α-ZEA. Although the results from both assays are comparable, the level of induction of ZR-gene and VTG mRNA exhibited a significant larger dynamic range that the level of expression of ZR-proteins and VTG. In the ELISA, maximal E_2 induction of ZR-proteins and VTG levels were 7.6- and 17-fold, respectively. In contrast, 10 mg/kg bw E_2 induced ZR-gene and VTG mRNA at levels of 925- and 4600-fold respectively.

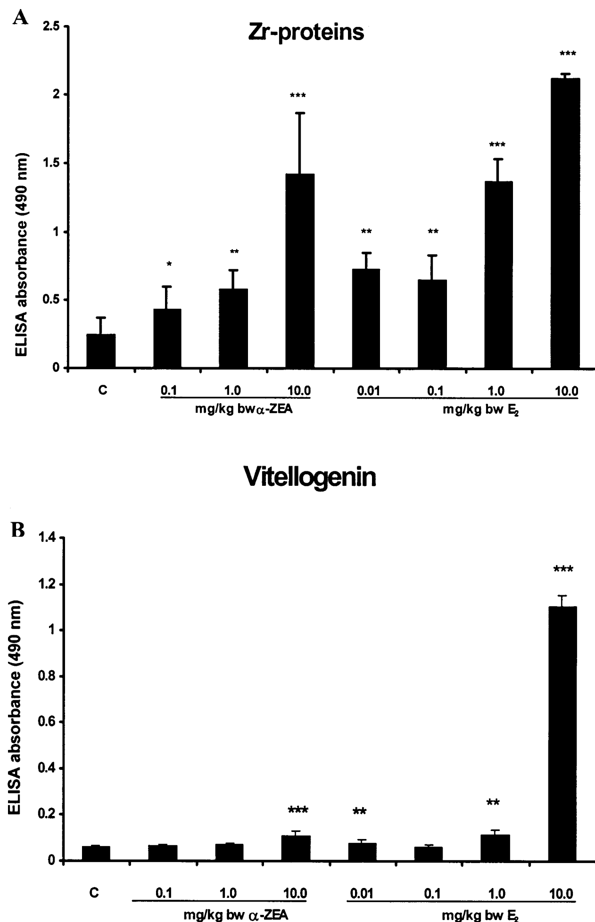

Fig. 4. ELISA analysis of relative levels of ZR-proteins (A) and VTG (B) in rainbow trout plasma after 10 days of exposure to α-ZEA (0.1, 1.0 and10.0 mg/kg bw) or E_2 (0.01, 0.1, 1.0, and 10.00 mg/kg bw). Absorbance was measured at 490 nm and each experimental group consisted of nine fish. Microsoft Excel was used to calculate P values, $^*P < 0.1$; $^{**}P < 0.05$; $^{***}P < 0.001$ compared with controls (t-test).

4. Discussion

The present study compares the ER-mediated induction of ZR-gene and VTG protein and mRNA levels in rainbow trout following exposure to E_2 or α-ZEA using ELISA and real-time PCR assays. Partial cloning of the rainbow trout ZR-gene resulted in a cDNA that exhibited high amino acid sequence identity when compared to ZR-proteins from salmon, winter flounder, carp (ZP2) and medaka choriogenine H (Figs. 1 and 2). The rainbow trout β-actin cDNA was also partially cloned and showed almost 100% amino acid sequence identity to β-actin from a number of other species (Fig. 3).

Previous studies report that ZR-proteins are a more sensitive marker of exposure to estrogenic substances than VTG, as measured in Atlantic salmon by ELISA using homologous antibodies to salmon ZR and VTG [21,33,22]. Furthermore, it was reported that ZR-proteins were detected in salmon plasma earlier than VTG after E_2-exposure [35]. In the present study, E_2 and α-ZEA exhibited an approximate dose-dependent induction of ZR-proteins and VTG in plasma analyzed 10 days after exposure. However, E_2 was found to be significantly more potent and efficacious in inducing both responses when compared to α-ZEA (Fig. 4). Similar differences in response between the two ligands were also observed in Atlantic salmon [36]. Moreover, ZR-protein induction was also found to be a more sensitive response when compared to VTG. However, differences in response between VTG and ZR maybe due to differences in affinity of the two antibodies used. Although the present results are comparable to previous reports, there were differences, which may be due to differences in species, epitope recognition, doses and route of exposure. Nevertheless, this study confirms that ZR-protein levels are a more sensitive marker than

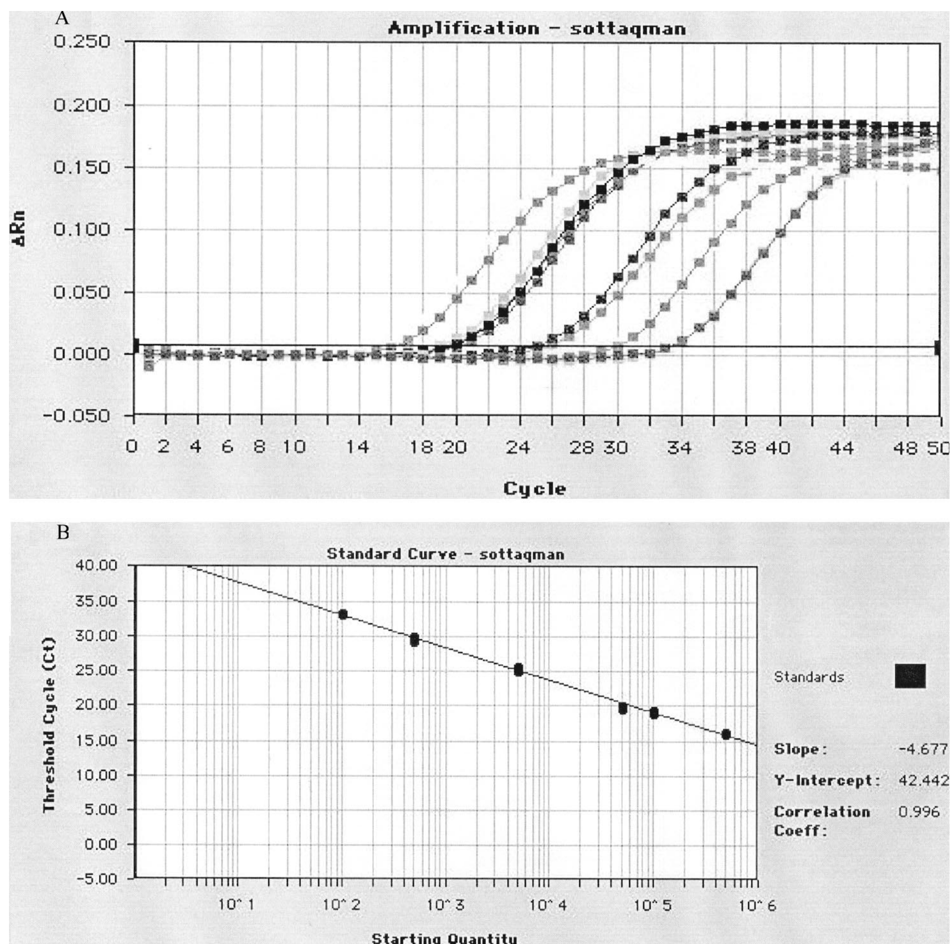

Fig. 5. Amplification plot (A) and standard curve (B) for VTG standards ranging from 100–500 000 copies of target sequence. The standards are amplified for 50 cycles, and the standard curve is made by plotting the number of starting copies of the target against the first cycle (C_T) that showed significant increase in fluorescence (ΔR). All standards were measured in duplicate.

VTG protein levels, and possibly a more useful and environmentally relevant bioindicator of exposure to estrogenic substances.

A comparison of the results indicates that the ZR-proteins and -gene are more sensitive to induction following low doses of estrogenic substances when compared to VTG. This was observed in both the ELISA and real-time PCR assays and is consistent with a previous published report where the amounts of ZR-proteins and VTG were measured by ELISA [22]. However, at high E_2-doses (10 mg/kg bw), VTG induction was greater than ZR-protein/gene induction as measured in both the ELISA and real-time PCR assays. This difference between the two genes may be due to differences in the regulation of gene expression and affects on mRNA stabilization. E_2 has been previously shown to stabilize numerous mRNAs including VTG [37]. In addition, in male fathead minnow, VTG protein levels were shown to remain high due to the lack of a mechanism for its removal from plasma [38]. The effect of estrogens on ZR-gene mRNA stabilization and ZR-protein clearance in male fish is unknown.

The use of ELISA to measure VTG and ZR-proteins in Atlantic salmon has been established and compared [21,35,22]. In contrast, real-time PCR is a relatively new and powerful technique for the sensitive measurement of mRNA levels. This method is based on the 5′ nuclease assay first described by Holland et al. [39] and uses the 5′ nuclease activity of Taq polymerase to cleave a fluorogenic non-extendible probe during the extension phase of PCR. The probe is dual labeled with a fluorogenic dye serving as a reporter at the 5′ end, and a quencher dye at the 3′ end. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, releasing a measurable fluorescent signal from the reporter (Fig. 5) [40,41]. This increase in fluorescence is measured following each cycle in the PCR reaction and can be reported as an amplification plot (Fig. 5).

A comparison between the two assays indicates that both methods have unique advantages and limitations. The ELISA is relatively inexpensive, however it is dependent on the availability of an antibody or antiserum that may exhibit a species bias. In contrast, the process

of designing probes and primers for real-time PCR is relatively easy provided the cDNA sequence is available. Real-time PCR is also inexpensive when compared to antibody production and the development of a robust ELISA. However, the real-time PCR method requires expensive specialized equipment but has the capability of higher and more efficient sample throughput.

Preliminary studies (data not shown) and published reports [38,42–45] suggest that the maximum induction of VTG and ZR-proteins occur between 7 and 12 days depending on the species, route of exposure and doses used. In contrast, the time course for maximum induction of VTG mRNA ranges from 16 h to 15 days [46,47]. Despite the fact that the optimal time point for mRNA induction may not have been used in the present study, real-time PCR appears to be more sensitive when compared to the ELISA. Examination of the results also reveals that the real-time PCR results exhibited higher fish to fish variability in regard to the number of copies of the different target genes when compared to the variability in absorbance observed in the ELISA. Several factors may have contributed to this variability including differences in the kinetics of induction and clearance of mRNA. Inaccuracy during delivery of the different compounds, due to the small size of the fish may also be a factor. Recent results investigating repeated doses using larger fish showed a lower variability within a treatment group (manuscript in preparation). In general both assays proved to be an acceptable approach. Selection of which method to use will be dependent on the objectives and design of the study, and the availability of antibodies, antiserum and DNA sequence.

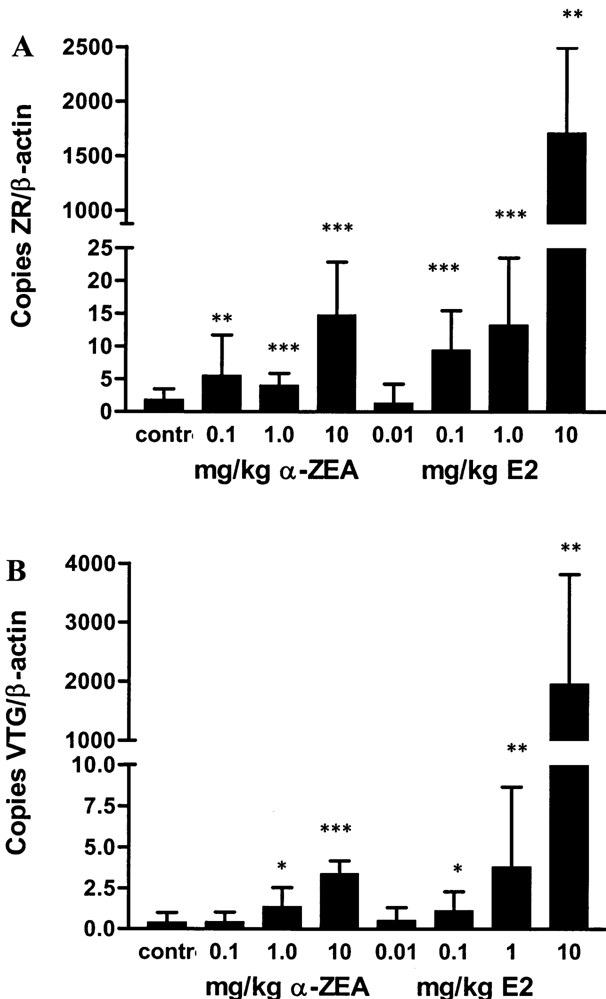

Fig. 6. Real-time PCR analysis of ZR-gene (A) and VTG mRNA (B) levels in rainbow trout liver after 10 days of exposure to α-ZEA (0.1, 1.0 and 10.00 mg/kg bw) or E_2 (0.01, 0.1, 1.0, and 10.00 mg/kg bw). The results are reported as the ratio of ZR-gene/β-actin or the ratio VTG/β-actin. Each experimental group consisted of at least six fish. Microsoft Excel was used to calculate P values, $*P < 0.1$; $**P < 0.05$; $***P < 0.001$ compared with controls (t-test).

In summary, the ZR-gene and proteins provide a sensitive and rapid marker to assess estrogenic activity of a test substance in rainbow trout. The induction of ZR-gene and proteins appear to be more sensitive than VTG and therefore may be a more suitable indicator of exposure to estrogen or estrogenic substances. Nevertheless, further studies are required to determinate if the induction of VTG and ZR-gene/protein are predictive of adverse effects resulting from exposure to an estrogenic substance.

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