VITELLOGENIN GENE TRANSCRIPTION: A RELATIVE QUANTITATIVE EXPOSURE INDICATOR OF ENVIRONMENTAL ESTROGENS

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Abstract—We report the development of a quantifiable exposure indicator for measuring the presence of environmental estrogens in aquatic systems. Synthetic oligonucleotides, designed specifically for the vitellogenin gene (Vg) transcription product, were used in a reverse transcription–polymerase chain reaction (RT-PCR) protocol. This extremely sensitive and rapid method was able to detect vitellogenin gene expression in male common carp (Cyprinus carpio) injected with 17β-estradiol. Sequence analysis of the induced mRNA product confirmed a vitellogenin gene transcript with homology to rainbow trout and fathead minnow vitellogenin cDNA sequences. Relative levels of vitellogenin gene induction among individuals were quantified by incorporating 18S ribosomal RNA universal primers and Competimers® in a PCR multiplex reaction with primers for vitellogenin. This method is more sensitive than current protocols, such as mortality, visible signs of stress, or other techniques that look for unscheduled gene expression, because it measures the appearance of primary transcripts at the nanogram level. In addition, this procedure does not sacrifice accuracy or reliability, even though the exposure to estrogen is within 1 d. This research will support the construction of regional stressor profiles, thereby providing a method for comparative environmental exposure assessment. It may also provide an in vivo screening method for potential endocrine-disrupting compounds.

Keywords—Carp Estrogen Gene expression Vitellogenin

INTRODUCTION

The biological and toxicological science literature continues to report evidence of adverse biological changes resulting from the exposure of laboratory animals, wildlife, and humans to a diverse class of chemical stressors collectively referred to as endocrine-disrupting compounds (EDCs) [1–4]. These reports add to the growing body of studies already in print that provide evidence both for and against the role of environmentally persistent chemicals in endocrine disruption [5–7]. To reduce uncertainties in the assessment of risk to both humans and wildlife from exposure to EDCs, government workshops and research plans during the last five years have identified critical research gaps that need to be filled. In 1998, the U.S. Environmental Protection Agency Office of Research and Development published a research plan for endocrine disruptors [8]. As a response to the key scientific question (i.e., “How and to what degree are human and wildlife populations exposed to EDCs?”), the U.S. Environmental Protection Agency Office of Research and Development as well as others [9] proposed to develop exposure screening tools and biomarkers for EDCs. The goal of the present study was to develop a highly specific, molecular indicator that is diagnostic for exposure of aquatic organisms to estrogenic compounds, which are one class of EDCs. This indicator will allow near–real time measurement of total estrogenic activity in aquatic systems and of the bioavailability of known estrogenic compounds, both alone and in mixtures.

Molecular tools applied to biological systems allow sensitive detection of subcellular events induced by EDCs. These events can be analyzed in the tissues of potentially impacted wildlife populations, permitting diagnostic exposure monitoring in ecosystems where appropriate sampling is possible. The present study describes the onset of vitellogenin (Vg) gene expression as an indicator of xenoestrogen exposure in teleosts. This method, which is predicated on examining the state of individual tissue-specific genes, detects de novo gene-specific transcriptional response to chemical exposure.

In laboratory studies, hormonally active and suspected estrogenic compounds have been linked to accumulation of vitellogenin protein in males of numerous teleost species [10–15]. In field-exposure monitoring for EDCs, elevated serum levels of vitellogenin protein in male common carp were detected in fish collected from an effluent channel downstream from a St. Paul, Minnesota, USA, sewage treatment plant [16]. Other studies have noted the xenobiotic induction of plasma egg protein precursor resulting from exposure to effluents of municipal wastewater treatment plants [17–19] and oil refineries [20]. However, the appearance of circulating vitellogenin protein is several cellular processes removed from the onset of gene transcription. Because the protein product is the terminal aspect in a biochemical pathway replete with numerous control mechanisms, determination of circulating proteins does not reflect the immediacy of chemical exposure and, as a method for detection of exposure, lacks sensitivity.

Because the Vg gene is normally quiescent in males, detection of the transcribed gene product (i.e., Vg mRNA) provides a sensitive exposure marker for environmentally present...
estrogenic compounds and other EDCs. The hormone 17β-estradiol has been used to study the induction of Vg gene transcription in rainbow trout (Oncorhynchus mykiss) [21,22] and fathead minnow (Pimephales promelas) [23]. These studies used the Northern blot analysis and ribonuclease protection assay to detect and measure the expression level of specific RNAs in cells or tissue. Inconsistencies in the procedures used to isolate and analyze the RNA samples, however, can introduce errors into the analytical process. In addition, these assays are difficult to perform and lessen the likelihood for the technology transfer of indicator methods to regional and state laboratories.

One method for minimizing these errors is to simultaneously measure a cellular RNA with a constant level of expression among samples. The 18S ribosomal RNA (rRNA) may be used as a standard against which other RNA values can be normalized. Ribosomal RNAs are ubiquitous and less likely to fluctuate under conditions that affect the expression of cellular mRNAs, because they are transcribed by RNA polymerase I in eukaryotic organisms. Some suppliers of molecular biological products have developed technologies for manipulating the amplification efficiency of intact RNA standards without affecting the detection efficiency for target cDNA sequences in the polymerase chain reaction (PCR). Levels of 18S and 28S pre-rRNAs remain at steady state in all tissues and cell types, whereas the profiles of cellular mRNA pools vary markedly [24,25].

This paper describes the development of a quantitative reverse transcription (RT)–PCR method for the detection of Vg gene expression in male common carp (and other aquatic species). The RT-PCR methods are rapid and more sensitive than other mRNA detection methods. Vitellogenin mRNA levels have been standardized relative to 18S rRNA expression levels. We describe the response of standardized vitellogenin mRNA levels (across dose and time) in fish injected with 17β-estradiol. In addition, we present the partial nucleic acid sequence of common carp cDNA for vitellogenin, as characterized from liver RNA following exposure to 17β-estradiol, and we discuss its homology with other published teleost vitellogenin sequences.

MATERIALS AND METHODS

Animals

Sexually mature common carp (Cyprinus carpio) were obtained from Aquatics Research Organisms (Hampton, NJ, USA), tagged for identification, weighed (range, 18–54 g), and maintained in Living Stream® flow-through tanks (Frigid Units, Toledo, OH, USA) at a rate of 1.2 L/min for one week of quarantine before the exposure phase of the experiment. Tank conditions, including pH, alkalinity, hardness, conductivity, dissolved oxygen, and temperature, were maintained at optimal levels before and during the experiment. The fish were fed trout pellets (Zigler Brothers, Elveron, PA, USA) ad libitum throughout the quarantine and exposure phases.

Fish exposure protocol

Fish were intraperitoneally injected with either 0.33, 0.1, 0.033, or 0.01 mg/kg body weight of 17β-estradiol (1,3,5[10]-estradiene-3,17β-diol) obtained from Sigma Chemical (St. Louis, MO, USA) in a dimethyl sulfoxide (DMSO)/peanut oil vehicle. Each fish received an injection volume of 100 μL. Control fish received either no injection or 100 μL of DMSO/peanut oil. Fish were sacrificed at either 24 or 48 h after injection, and sex determination was made at necropsy. Livers from all fish were removed and snap-frozen in liquid nitrogen, although only those of the males were processed for this study. Of the 98 fish injected for the study, 55 were found to be male at necropsy.

Initial weights of fish were used to create experimental design blocks within which fish were assigned randomly to treatment dose and duration. This blocking ensured that the fish biomass in each tank remained constant throughout the experimental exposure. Fish of each dose group were maintained in separate tanks to control for secondary exposure due to seepage from the injection site or excretion of the test substance.

Analysis of nucleic acid sequence

Preliminary indicator development studies were conducted with male carp injected at a dose of 10 mg/kg of 17β-estradiol. The RT-PCR analysis, using the oligonucleotide primers described in the next section, revealed the presence of an amplified cDNA band at approximately 1 kb. To confirm that this PCR product amplified from the primer set was specific for vitellogenin mRNA, the amplified cDNA was electrophoresed and isolated from low-melt GTG agarose (FMC, Rockland, ME, USA). Following spectrophotometric quantification, the recovered product was cloned in the Invitrogen TA vector PCR 2.1® (Invitrogen, Carlsbad, CA, USA).

The DNA sequencing was performed using a d-Rhodamine dye terminator cycle sequencing kit obtained from PE Applied Biosystems (Foster City, CA, USA). Forward- and reverse-primer sequencing was performed on a DNA Thermal Cycler 480® (PE Applied Biosystems). The products of the cycle sequencing reaction were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer® (PE Applied Biosystems). The DNA sequence of the clone was then generated with the automated sequencing analysis software program of the ABI Prism 310.

Analysis of Vg gene transcription

Isolation of total RNA from carp liver. Total RNA was isolated from individual livers by the standard guanidinium isothiocyanate method [26] using American Chemical Society–grade reagents purchased from Sigma Chemical. Briefly, snap-frozen liver was homogenized in 1 ml of 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), and 0.1 M β-mercaptoethanol. The homogenate was then acidified by the addition of 0.1 ml of 2.0 M sodium acetate (pH 4.0), after which the homogenate was briefly extracted with H2O-equilibrated phenol (pH 4.3) and chloroform:isoamyl alcohol (24:1 v/v), with the respective phases separated by centrifugation. Next, the aqueous phase was extracted a second time with 1 ml of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v). The re-extracted aqueous phase was then removed in 500-μl aliquots, to which equal volumes of isopropanol were added. Following incubation at room temperature for 30 min, total RNA was precipitated by centrifugation at 15,000 rpm for 15 min at 4°C. Supernatants were discarded, and precipitated pellets were washed twice with 70% ethanol. A final wash of 95% ethanol was then briefly applied to the RNA and, following decanting of supernatants, pellets were dried in a Laminar flow hood and resuspended in 35 to 50 μL of diethyl pyrocarbonate–treated water, depending on the relative size of the recovered pellet.
The RNA samples were evaluated for purity and spectrophotometrically quantified with a Shimadzu ultraviolet-1601PC (Shimadzu Scientific Instruments, Columbia, MD, USA). Total RNA was subsequently analyzed for structural integrity of 28S and 18S rRNA species by electrophoresis in formaldehyde/agarose (1% 3-(N-morpholino)propanesulfonic acid) gels. Before RT-PCR analysis, RNA samples were diluted to a concentration of 1 μg/μl in diethyl pyrocarbonate—treated water.

Optimization of procedure to quantify Vg gene transcripts. Quantification of Vg gene expression was accomplished with a multiplex PCR reaction using vitellogenin-specific oligonucleotides and Competimer/18S rRNA oligonucleotides (Ambion, Austin, TX, USA). Accurate measurement of the amount of mRNA present can only be accomplished if the amplification cycle number is within the log-linear range of formation of the gene-specific product. To verify the linear nature of amplified product formation, an individual male liver RNA sample with an apparent vitellogenin mRNA copy number in the intermediate range was used. Several identical RT-PCR reactions were simultaneously prepared, and individual tubes were sequentially removed from the PCR machine at single-cycle intervals between cycles 26 and 38. Based on gel electrophoresis and subsequent image analysis, the number of amplification cycles required for detection of low-copy-number vitellogenin mRNA, while still within the log-linear range of intermediate copy number, was empirically determined to be 34 cycles.

The Thermal Cycler 480 and GeneAmp 9600® (PE Applied Biosystems) are used in our laboratory; therefore, the protocol was optimized for both PCR systems. The theoretical optimal annealing temperature (Tm) of the designed oligonucleotides is 59°C; however, no reduction in specific product was observed up to a temperature of 62°C. Because the Competimer/18S rRNA oligonucleotides function efficiently up to 68°C, the selected temperatures of 58 and 60°C for the GeneAmp 9600 and DNA Thermal Cycler 480 models, respectively, are well within the acceptable temperature range.

Following cycle number determination for log-linear formation of vitellogenin cDNA, 18S rRNA universal primers were added to the PCR reaction, with increasing concentrations of 18S Competimers. Although identical in nucleotide sequence to the 18S primers, the Competimer oligonucleotides have a chemically modified 3' end that inhibits polymerization by Taq polymerase. For every attempted experimental quantification across an unknown range of exposures, the ratio of 18S universal primers and Competimers in the PCR reaction must be titrated and the relative amount of amplifiable 18S rRNA adjusted, such that the 18S gel band is of the same relative intensity as that of the vitellogenin-specific PCR product. Because the relative level of rRNA is constant between total RNA preparations, a comparison of the ratio of vitellogenin amplification product and 18S rRNA product permits quantifiable comparison of gene expression among individuals. An empirically determined Competimer/18S ratio of 6:4 allowed quantification through the range of amplified vitellogenin products observed in this study at 34 cycles.

Thermal amplification (RT-PCR). We redesigned the 5' Vg gene primer based on a primer pair that was designed and used in a previous study [22]. Although the original oligonucleotide primers were designed using the rainbow trout Vg gene sequence [27] and demonstrated a species-specific detection of vitellogenin mRNA, the modified primer pair was shown to detect the Vg gene transcript across teleost species.

Oligonucleotide primers used in RT-PCR amplification of Vg gene transcription products were synthesized by New England BioLabs (Beverly, MA, USA) and comprised the following sequences:

5' Vg primer 1: 5'-TGTTGTTGATGCTGGTTCTG-3'
3' Vg primer 2: 5'-GGCTCAACAGTAGCATTGG-3'

Final mass and concentrations for RT reactions followed the manufacturer's recommendation (PE Applied Biosystems): 1 μg of total RNA, 5 mM MgCl2, 1 mM deoxy(guanosine, adenosine, thymidine, cytidine) triphosphates, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 20 U RNase inhibitor, 2.5 mM random hexamers, and 2.5 μl of Moloney murine leukemia virus reverse transcriptase. All components, except for individual sample RNAs and reverse transcriptase, were combined into a master mix and aliquoted into individual, thin-walled tubes.

Before addition of the reverse transcriptase, reactions were heated to 70°C for 5 min, followed by cooling to 23°C over a ramp period of 15 min. This ensured the denaturation of RNA secondary structures and facilitated efficient annealing of random hexamers. The RT reactions were incubated for 60 min at 42°C, followed by 5 min at 99°C.

To increase sensitivity and specificity and to efficiently generate amplification products with two dissimilar sets of primers in PCR reactions (i.e., multiplex), it was necessary to use a hot-start PCR protocol. Components of the PCR reaction, specifically oligonucleotides and target cDNAs, remained physically sequestered by a wax barrier until the entire reaction mix was above the temperature at which nonspecific PCR priming may occur.

The 100-μl volume secondary multiplex reactions contained the following final concentrations: 2 mM MgCl2, 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 2.5 U of AmpliTaq (PE Applied Biosystems), 150 nM of each Vg oligonucleotide primer, and 1 μl of Competimer/18S oligonucleotides in a 6:4 ratio.

Secondary PCR mix I (MgCl2, one-half volume of water and AmpliTaq) was added (36.5 μl) to the RT reaction, and a single AmpliWax PCR Gem 100 bead (PE Applied Biosystems) was dispensed into each reaction tube. The wax bead was melted for 3 min at 78°C, then resolidified at 12°C for 4 min.

Secondary PCR mix II (10× PCR buffer II [PE Applied Biosystems] one-half volume of water, vitellogenin oligonucleotide mix, and appropriate ratio of Competimer/18S rRNA oligonucleotides) was overlaid on the wax barrier (43.5 μl). The PCR reactions were carried out with the following thermal cycle profiles:

DNA Thermal Cycler 480 protocol: 94°C for 2 min; followed by 34 cycles at 94°C for 45 s, 60°C for 30 s, and 72°C for 45 s; and then one cycle at 94°C for 45 s, 60°C for 30 s, and 72°C for 10 min to complete unfinished transcripts.

GeneAmp 9600 protocol: 94°C for 2 min; followed by 34 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and then one cycle of 94°C for 30 s, 58°C for 30 s, and 72°C for 10 min to complete unfinished transcripts.

Electrophoresis and image analysis. Amplified cDNA products were electrophoretically separated in 1.8% SeaKem LE agarose gels (FMC) prepared with 1× TBE (89 mM Tris base, 89 mM boric acid, 2 mM Na2-ethylenediaminetetraacetic acid [pH 8.0]). A volume of 20 μl was removed from each
100-µl PCR amplification reaction and combined with 5.8 µl of gel loading dye (15% Ficoll 400, 0.05% xylene cyanol FF). Agarose gels and electrophoresis buffer (0.6× TBE) were prepared with 5× TBE. Following electrophoresis, agarose gels were stained for 30 min in 1 L of 1× TAE (40 mM Tris acetate, 2 mM Na₂-ethylenediaminetetraacetic acid [pH 7.4]) containing SYBR® Green I Nucleic Acid Gel Stain (Molecular Probes, Eugene, OR, USA) per the manufacturer’s recommendation with no destaining step. Gels were scanned digitally on a FluorImager 595® (Molecular Dynamics, Sunnyvale, CA, USA), and resultant images were analyzed with ImageQuant® (Molecular Dynamics) software. The ImageQuant software quantifies and reports the magnitude of the fluorescence within a user-defined rectangle on the gel image. For consistency of fluorescence measurement across individual lanes on one gel and for lanes on different gels, rectangles of equal size were located at the appropriate known location representing the migration of the vitellogenin and 18S rRNA RT-PCR products from each sample. The size of the rectangle was designed to be large enough to completely encompass the largest vitellogenin band observed, and rectangles were positioned for vitellogenin even when no band was visually apparent. Because of the large amount of background present in the rectangle relative to the band (for all but the brightest bands), the total background fluorescence value for each rectangle reported by the ImageQuant program was corrected by subtracting the background for the pixels above background in the rectangle.

Data analysis and statistics

Minor variations in gel loading quantities for each sample, current applied during gel electrophoresis, and staining times as well as efficiencies can result in variations of band intensities that can be considerable among gels. Such gel-to-gel variation, along with any inconsistencies in isolation of RNA and subsequent RT-PCR, might obscure any differences among individuals due to dose and duration of exposure to 17β-estradiol. Thus, rather than use the intensity values for the vitellogenin band alone, the ratio of the vitellogenin band to that of the large amount of background present in the rectangle relative to the band (for all but the brightest bands), the total background fluorescence value for each rectangle reported by the ImageQuant program was corrected by subtracting the background for the pixels above background in the rectangle.

RESULTS

Identification of 17β-estradiol–induced liver mRNA

The PCR amplification product induced by 17β-estradiol was confirmed to have identity with vitellogenin mRNA by nucleic acid sequencing. The amplified partial cDNA sequence for common carp vitellogenin is shown in Figure 1. The BLAST [28] analysis (National Center for Biotechnology Information, Bethesda, MD, USA) of this sequence indicated that the 5’ portion (nucleotides 3–418) of the common carp partial vitellogenin cDNA exhibits 82% homology with the 3’ region of fathead minnow (Pimephales promelas) vitellogenin precursor mRNA sequence (nucleotides 3,574–3,986 of 4,020 nucleotides) [23]. The partial coding sequence also has 82% homology with a 253-base region of the rainbow trout 3’ vitellogenin mRNA. Other minor regions of sequence identity were noted in Fundulus heteroclitus [29] and Oreochromis aureus [27,30].

Measurement of 17β-estradiol-induced liver mRNA levels

Following exposure to 17β-estradiol, total RNAs isolated from livers of male common carp were analyzed for expression of vitellogenin mRNA. Figure 2 shows an agarose gel displaying the vitellogenin and 18S rRNA products generated in the multiplex RT-PCR reaction. The vitellogenin cDNA bands migrate electrophoretically with a mass of 1,081 bp; the 18S amplification products have a mass of approximately 468 bp. The range in the magnitude of Vg gene expression is readily apparent among individuals, especially when compared to the nearly equivalent amplification of 18S cDNA across all treatment groups.

A priori, it was of interest to study sources of variation in various components of these analyses (e.g., different electrophoretic runs [gels]). After fish samples were randomly assigned to particular gels, the average intensity of both the vitellogenin and the 18S rRNA bands was observed to differ significantly among gels (p = 0.03 and 0.001, respectively). These differences were independent of the distribution of samples by estradiol dose or duration of exposure among gels. Expressing the vitellogenin response as the ratio of vitellogenin to 18S rRNA eliminated this gel-to-gel variation (p = 0.60), thus reducing uncertainty of comparison of vitellogenin response for samples run on different gels.

Comparison of carrier-injected and noninjected controls showed no effect of dose, duration of exposure, or interaction (p > 0.35). Thus, the two types of controls were pooled for all further analyses.

The vitellogenin:18S ratios were unchanged by duration of exposure (24 vs 48 h; data not shown), nor did the duration of exposure influence the significant dose trends observed. Thus, the data for both durations were pooled for the final analysis of dose effect. Relative fluorescence values are presented for the control oil-injected fish (Table 1). These values were essentially background fluorescence of the agarose gel, because at no time were vitellogenin-specific amplification

Fig. 1. Partial cDNA sequence of vitellogenin gene (Vg) reverse transcription-polymerase chain reaction (RT-PCR) amplification product obtained from male common carp exposed to 17β-estradiol.

1 tggtxctgta tggtxctgta tggtxctgta cacacagctac acaaatgccac tggctcggtt 121 cccctcgtgc ctgtxctgta tggtxctgta attttacca caatactgc
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781 cttcctctgc tggctgctgag gcgtgctgaa gcgtgctgag gcgtgctgag gcgtgctgag 841 cagctcctctgc cgcgtgctgaa tcgtcctctgc cgcgtgctgaa gcgtgctgaa gcgtgctgaa 901 aggccacacgt cgggttgaa cgggttgaa cgggttgaa cgggttgaa cgggttgaa 961 gggctgctgag gcgtgctgag gcgtgctgaa gcgtgctgaa gcgtgctgaa gcgtgctgaa 1021 tggctgctgaa gcgtgctgaa gcgtgctgaa gcgtgctgaa gcgtgctgaa gcgtgctgaa 1081 c
products observed in control animals. The ratio of Vg gene expression to 18S rRNA expression for the lowest dose was not statistically different from that of the controls. At the lowest 17β-estradiol dose (0.01 mg/kg), 6 of 11 fish showed clear vitellogenin bands. The 0.1-mg/kg dose showed the highest mean ratio, 1.708, which was significantly different from the means of each of the other doses (Table 1). The 0.33-mg/kg dose had the next highest mean ratio, 0.756, which was not significantly different from the mean ratio of 0.409 obtained in the 0.033-mg/kg dose group. The mean ratio of the 0.033-mg/kg dose also was not statistically distinguishable from either that of the 0.01-mg/kg dose group (mean ratio, 0.089) or of the pooled control group (mean ratio, 0.071) (Table 1).

**DISCUSSION**

A straightforward and effective method has been developed to assess the immediate activity of synthetic estrogenic chemical compounds and xenostrogens. We have applied the method of thermal cycle amplification (i.e., PCR) with synthetic oligonucleotides to detect the appearance of the Vg gene transcription product in male common carp. Under normal conditions, activation of this gene does not occur in male fish; therefore, detection of the transcriptional switch that results in the onset of vitellogenin mRNA provides a sensitive indicator of exposure to estrogenic compounds. Using the nucleotide sequence available from rainbow trout [21], Vg gene-specific synthetic oligonucleotides were designed, using Oligo Primer Analysis Software® [31], that detects the onset of gene expression in teleosts from families as diverse as Salmonidae and Cyprinidae. We have observed Vg gene expression in 17β-estradiol-injected rainbow trout (Oncorhynchus mykiss) and yellow perch (Perca flavescens) with the same molecular methods described herein (data not shown).

We have overcome the difficulty in quantification, which historically has limited the application of RT-PCR methods, by use of a multiplex control PCR approach. The extreme sensitivity of the PCR method renders the technique subject to influence of its kinetics by a host of factors. Among these are variations in RNA isolation, initial quantification errors, and between-tube variations in both RT and secondary PCR reactions. It is possible to control for the efficiency of thermal amplification by spiking reactions with exogenous control DNA. This necessitates that the control sequences be present at a level similar to that of the specific test sequences. For this reason, we have adopted a method developed by Ambion for quantitative comparison to the endogenous sequence standard for the structural transcripts of 18S rRNA. The expression of rRNA, which is the primary component of total RNA, is ostensibly invariant during the cell cycle and between cell types; therefore, the relative level of rRNA remains essentially constant from sample to sample. Ribosomal RNA has also been shown to be refractory to experimental treatment with numerous toxic and biochemical reagents [24,25].

The intraperitoneal-injection experiments were designed to show the feasibility of new RT-PCR methods to detect quantifiable Vg gene expression. Significant increases in relative

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Table 1. Dose group sample sizes, means, and standard deviations for values used to quantify vitellogenin gene induction in male carp injected with 17β-estradiol

<table>
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<th>Dose (mg/kg)</th>
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<th>Mean (fluorescence units × 10⁶)</th>
<th>SD</th>
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*Within a column, means not sharing a common letter are significantly different from one another (p < 0.05); p values were derived from simple pair-wise t tests, unadjusted for multiple comparisons.

* SD = standard deviation.
Vg gene expression were observed with increasing 17β-estradiol dose up to 0.1 mg/kg. Our statistical model, which is based on vitellogenin expression relative to 18S rRNA expression, showed no significant differences between the controls and the lowest dose (0.01 mg/kg), but vitellogenin-specific amplification products were visible in some of the lowest-dose fish (no bands were present in the controls). It was understood that our ability to detect significant differences at the lowest dose might be compromised by the variability inherent to steroid emulsion injections. As addressed earlier, preliminary studies with Vg gene expression in fathead minnows (Pimephales promelas) confirm the ability of this method to detect changes in gene expression at extremely low levels of aquatic exposure (parts per billion to parts per trillion).

Our investigations indicate that the highest concentration of 17β-estradiol tested results in somewhat less vitellogenin amplification product than the next highest dose. Again, the biological relevance of these highest estradiol concentrations is open to question. This attenuation could result from the intraperitoneal route of exposure and the concomitant metabolic pathway. Because the injection volume was constant, the lower dose of estradiol was present at a lower concentration in the peanut oil carrier. A metabolic breakdown of the greater relative amount of peanut oil could cause the estradiol to be delivered in a time-release manner, thus maintaining Vg gene induction for a longer period of time. Although the kinetics of induced mRNA synthesis in this study are unknown, the decrease in Vg message pool at higher dose concentrations could also reflect an early temporal spike in gene transcription products, with a concomitant, rapid rate of mRNA turnover before the sampling time points. Other experiments have demonstrated that exposure to cortisol induced immediate, but short-lived, Vg gene expression in male Oreochromis aureus [32].

A number of laboratory and field studies have focused on measurement of circulating vitellogenin protein in fish plasma. As stated earlier, changes in levels of vitellogenin protein occur following a number of cellular events after the induction of gene expression. These events themselves are regulated by other factors. A recent study [33] reported a lack of correlation between levels of the xenoestrogen nonylphenol and those of plasma vitellogenin in the fathead minnow (Pimephales promelas). Those authors state, with reference to their own and other studies, that whereas induction of vitellogenin synthesis by the liver for release into the plasma is controlled by the estrogen receptor, the relationship between concentrations of 17β-estradiol and vitellogenin in the plasma may not be as strong. The present study on the common carp provides evidence of the close correlation between estrogenic exposure and changes in Vg gene expression. This initial study, which likely is subject to extra variation because of the intraperitoneal-injection protocol used, has been followed in our laboratory by studies of Vg gene expression in the fathead minnow. In an identical approach to the one described here, we have designed vitellogenin-specific oligonucleotides for this commonly found, U.S. Environmental Protection Agency toxicological standard model. Preliminary results from laboratory validation studies show a clear, dose-dependent increase in Vg gene expression at environmentally relevant doses of 17α-ethynylestradiol. These data suggest that the fathead minnow is favorable for use as an EDC indicator, both in field paradigms and in laboratory testing of water from environmental sources. We are currently investigating the fathead minnow model as a indicator of chemical exposure in surrogate ecosystems (i.e., mesocosms) and in numerous environmentally contaminated locations.

Thus far, the long-term physiological effects and ecological relevance of vitellogenesis resulting from exposure to hormonal mimics are subject to debate and speculation. Others [34,35] have suggested, and it is widely held, that an acute need exists for continued investigation focusing on organismal and population effects of environmental EDCs. The method described here provides not only a sensitive indicator of aquatic exposure but, also, a means to link observed changes in expression of the Vg gene with long-term effects, including physiological, reproductive, and behavioral anomalies [18,19,36]. This method, in contrast to cell- or yeast-based assays, also provides an important link to interpreting biological effects and physiological relevance of chemical exposure. Although cell-based [37,38] in vitro screening systems are capable of indicating chemical exposure and the possible degree thereof, extrapolating bioavailability and potential effects on biological endpoints from such approaches is more difficult.

The method described here presents an expedient strategy for assessing the in vivo estrogenic potential of chemicals, including pesticides, in fish. This is accomplished by quantifying the degree to which the Vg gene is induced to transcribe mRNA. The analytical scheme detailed herein is designed for rapid assessment and is accomplished without use of radio-nucleides. For this method to have regional environmental applicability, it is necessary to quantify expression profiles for other chemical compounds and genes of interest. If suitable baselines for exposure limits and dose relationships can be described, then immediate quantities of vitellogenin mRNA will serve as a reliable indicator of EDC exposure levels.

REFERENCES


