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DYNAMICS OF ESTROGEN BIOMARKER RESPONSES IN RAINBOW TROUT EXPOSED TO 17β-ESTRADIOL AND 17α-ETHINYLESTRADIOL

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Abstract—We have investigated the response dynamics of the estrogen-dependent genes vitellogenin (VTG) and the vitelline envelope proteins (VEPs) as well as circulating VTG in immature female rainbow trout (*Oncorhynchus mykiss*) exposed to 17β-estradiol (E_2) and 17α-ethinylestradiol (EE_2) for periods of 7 and 14 d. Gene responses were quantified by measurement of messenger RNA (mRNA) in liver extracts using a chemiluminescent hybridization protection assay. Circulating VTG was measured by a homologous enzyme-linked immunosorbent assay. Exposure to both E_2 and EE_2 induced concentration-dependent increases in all biomarkers. The data presented indicate that VEP genes may be more sensitive to estrogens than the VTG gene. The biomarker lowest-observed-effect concentrations (^{biomarker}LOEC) in the 14-d study with E_2 were 14 ng/L (VTG protein, VTG mRNA, VEPβ, and VEPγ) or 4.8 ng/L (VEPα). The EE_2 was 5- to 66-fold more potent depending on the biomarker studied. In the 7-d study, all biomarkers were elevated after 48-h exposure to E_2, with ^{biomarker}LOECs of 30 ng/L (VTG protein, VTG mRNA, and VEPγ) or 9.7 ng/L (VEPα and VEPβ). Vitellogenin mRNA was induced up to 1,000-fold above baseline, and this translated into an increase of approximately 50,000-fold in circulating VTG. In conclusion, all biomarkers responded to estrogen exposure at environmentally relevant concentrations.

Keywords—Hybridization protection assay Endocrine disruption Rainbow trout Vitellogenin Gene expression

INTRODUCTION

It is well established that many chemicals discharged into the environment (both natural and man-made) have the potential to interfere with the endocrine system of organisms, and in turn, this can lead to alterations in growth, development, and/or reproduction [1,2]. Research into these endocrine-disrupting chemicals (EDCs) has focused mainly on compounds that act as estrogens. A valuable biomarker for assessing exposure to estrogens in oviparous vertebrates is the induction of the yolk protein precursor vitellogenin (VTG) [3,4]. Vitellogenin is normally synthesized in response to endogenous estrogen and is present only in the plasma of female fish, although in males, the VTG gene can be readily induced by exposure to exogenous estrogens [5]. Useful features of VTG induction are the specificity for estrogens and the sensitivity and magnitude of the response [6]. In fish, VTG messenger RNA (mRNA) expression occurs rapidly in response to steroid estrogens [7,8], and its measurement can be effective in quantifying responses to such estrogens and their mimics [8,9].

Other potential estrogen-sensitive genes are those coding for the family of egg envelope proteins that correspond to the zona radiata/pellucida proteins of higher vertebrates. In rainbow trout, three such proteins have been identified: The vitelline envelope proteins (VEPs) α, β, and γ [10,11]. The VEPs are normally synthesized in females during oogenesis, starting before the onset of vitellogenesis. Their concentration increases rapidly in the plasma during early vitellogenesis, because plasma concentrations of 17β-estradiol (E_2) increase [11–13]. As with VTG, males can synthesize the VEPs in response to steroid estrogens and their mimics [11–14]. It has been suggested that VEPs may be more responsive to estrogen than VTG [15,16]. Thus, they may serve as earlier biomarkers for estrogenic disruption, although to date, responses have only been determined semiquantitatively.

Vitellogenin protein, VTG mRNA, and to a lesser extent, VEPs are now being investigated in the context of routine screening and testing of chemicals for estrogenic activity. Furthermore, the Organisation of Economic Cooperation and Development and the U.S. Environmental Protection Agency are presently considering VTG induction as an endpoint in the development of standardized tests for EDCs [17]. Recent studies have started to assess the dynamics of the vitellogenic response in fish exposed to estrogen [8,18]. As yet, however, no comprehensive investigations have been reported that have quantified the comparative dynamics of VTG and VEP induction in any fish species, and this information is needed for the development of standardized assays for estrogens.

In the present study, we first employed a 14-d screen based on immature female rainbow trout (*Oncorhynchus mykiss*) exposed to E_2 and 17α-ethinylestradiol (EE_2) to establish the relative induction of VTG mRNA and protein and of VEPα, VEPβ, and VEPγ mRNA transcripts. In the second experiment, a time-course study was performed to quantify the temporal induction dynamics of these estrogenic biomarkers. A homologous enzyme-linked immunosorbent assay (ELISA) was used to quantify VTG induction in plasma, and hybridization protection assays (HPA) were used for mRNA quantification.

Chemiluminescent HPAs are used routinely in the field of clinical diagnostics, primarily in the identification of infectious

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agents [19–22]. They involve the hybridization of acridinium ester (AE)-labeled oligonucleotide probes to target RNA, followed by a selection step in which AE associated with unhybridized probe is hydrolyzed to a nonchemiluminescent derivative while that associated with hybridized probe remains unaffected. The stringency of the hybridization conditions renders this method highly specific. Moreover, the protection of AE against hydrolysis is strictly dependent on a homologous fit between probe and target, such that disruption of the duplex caused by a single mismatch will result in complete loss of the chemiluminescent signal [21,22]. Differential hydrolysis of the AE produces a very low background signal, thereby producing a highly sensitive detection system. This technique is ideally suited to measuring specific mRNA transcripts where closely related gene products exist.

MATERIALS AND METHODS

Test organisms

Fish used in experiment 1 (14-d exposures to E_2 and EE_2) were obtained from West Country Trout (Trafalgar, Cornwall, UK). The body weight (mean ± standard error [SE]) of the fish at the start of the study was 10.5 ± 0.71 g (n = 24). For experiment 2, 7-d exposure to E_2 was performed with female juvenile rainbow trout obtained from Houghton Springs Fish Farm (Dorset, UK). The body weight (mean ± SE) of the fish at the start of the study was 6.9 ± 0.23 g (n = 30). Throughout both experiments, fish were maintained under flow-through conditions in dechlorinated water at 15 ± 1°C, with a 16:8-h light:dark photoperiod and 20-min dawn and dusk transition periods. Fish were fed a maintenance ration of 1% body weight per day of Keystart Hatchery 1200 fish food pellets (BOCM Pauls, Renfrew, UK). Before the start of the experiment, fish were acclimated in the same conditions for 10 d.

Water supply and test apparatus

Dilution water from the municipal supply to Brixham Environmental Laboratory (Devon, UK) was filtered through activated carbon and dechlorinated using sodium thiosulfate. The treated water was passed through an ultraviolet sterilizer and a series of Hytrex cartridge filters (25- and 10-μm rating; L.H. Loveless, Harrogate, Yorkshire, UK) and fed into a temperature-controlled, aerated header tank (15 ± 1°C). Flow-control devices were used to feed the dilution water from the header tank to glass mixing chambers and into the test vessels. The test vessels had a working volume of 45 L and were constructed of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions. Dilution water and test chemical flow rates were checked at least three times per week. The flow rate provided a 99% replacement time of approximately 7 h.

Experimental design

In experiment 1, groups of 12 juvenile female rainbow trout were exposed for 14 d to a dilution water control (DWC), methanol solvent control (SC), and nominal concentrations of 1.0, 3.2, 10, 32, 100, and 320 ng/L of E_2 and 0.1, 0.32, 1.0, 3.2, 10, and 32 ng/L of EE_2. Each dose involved a single tank of fish. Estradiol (98% purity) and EE_2 (98% purity) were purchased from Sigma (Poole, Dorset, UK). Stock solutions of E_2 and EE_2 were prepared weekly in a high-performance liquid chromatography-grade methanol (Fisher Scientific, Loughborough, UK) and dosed to glass mixing vessels by means of a peristaltic pump at a rate of 0.04 ml/min to mix with the dilution water flowing to the test vessels at a rate of 400 ml/min. The SC vessel received the same rate of addition of methanol, such that the water in all test vessels, except the DWC, contained 0.1 ml/L of methanol. The E_2 and EE_2 exposures were run simultaneously and, therefore, shared a common group of control fish. For full experimental details, see Thorpe et al. [23].

In experiment 2, groups of 45 juvenile female rainbow trout were exposed for 7 d to a DWC, a methanol SC, and nominal concentrations of 10, 32, and 100 ng/L of E_2. The E_2 (98% purity) was purchased from Sigma. Stock solutions of E_2 were prepared and dosed as described for experiment 1, with the exception that dosing to the glass mixing vessels occurred at a rate of 0.08 ml/min to mix with the dilution water flowing to the test vessels at a rate of 800 ml/min. Each dose involved a single tank of fish, and the higher flow rate was required to ensure adequate oxygenation for the greater biomass of fish in this experiment compared with experiment 1.

Analytical chemistry

Concentrations of E_2 and EE_2 were monitored throughout all experiments. Water samples were taken at the start of the experiment and, thereafter, weekly and directly from the center of each tank into solvent-cleaned flasks. For these measurements, 500-ml water samples were extracted under vacuum (5 ml/min) onto preconditioned, solid-phase extraction columns. Both E_2 and EE_2 were eluted from the columns using 5 ml of methanol. The solvent was removed under a stream of nitrogen, and the extracts were redissolved in ethanol. In experiment 1, E_2 and EE_2 were measured using established radioimmunoassays [24,25]. In experiment 2, concentrations of E_2 were determined by gas chromatography-mass spectrometry. Water samples were spiked with deuterated E_2 and then extracted under vacuum (50 ml/min) onto preconditioned, 47-mm C_{18} Envi-disks (Supelco, Bellefonte, USA). The E_2 was eluted from the disks using 30 ml of methanol, and residual solvent was removed under a stream of nitrogen. The extracts were derivatized by heating to 120°C for 30 min with 200 μl of pyridine and 300 μl of *N*-(*tert*-butyldimethylsily)-*N*-methyltrifluoroacetamide. After cooling, 300 μl of bis(trimethylsily)trifluoroacetamide were added, and the vial was heated to 60°C for 240 min. The reagents were removed under nitrogen, and the extracts were redissolved in 250 μl of dichloromethane. The derivatized samples were analyzed on a Polaris ion-trap gas chromatograph-mass spectrometer (Thermoquest, San Jose, CA, USA). The analysis conditions were as follows: Sample volume, 2.5 μl; gas chromatography column, HP-5MS, 30 m × 0.25 mm (inner diameter), fused silica with 0.25-μm stationary-phase film thickness (Hewlett-Packard, Avondale, PA, USA) using helium as the carrier gas at 1 ml/min; injector temperature, 300°C; column program, 50°C for 10 min, then an increase to 300°C at 8°C per min, and then isothermal at 300°C for 10 min. The mass spectrophotometer was operated in the electron-ionization mode (70 eV) and set up to carry out mass spectrophotometry–mass spectrophotometry experiments. Precursor (parent) ions *m*/*z* 458 and *m*/*z* 460 (trimethylsilyl/*tert*-butyldimethylsilyl derivative of E_2 and its deuterated analogue, respectively) were stored in the ion trap, fragmented, and then scanned out to give daughter-ion spectra for each compound. The major daughter ion for each compound was the $[M + H_2O\text{-}57]^+$—that is, *m*/*z* 419 and *m*/*z* 421 (trimethylsilyl/*tert*-butyldimethylsilyl derivative of E_2 and its deuterated analogue, respectively). These ions were profiled,

and the resulting peaks were integrated to give peak areas, which were used for the calculations, using deuterated E_2 as the internal standard.

Fish sampling

In experiment 1, a subgroup of fish ($n = 24$) was sampled at the outset (day 0) of the experiment, and then all the exposed fish were sampled on day 14 ($n = 12$ for each treatment). In experiment 2, a subgroup of fish ($n = 30$) was sampled at the outset (day 0) of the experiment, and then subgroups ($n = 15$) were randomly sampled after 2, 4, and 7 d. Fish were sacrificed by administration of a lethal dose (200 mg/L) of MS222, buffered with 1 M NaOH to pH 7.3. Total wet body weight and gutted body weight of the fish were recorded to the nearest 0.01 g, and both standard and fork lengths were measured to 0.1 cm. Blood was collected by cardiac puncture using a heparinized syringe (5,000 U heparin/ml) and centrifuged (7,000 *g*, 5 min, 4°C), then the plasma was removed and stored at −80°C until required for VTG analysis. Plasma samples were assayed for VTG using a rainbow trout ELISA [25]. The liver was removed, wet-weighed to the nearest 0.01 mg, snap-frozen in liquid nitrogen, and stored at −80°C for determination of liver VTG and VEP mRNA concentrations.

Preparation of RNA

Total RNA was prepared from rainbow trout liver tissue using TRI reagent (Sigma). Resulting RNA pellets were dissolved in 100 μl of sterile, deionized water. The RNA samples were evaluated for purity and quantified spectrophotometrically (GeneQuant; Amersham Pharmacia Biotech, Cardiff, UK). The RNA integrity was inspected visually using the appearance of the 28S and 18S ribosomal RNA bands by electrophoresis analysis with a 1% (w/v) agarose gel prepared using sterile 1× TBE (Tris-borate-EDTA buffer; Gibco BRL, Life Technologies, Paisley, UK). Samples of RNA were stored at −80°C until expression levels of the target genes were analyzed.

Synthesis of chemiluminescent oligonucleotide probes

A complementary region of each target gene was selected for probe design (see below). The probes were synthesized to contain a nonnucleotide, amine-terminated linker to which a chemiluminescent acridinium salt was attached (# indicates position of the nonnucleotide linker). The following probe sequences were used:

VTG probe: 5′-GCA CCC AGG AAT#GGG CAA AGC TGA CT-3′ (GenBank accession no. U26703)
VEPα probe: 5′-GGG TAT CTG TTG AGG TAT#GTT TTG GGC AGG-3′ (GenBank accession no. AF231706)
VEPβ probe: 5′-CAG GGT CAG CAC AAT#GTT GGG ATC TGT C-3′ (GenBank accession no. AF231707)
VEPγ probe: 5′-GGC ATG AAC T#G GGA GTG GGA ACC TGT C-3′ (GenBank accession no. AF231708)
β-Actin probe: 5′-CAT GAT GGA GTT GAA GTT#GGTCTC GTG G-3′ (GenBank accession no. AF157514)

Hybridization protection assay

Acridinium salts can be readily hydrolyzed to yield nonchemiluminescent products. However, the formation of a duplex between the labeled probe and its complementary target protects the acridinium salt from contrived hydrolytic degradation. The assay method comprises three basic steps: The first involves hybridization of the probe to target present in samples. The second involves selective hydrolysis to destroy chemiluminescent signal from any unhybridized probe. The third comprises initiation of the chemiluminescent reaction. In this system, the intensity of the measured chemiluminescence is directly proportional to the amount of transcribed mRNA present in the sample. To minimize any reduction in the efficiency of hybridization caused by secondary structure of RNA transcripts, unlabeled oligonucleotides (30 –mers), complementary to the flanking regions of the target sequences, are incorporated into each hybridization reaction [26].

Hybridization. Lyophilized AE-labeled probe was reconstituted in 6 ml of hybridization buffer (0.1 M lithium succinate [pH 5.0], 2 mM ethylenediaminetetraacetic acid, 2 mM ethylene glycol-bis [β-aminoethyl ether]-*N,N,N′,N′*-tetraacetic acid, and 10% [w/v] lithium lauryl sulfate). Dilutions of synthetic oligonucleotide complementary to the AE probe were used to generate a calibration curve (0, 10, 40, and 400 fmol/ml). Rainbow trout liver total RNA samples were prediluted to approximately 0.2 to 2.0 μg/μl. In 12- × 75-mm tubes (Sarstedt 55.476, Leicester, UK), duplicate 100-μl hybridization reactions were prepared containing 50 μl of AE probe (0.1 pmol) and 50 μl of nucleic acid target (for calibration) or total RNA sample. Tubes were mixed by vortexing and then were covered and incubated in a water bath for 30 min at 60°C.

Hydrolysis. Three-hundred microliters of selection reagent (0.15 M sodium tetraborate [pH 7.6] and 5% [v/v] Triton X-100; Sigma, Poole, Dorset, UK) were added to each tube and then mixed by vortexing. Tubes were incubated for a further 15 min at 60°C.

Detection. Immediately after removal from the water bath, tubes were placed in an ice/water slurry for 2 min, then removed to stand at room temperature for a further 2 min. Chemiluminescence was measured in a luminometer (Stratec Electronic, Birkenfeld, Germany) by automatic sequential injection of 200 μl of 0.032 M hydrogen peroxide in 0.001 M nitric acid and 200 μl of 1.575 M sodium hydroxide. The chemiluminescent emission was integrated over a 5-s time period. Target mRNA concentrations were determined by interpolation from the calibration curve.

Statistical analyses

Vitellogenin protein (μg/ml) as well as VTG mRNA and VEP mRNA concentration (fmol/ml) data were tested for the assumption of normality using the Anderson-Darling method. The data failed to meet this criterion, so a nonparametric Mann–Whitney test was used for all data sets in which data groups were compared to the initial control group.

RESULTS

Analytical chemistry

Mean measured concentrations of the chemicals used in experiments 1 and 2 are given in Table 1. In experiment 1, the mean measured concentrations of E_2 were between 100% and 150% of nominal, with the exception of the lowest concentration of E_2 (1.0 ng/L), for which the mean measured concentration was 230% of nominal. This lowest concentration of E_2 was at the limit of detection for the analytical method and, therefore, could not be reliably assessed. For EE_2, the mean measured concentrations were between 45% and 130% of nominal. In experiment 2, the mean measured concentrations of E_2 were equal to or greater than 94% of nominal.

Table 1. Mean measured tank concentrations of 17β-estradiol (E_2) and 17α-ethinylestradiol (EE_2) for experiment 1 and 2[a]

Nominal (ng/L)	Experiment 1		Experiment 2
	E_2 (ng/L)	EE_2 (ng/L)	E_2 (ng/L)
0.1	NT[b]	0.13 ± 0.1	NT
0.32	NT	0.21 ± 0.1	NT
1.0	2.3 ± 0.8	1.0 ± 0.3	NT
3.2	4.8 ± 1.5	1.5 ± 0.3	NT
10	14.0 ± 4.0	5.4 ± 0.2	9.7 ± 1.8
32	47.0 ± 14.1	26.0 ± 4.7	30 ± 2.8
100	99.0 ± 12.1	NT	94.5 ± 21.9
320	463.0 ± 155.9	NT	NT

[a] The test chemicals were not detected in the dilution water or solvent controls. Data are presented as the mean ± standard deviation (n = 2).

[b] NT = not tested; these concentrations were not included in the experimental design.

Mean measured concentrations for each chemical are reported for protein and mRNA induction throughout.

The concentration of β-actin mRNA varied little between individual samples (76 ± 1.54 fmol/ml), indicating that the quality of RNA used for the measurement of the relevant mRNAs was satisfactory. Given such little variability in the control gene measurements, all results of specific mRNA measurements are expressed as individual concentrations.

Experiment 1

No effects of estrogen treatment were observed on survival, growth, or condition of the exposed fish.

Concentrations of liver VTG mRNA in juvenile female fish at the onset of the experiment were below the detection limit of the assay (<2 fmol/ml), and no increase was detectable in the DWC or SC fish after 14-d exposure. Exposure to E_2 produced concentration-dependent increases (Fig. 1) with a lowest-observed-effect concentration ([biomarker]LOEC) of 14 ng/L. The measured concentration of liver VTG mRNA at this exposure dose (8.16 ± 1.34 fmol/ml; $p < 0.05$) was approximately fourfold the detection limit of the assay. Exposure to

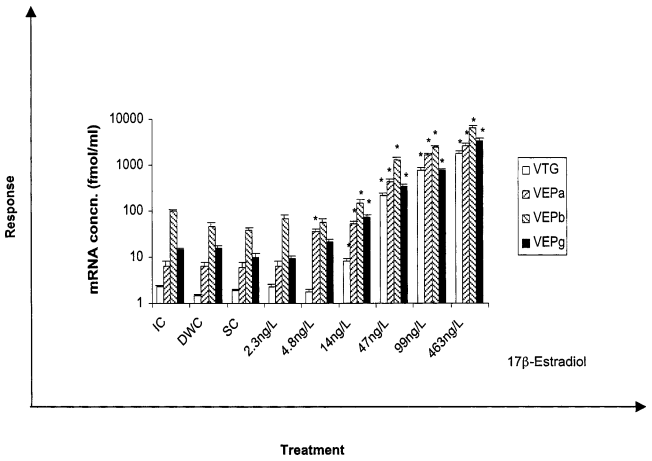

Fig. 1. Concentrations of vitellogenin (VTG) messenger RNA (mRNA) and vitelline envelope proteins (VEP; α, β, and γ) mRNAs in liver extracts of juvenile female rainbow trout exposed to 17β-estradiol for a period of 14 d. Each column represents the mean ± standard error. Asterisks indicate significant differences from initial control values ($p < 0.05$, Mann–Whitney test). DWC = dilution water control; IC = initial control; SC = solvent control.

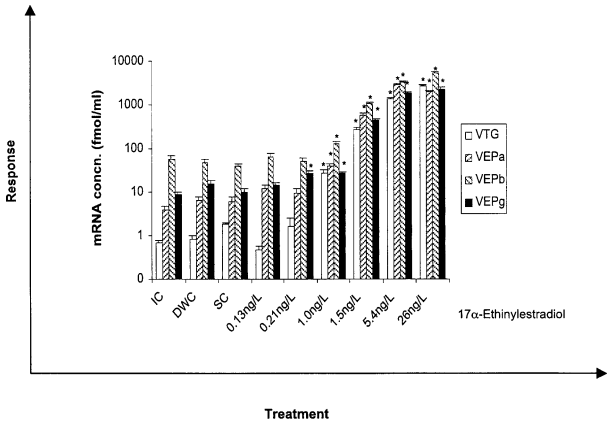

Fig. 2. Concentrations of vitellogenin (VTG) and vitelline envelope proteins (VEP; α, β, and γ) messenger RNAs (mRNAs) in liver extracts of juvenile female rainbow trout exposed to 17α-ethinylestradiol for a period of 14 d. Each column represents the mean ± standard error. Asterisks indicate significant differences from initial control values ($p < 0.05$, Mann–Whitney test). Refer to Figure 1 for definition of acronyms.

EE_2 produced concentration-dependent increases in liver VTG mRNA (Fig. 2), with a [biomarker]LOEC of 1.0 ng/L (VTG mRNA concentration of 27.7 ± 5.1 fmol/ml; $p < 0.05$).

Plasma VTG was 370 ± 50 ng/ml at the start of the experiment and remained unaltered in the DWC and SC fish. The [biomarker]LOECs for both E_2 and EE_2 were the same as those observed with VTG mRNA. The magnitude of the responses in plasma VTG was between 25,000- and 50,000-fold higher than basal concentrations.

Liver VEPα, VEPβ, and VEPγ mRNA concentrations at the onset of the experiment were 6.4 ± 2.0, 97.7 ± 6.3, and 14.7 ± 1.3 fmol/ml, respectively, in the E_2 exposure group and 3.9 ± 1.0, 58.5 ± 12.4, and 9.0 ± 0.9 fmol/ml, respectively, in the EE_2 exposure group. No increases were detectable in VEPα, VEPβ, or VEPγ mRNA in the DWC or SC fish after 14 d for either experiment.

Exposure to E_2 produced concentration-dependent increases in liver VEPα, VEPβ, and VEPγ mRNA (Fig. 1), with a LOEC of 4.8 ng/L for VEPα (37.1 ± 3.9 fmol/ml) and 14 ng/L for VEPβ (150.5 ± 22.3 fmol/ml) and VEPγ (73.5 ± 10.5 fmol/ml; $p < 0.05$). Exposure to EE_2 similarly produced concentration-dependent increases in liver VEPα, VEPβ, and VEPγ mRNA (Fig. 2), with a LOEC of 0.21 ng/L for VEPγ (27.5 ± 2.7 fmol/ml; $p < 0.05$) and 1.0 ng/L for VEPα and VEPβ (38.9 ± 6.3 and 128.3 ± 16.7 fmol/ml, respectively; $p < 0.05$).

The highest dose of E_2 (463 ng/L) induced increases in expression of approximately 400-, 65-, and 230-fold for VEPα, VEPβ, and VEPγ, respectively. Similar levels of induction of 450-, 90-, and 230-fold for VEPα, VEPβ, and VEPγ, respectively, occurred for the exposures to the highest dose of EE_2 (26 ng/L). In the EE_2 exposure, expression levels of VEP mRNAs differed little at 14 d between the two highest doses used (5.4 and 26 ng/L), suggesting that these responses may be close to maximal.

Experiment 2

No effects of exposure to E_2 for 7 d were observed on either growth or condition of the exposed fish.

Liver VTG mRNA concentrations in juvenile female fish at the onset of the experiment were below the detection limit

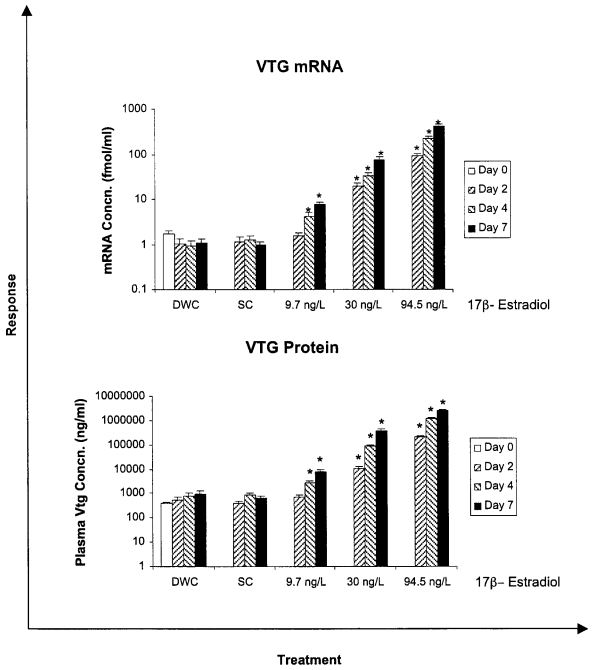

Fig. 3. Concentrations of vitellogenin (VTG) mRNA in liver extracts and plasma VTG in juvenile female rainbow exposed to 17β-estradiol for a period of up to 7 d. Each column represents the mean ± standard error. Asterisks indicate significant differences from day 0 control values ($p < 0.05$, Mann–Whitney test). Refer to Figure 1 for definition of acronyms.

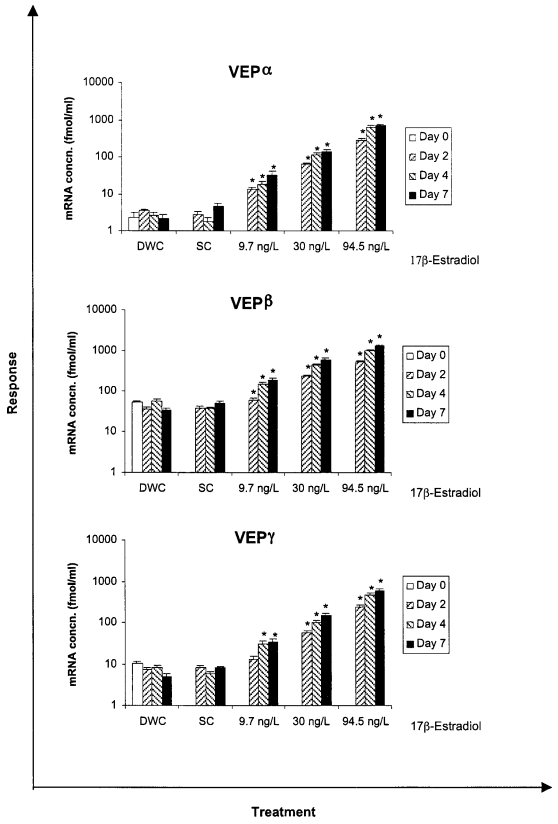

Fig. 4. Concentrations of vitelline envelope protein (VEP; α, β, and γ) messenger RNAs (mRNAs) in liver extracts of juvenile female rainbow trout exposed to 17β-estradiol for a period of up to 7 d. Each column represents the mean ± standard error. Asterisks indicate significant differences from day 0 control values ($p < 0.05$, Mann–Whitney test). Refer to Figure 1 for definition of acronyms.

of the assay (<2 fmol/ml), whereas the plasma VTG concentration at the start of the experiment was 397.2 ± 43.6 ng/ml. No increases were detectable in VTG mRNA or protein concentrations in the DWC or SC fish over the 7-d exposure period ($p > 0.05$).

Exposure to E_2 (9.7–94.5 ng/L) produced concentration- and time-dependent increases in both VTG mRNA and protein concentrations after 2, 4, and 7 d (Fig. 3). The period of exposure affected the threshold concentration of the response to E_2 for both VTG mRNA and protein. The $^{\text{biomarker}}$LOEC for E_2 was 30 ng/L on day 2, whereas on day 4, it was 9.7 ng/L (the lowest dose included in the present study; $p < 0.05$).

At the highest E_2 exposure dose (94.5 ng/L), a 200-fold increase occurred in VTG mRNA by day 7, which translated into an approximately 5,000-fold increase in circulating VTG protein. Comparison of the data as a whole revealed a correlation between the concentration of VTG mRNA (fmol/ml) measured in the liver and the concentration of VTG circulating in the blood. This linear correlation (slope = 5,794, $r^2 = 0.855$) held true for the full magnitude of the vitellogenic response. However, it was noted that individual regression plots for each of the time points in the present study exhibited a different slope (day 2 slope = 2,314, $r^2 = 0.817$; day 4 slope = 4,102, $r^2 = 0.620$; day 7 slope = 6,207, $r^2 = 0.877$).

Liver VEPα, VEPβ, and VEPγ mRNA concentrations at the onset of the experiment were 2.3 ± 0.8, 51.9 ± 5.8, and 10.4 ± 1.1 fmol/ml, respectively. As in experiment 1, VEPβ had a higher basal level of expression compared with the other VEP mRNAs. No changes were detectable in VEPα, VEPβ, or VEPγ mRNA in the DWC or SC fish over the 7-d exposure period ($p > 0.05$).

Exposure to E_2 (9.7–94.5 ng/L) produced concentration-dependent increases in VEPα, VEPβ, and VEPγ mRNA after 2, 4, and 7 d (Fig. 4). The magnitude of change in expression levels equated to 120-, 10-, and 23-fold increases for VEPα, VEPβ, and VEPγ, respectively, for the highest dose on day 2. On day 4, the mRNA concentrations increased by 269-, 19-, and 44-fold, respectively, and by day 7, the mRNA concentrations had increased by 300-, 25-, and 55-fold, respectively.

The increase in VEPα, VEPβ, and VEPγ mRNA expression levels between days 2 and 4 were significant ($p < 0.05$). As for VTG induction, the time period of exposure affected the threshold of response for VEP mRNA induction. The $^{\text{biomarker}}$LOEC for VEPα mRNA (13.1 ± 2.3 fmol/ml) was 9.7 ng/L from day 2 ($p < 0.05$). For VEPβ and VEPγ mRNA on day 2, the $^{\text{biomarker}}$LOEC was 30 ng/L (233.2 ± 20.5 and 55.9 ± 6.2 fmol/ml, respectively; $p < 0.05$), whereas on both day 4 and day 7, the LOEC was 9.7 ng/L (the lowest dose included in the present study; $p < 0.05$).

DISCUSSION

Many chemicals have been implicated as estrogen mimics and are thought to have adverse effects on fish reproduction. For many estrogenic chemicals, however, the mechanisms by which they exert an effect on reproductive parameters have yet to be fully understood. Induction of VTG and VEPs by steroid estrogens and their mimics occurs via binding to, and activation of, the estrogen receptor(s) [27]; thus, these responses potentially provide valuable biomarkers for both the development of in vivo screens to identify estrogen-receptor agonists and the assessment of the estrogenic potency of environmental chemicals. In recent years, measurement of cir-

culating VTG protein has been used widely as a biomarker for estrogen exposure, but to date, relatively few studies of VTG gene expression in fish have been performed [7,8,28,29]. This may be explained, at least in part, by the lack of convenient methods for mRNA quantification. We have demonstrated that the measurement of VTG and VEP gene expression by means of HPAs can provide comparable endpoints to circulating VTG protein for assessing the estrogenic activity of chemicals.

In the experiments described above, the concentrations of plasma VTG were consistent with those previously reported for immature females of a similar age and size [23,30,31]. In male rainbow trout, plasma VTG concentrations are often below the detection limit of most VTG assays (<10 ng/ml) and, in this regard, are arguably preferable for studies detecting weak estrogens. However, the very limited supply of male rainbow trout makes them an impractical resource for the development of routine tests and screens at this time [30]. In contrast to circulating VTG protein, the level of VTG mRNA expression in juvenile females was extremely low, with the concentrations in liver extracts being at or below 2 fmol/ml (the detection limit of VTG mRNA in the HPA). In juvenile female trout, a constitutive synthesis of VTG protein occurs, but this remains at a low level until they reach the vitellogenic phase of development. Only at this time does a rapid activation of the genetic machinery for VTG production occur, induced by rapidly increasing concentrations of endogenous E_2. This is followed by a rapid rise in plasma concentrations of VTG, which may increase by more than 10,000-fold in only a few weeks [6].

In contrast to VTG, the expression level for all VEP mRNAs in juvenile female trout was detectable under basal conditions. To our knowledge, the present study provides the first direct demonstration of this difference, although previous researchers using qualitative/semiquantitative approaches have suggested that such a difference occurs [12–14]. The basal expression of VEPβ mRNA in juvenile rainbow trout was higher than that for either VEPα and VEPγ, with a VEPα:VEPβ:VEPγ ratio of approximately 1:15:2. Higher levels of VEPβ have been previously reported for the Arctic char [16]. Studies of the expression levels of the equivalent zona pellucida (ZP) transcripts in the mouse show that ZP2 transcripts, but not ZP1 or ZP3 transcripts, are detected in resting oocytes and that all three ZP mRNAs coordinately accumulate as the oocytes begin to grow [32]. The relative abundance of the ZP1, ZP2, and ZP3 mRNA transcripts was reported as 1:4:4.

Exposure to steroid estrogens induced concentration- and time-dependent increases in both liver VTG mRNA and circulating VTG protein. No differences were observed in the effective dose for induction of VTG mRNA as compared with VTG protein or in the timing of these responses. Our first sampling point, however, was 2 d after estrogen stimulation, and there may be measurable differences between induction of VTG mRNA and the presence of VTG in the circulation at earlier intervals [33,34]. Determining quantitative relationships between hepatic VTG mRNA and concentrations of plasma VTG is complicated by a number of factors, including differences in the half-lives of the VTG message compared with the VTG protein; the half-life of VTG mRNA in the fathead minnow has been estimated at 20 to 30 h, compared with approximately 21 d for circulating VTG [8]. It has been reported that VTG mRNA can be stabilized by estrogen after its induction [35] and also that a more efficient translation of VTG mRNA to VTG protein can occur on secondary stimulation by estrogen [9,34,36]. In the present study, a positive correlation was found between the level of mRNA induction and the amount of protein in the circulation for exposures up to 14 d. This is in contrast to the results of some previous studies, in which no such correlation was found on exposure to estrogen [7,28]. Various hypotheses have been put forward to explain these discrepancies, including the longer half-life of the VTG protein compared with that of the VTG mRNA. A major difference in the present study compared with the previous work was in the use of HPA for measuring VTG mRNA concentrations. A significant advantage of HPA is that it is sufficiently sensitive to measure mRNA levels in liver extracts without amplification and, thus, can yield quantitative results.

In the present study, the increase in circulating VTG in response to estrogen in immature female fish was between 5,000-fold (94.5 ng E_2/L, experiment 2) and 50,000-fold (463 ng E_2/L, experiment 1) over baseline, whereas in the liver, the respective VTG mRNA increases were 200- and 1,000-fold. Differences in the magnitude of the responses between the VTG protein and VTG mRNA might be expected, reflecting differences in the dynamics of synthesis and turnover in mRNA as compared with those of the storage protein that it produces. The huge induction capacity for VTG protein that can occur over time periods that are conventionally used for chemical testing in fish (7–14 d) would appear to favor circulating VTG over VTG mRNA as a biomarker for estrogens. However, regression analysis of data for liver VTG mRNA and circulating VTG protein drawn for each separate sampling time point showed a progressive increase in the slope of the line during the course of the experiment (day 2 < day 4 < day 7), suggesting that the proportional increase in liver mRNA was greater than that in circulating VTG protein during the earlier stages of the exposure. These data imply that mRNA might prove to be more sensitive as a biomarker than the more established VTG protein for rapid screens for estrogens, although further studies are needed to validate this hypothesis. In field studies, the long half-life of VTG protein means that influences can be detected that occurred long before the fish were sampled, whereas the measurement of VTG mRNA may be used to indicate more acute estrogenic exposure.

The use of VEP proteins as biomarkers for estrogen exposure has been proposed by previous researchers [12–14], who developed an immunoassay to monitor expression levels in Atlantic salmon exposed to various environmental pollutants, including E_2, nonylphenol, *o′,p′*-DDT, lindane, bisphenol A, and a technical mixture of PCBs. In those studies, however, accurate quantification was hampered by the requirement to separate the individual egg protein components by gel electrophoresis before measurement as a consequence of the close structural homology between them. Furthermore, the ELISAs used were semiquantitative, making comparisons between responses in VEP and VTG induction difficult to validate. Nevertheless, on the basis of those studies, it was suggested that VEPβ was more responsive to xenoestrogen than VTG. In the Arctic char, mRNAs for VEPα and VEPβ responded more rapidly than mRNA for VEPγ to estrogen induction [16]. However, a recent study that quantified both VTG and VEP in the plasma in juvenile rainbow trout did not find VEPs to be more sensitive than VTG in response to nonylphenol [28]. Our findings in the rainbow trout indicated that the VEP mRNAs provided a more sensitive endpoint for estrogen stimulation compared with VTG mRNA. In the 14-d exposure (experiment 1),

VEPα was the only biomarker to show a significant increase at a concentration of E_2 of 4.8 ng/L, although VEPγ appeared to be the most sensitive to EE_2. In the second experiment, both VEPα and VEPβ were induced at the lowest test E_2 concentration (9.7 ng/L) after 2 d, although the response in VEPα mRNA was considerably higher than that of VEPβ (a sixfold increase compared with a 10% increase, respectively). It should be emphasized, however, that in these juvenile female trout, the basal expression level of VEPα was lower than that for VEPβ and VEPγ. The quantitative data generated here, through the use of HPAs, provide support for the concept that VEP genes are the most sensitive estrogen biomarker of those tested in the female rainbow trout. It would be valuable to examine if the same comparative responses for VEP and VTG mRNA occur in male fish.

The HPA system described here for measuring mRNA transcripts for VTG and VEPs was shown to be a highly sensitive and robust method for quantifying gene expression. Hybridization protection assay is an extremely specific method, because even a single base mismatch in the target sequence can frequently lead to complete loss of the chemiluminescent signal through differential hydrolysis. Such high specificity is of importance in monitoring the activity of the VEP genes, for example, in which the protein products show close sequence and structural homology. The measurement of mRNA is a suitable endpoint for both in vivo and in vitro test systems, and once the appropriate gene has been identified, the design and synthesis of a labeled probe become a relatively simple procedure. The technology can potentially be applied to the routine measurement of a range of environmentally relevant gene products and should provide a valuable tool in the quantification of biological responses to chemicals.

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REFERENCES

1. Colburn T, vom Saal FS, Soto AM. 1996. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 101:378–383.
2. Tyler CR, Jobling S, Sumpter JP. 1998. Endocrine disruption in wildlife: A critical review of the evidence. *Crit Rev Toxicol* 28: 319–361.
3. Sumpter JP, Jobling S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ Health Perspect* 103(Suppl. 7):173–178.
4. Tyler CR, van der Eerden B, Jobling S, Panter G, Sumpter JP. 1996. Measurement of vitellogenin, a biomarker for exposure to estrogenic chemicals, in a wide variety of cyprinid fish. *J Comp Physiol B* 166:418–426.
5. Chen TT. 1983. Identification and characterization of estrogen-responsive gene products in the liver of rainbow trout. *Can J Biochem Cell Biol* 61:802–810.
6. Tyler CR, Sumpter JP, Witthames PR. 1990. The dynamics of oocyte growth during vitellogenesis in the rainbow trout, (*Oncorhynchus mykiss*). *Biol Reprod* 43:202–209.
7. Folmar LC, Hemmer M, Hemmer R, Bowman C, Kroll K, Denslow ND. 2000. Comparative estrogenicity of estradiol, ethinyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. *Aquat Toxicol* 49:77–88.
8. Schmid T, Gonzalez-Valero J, Rufli H, Dietrich DR. 2002. Determination of vitellogenin kinetics in male fathead minnows (*Pimephales promelas*). *Toxicol Lett* 131:65–74.
9. Pakdel F, Feon S, Le Gac F, Le Menn F, Valotaire Y. 1991. In vivo estrogen induction of hepatocyte estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. *Mol Cell Endocrinol* 75:205–212.
10. Oppen-Berntsen DO, Gram-Jensen E, Walther BT. 1992. Zona radiata proteins are synthesized by rainbow trout (*Oncorhynchus mykiss*) hepatocytes in response to 17β-estradiol. *J Endocrinol* 135:293–302.
11. Oppen-Berntsen DO, Olson SO, Rong C, Swanson P, Taranger GL, Walther BT. 1994. Plasma levels of Zr-proteins, 17β-estradiol and gonadotropins during the annual cycle of Atlantic salmon (*Salmo salar*). *J Exp Zool* 268:59–70.
12. Celius T, Walther BT. 1998. Oogenesis in Atlantic salmon (*Salmo salar* L.) occurs by zonagenesis preceding vitellogenesis in vivo and in vitro. *J Endocrinol* 158:259–266.
13. Celius T, Walther BT. 1998. Differential sensitivity of zonagenesis and vitellogenesis in Atlantic salmon (*Salmo salar* L.) to DDT pesticides. *J Exp Zool* 281:346–353.
14. Arukwe A, Celius T, Walther BT, Goksoyr A. 2000. Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). *Aquat Toxicol* 49: 159–170.
15. Arukwe A, Knudsen FR, Goksoyr A. 1997. Fish zona radiata (eggshell) protein: A sensitive biomarker for environmental estrogens. *Environ Health Perspect* 105:418–422.
16. Westerlund L, Hyllner SJ, Schopen A, Olsson PE. 2001. Expression of three vitelline envelope protein genes in Arctic char. *Gen Comp Endocrinol* 122:78–87.
17. Organization for Economic Cooperation and Development. 1999. Final report of the fish expert consultation meeting, London, October 28–29, 1998. Report 9906. Environmental Health and Safety Division, Paris, France.
18. Harries J, Jambakhsh A, Jobling S, Matthiessen P, Sumpter JP, Tyler CR. 1999. Estrogenic potency of effluent from two sewage-treatment works in the United Kingdom. *Environ Toxicol Chem* 18:932–937.
19. Arnold LJ, Hammond PW, Wiese WA, Nelson NC. 1989. Assay formats involving acridinium ester-labeled DNA probes. *Clin Chem* 35:1588–1594.
20. Nelson NC, Reynolds MA, Arnold LJ. 1995. Detection of acridinium esters by chemiluminescence. In Kricka LJ, ed, *Nonisotopic Probing, Blotting and Sequencing.* Academic, San Diego, CA, USA, pp 391–428.
21. Nelson NC, Hammond PH, Matsuda E, Goud AA, Becker MM. 1996. Detection of all single base mismatches in solution by chemiluminescence. *Nucleic Acids Res* 24:4998–5003.
22. Nelson NC, Cheikh AB, Matsuda E, Becker MM. 1996. Simultaneous detection of multiple nucleic acid targets in a homogeneous format. *Biochemistry* 35:8429–8438.
23. Thorpe KL, Cummings RI, Hutchinson TH, Scholze M, Brighty G, Sumpter JP, Tyler CR. 2003. Relative potencies and combination effects of steroidal estrogens in fish. *Environ Sci Technol* 37:1142–1149.
24. Länge R, Hutchinson TH, Croudace C, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP. 2001. Effects of the synthetic estrogen 17α-ethinylestradiol over the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20: 1216–1227.
25. Tyler CR, van Aerle R, Nilsen M, Maddix S, Nilsen B, Berg K, Hutchinson TH, Goksoyr A. 2002. Development and validation of a monoclonal ELISA to quantify vitellogenin in the rainbow trout, *Oncorhynchus mykiss*, and its application for studies on environmental estrogens. *Environ Toxicol Chem* 21:47–54.
26. Hogan JJ, Milliman CL. 1991. Means and method for enhancing nucleic acid hybridization. U.S. Patent 5,030,557. U.S. Patent Office, Washington, DC.
27. Wahli W, Dawid IB, Ryffel GU, Weber R. 1981. Vitellogenesis and the vitellogenin gene family. *Science* 212:298–304.
28. Ackermann GE, Schwaiger J, Negele RD, Fent K. 2002. Effects of long-term nonylphenol exposure on gonadal development and biomarkers of estrogen exposure in juvenile rainbow trout *Oncorhynchus mykiss*. *Aquat Toxicol* 60:203–221.
29. Hemmer MJ, Hemmer BL, Bowman CJ, Kroll KJ, Folmar LC, Marcovich D, Hoglund MD, Denslow ND. 2001. Effects of *p*-nonylphenol, methoxychlor and endosulfan on vitellogenin induction and expression in sheepshead minnows (*Cyprinodon variegatus*). *Environ Toxicol Chem* 20:336–343.

30. Thorpe KL, Hutchinson TH, Hetheridge MJ, Sumpter JP, Tyler CR. 2000. Development of an in vivo screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 19:2812–2820.
31. Purdom CE, Hardiman PA, Bye V, Eno NC, Tyler CR, Sumpter JP. 1994. Estrogenic effects of effluent from sewage treatment works. *Chem Ecol* 8:275–285.
32. Epifano O, Liang LF, Familari M, Moos MC, Dean J. 1995. Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* 121:1947–1956.
33. Maitre JL, Mercier L, Dolo L, Valotaire Y. 1985. Characterization of specific receptors for estradiol and induction of vitellogenin and its mRNA in the liver of rainbow trout (*Salmo gairdneri*). *Biochimie* 67:215–225.
34. LeGuellec K, Lawless K, Valotaire Y, Kress M, Tenniswood M. 1988. Vitellogenin gene expression in male rainbow trout (*Salmo gairdneri*). *Gen Comp Endocrinol* 71:359–371.
35. Brock ML, Shapiro DJ. 1983. Estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation. *Cell* 34:207–214.
36. Flouriot G, Pakdel F, Ducouret B, Valotaire Y. 1995. Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. *J Mol Endocrinol* 15:143–151.