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Evaluation Of Municipal Wastewater In The Vicinity Of Birmingham, Alabama For Estrogens Using Vitellogenin Gene Expression In Largescale Stonerollers (Campostoma Oligolepis)

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EVALUATION OF MUNICIPAL WASTEWATER IN THE VICINITY OF BIRMINGHAM, ALABAMA FOR ESTROGENS USING VITELLOGENIN GENE EXPRESSION IN LARGESCALE STONEROLLERS (*CAMPOSTOMA OLIGOLEPIS*)

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BIOLOGY

ABSTRACT

Environmental endocrine disrupting compounds (EDCs) have become a growing threat to the health of humans and wildlife. Numerous studies have demonstrated the presence of EDCs in freshwater aquatic environments. Environmental estrogens, both natural and synthetic, appear to be posing a threat to the reproductive fitness of aquatic organisms. Wastewater treatment plants (WWTPs) have been identified as significant routes by which the EDCs enter waterways. The egg yolk precursor protein vitellogenin (VTG) has proven to be a useful biomarker that can be used to identify organisms that have been exposed to environmental estrogens. In this study, a quantitative RT-PCR assay for expression of the VTG gene in the largescale stoneroller (*Campostoma oligolepis*) was developed. The dose-response pattern of VTG gene expression in largescale stonerollers exposed to various known concentrations of 17α-ethynylestradiol (EE2) was characterized. The newly developed assay was used to investigate the effects of the effluents of three WWTPs in the vicinity of Birmingham, Alabama on VTG gene expression in the largescale stoneroller. The largescale stoneroller has not previously been used as a biomonitor organism for the effects of environmental estrogens, and therefore one purpose of this study was to establish it as a potential model organism for future studies, particularly in the southeastern region of the United States, where this species is native. In order to design RT-PCR primers specific to largescale stoneroller

VTG, a partial sequence of the VTG gene was obtained. This obtained sequence showed high homology to the VTG sequences of other fish in the Cyprinidae family. Next RT-PCR primers were designed for the obtained VTG sequence and for ribosomal protein L8 (RPL8), which was used as a normalizing gene. This RT-PCR assay was effective in detecting increased VTG gene expression in largescale stonerollers exposed to dietary EE2. It also showed that largescale stoneroller VTG expression was affected by EE2 in a dose-dependent manner. This assay was also effective in detecting increased VTG gene expression in stonerollers collected below the outfall of one of the three WWTPs included in this study.

Keywords: Endocrine Disruption, Wastewater Treatment Plant, Vitellogenin, Real-Time Polymerase Chain Reaction, Environmental Estrogens, 17α-ethynylestradiol

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LIST OF ABBREVIATIONS

BP	base pair
BPA	bisphenol A
EDC	endocrine disrupting compound
EPA	Environmental Protection Agency
E2	estradiol
E1	estrone
EE2	17α-ethynylestradiol
RPL8	ribosomal protein L8
VTG	vitellogenin
WWTP	wastewater treatment plant

INTRODUCTION

In recent years, it has become apparent that endocrine disrupting compounds (EDCs) have become a growing threat to the health of humans and wildlife. The term endocrine disrupting compound refers to "any exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (Kavlock et al., 1996). Because of the potential health threats that EDCs pose, it is important to examine potential sources of these contaminants. Numerous studies have shown that the effluent from wastewater treatment plants (WWTPs) is a point source of EDCs (Snyder et al., 2001; Cargouet et al., 2004; Sarmah et al., 2006). In particular, WWTPs have been identified as significant routes by which environmental estrogens enter waterways (Fernandez et al., 2007). This growing source of EDCs, appears to be posing the greatest threat to the reproductive fitness of aquatic organisms (Shi, Kujawa-Roeleveld, 2007; Colborn et al., 1993; Jobling et al., 1998; Tyler et al., 1998; Lange et al., 2009). Freshwater systems are particularly vulnerable to environmental estrogens coming from WWTPs due to their proximity to these sources (Solè et al., 2001).

Since they are restricted to the aquatic environment, fish serve as good indicators of the quality of an aquatic ecosystem. There is increasing evidence for endocrine disruption in freshwater fish downstream of WWTP discharges. In the original endocrine disruption studies, conducted on freshwater fish in the United Kingdom, it was shown

that effluents from WWTPs stimulated expression of the estrogen-responsive gene that produces the protein vitellogenin (VTG) in male fish (Purdom et al., 1994). VTG is the egg yolk precursor protein that is normally produced in the liver of oviparous females in response to endogenous estrogen circulating in the blood plasma, and the VTG protein is then secreted into the plasma. Although males possess the VTG gene, it is not usually expressed in males due to very low levels of estrogen present in the plasma. However, exposure to estrogen in the aquatic environment is well known to induce expression of this gene. Thus, the presence of VTG in the plasma as well as the presence of VTG mRNA in the liver of male fish is widely accepted as a biomarker of estrogen exposure in the aquatic environment (Sumpter and Jobling, 1995; Heppell et al., 1995).

In addition to VTG production, exposure to treated wastewater effluents has also been associated with an increased incidence of intersex males in various species of fish. One of the most intensively studied is the roach (*Rutilus rutilus*), a cyprinid fish common throughout lowland rivers in the United Kingdom and Europe. Most intersex roach have female germ cells, or oocytes, within a predominantly male gonad and malformed or intersex reproductive ducts (Nolan et al., 2001). The number, pattern, and developmental stage of oocytes within testicular tissue in intersex roach vary greatly, and the more severely feminized fish contain large areas of ovarian tissue that are clearly separated from testicular tissue (Nolan et al., 2001). Compared to normal male fish, intersex male roach have reduced sperm counts, reduced sperm motility, and lower fertilization rates; a small number of wild roach in UK rivers were found that could not produce any gametes at all due to the presence of severely disrupted gonadal ducts (Jobling et al., 2002). Intersex roach also often have altered plasma sex steroid hormone concentrations,

elevated concentration of plasma VTG, and inhibited gonadal growth, suggesting that these effects are all caused by estrogenic chemicals in their environment (Jobling et al., 2002).

The naturally occurring estrogenic hormones, estrone (E1) and estradiol (E2), secreted by humans and animals and the synthetically prepared contraceptive, ethynylestradiol (EE2), used by women show the highest degree of estrogenic activity in aquatic environments (Desbrow et al., 1998; Fang et al., 2000). These hormones, when secreted by humans, are not always completely removed from the wastewater by current treatment methods and have been shown to enter the aquatic environment in the effluents of WWTPs. Steroid hormones, including E1 and E2, are excreted in urine of humans in the biologically inactive conjugated forms of glucuronides and sulfates (Shackleton, 1986). The conjugates are more polar and water-soluble than free estrogens, and estrogenic activity decrease remarkably with the addition of conjugate groups. However, once the conjugated estrogens reach WWTPs, they can undergo chemical or enzymatic dissociation in bacterial sludge and re-form active estrogens (Ternes et al., 1999; Ternes, Mueller, 1999).

The presence of estrogenic steroids in WWTP effluent has become a worldwide problem. Estrogenic steroids have been detected in influents and effluents of WWTPs in several different countries, including Italy, the Netherlands, Germany, Canada, the UK, Japan, and the United States (Desbrow et al., 1998; Ternes, Mueller, 1999; Baronti et al., 2000; Belfroid et al., 1999; Kuch, Ballschmitter, 2001; Nasu et al., 2000; Snyder et al., 1999). Furthermore, laboratory experiments have shown that environmentally relevant concentrations of these estrogenic steroids can impair the reproductive fitness of aquatic

organisms. For example, zebrafish (*Danio rerio*) exposed to E2 (100ng/L) were shown to have elevated VTG levels, modified secondary sexual characteristics and decreased egg production (Brion et al., 2004). It has also been shown that EE2 (0.3 ng/L and 4 ng/L) can have a dose-dependent effect on gonadal development and the production of sex responsive proteins in wild roach and that EE2 exposure during early life can sensitize the fish and make them more responsive if exposed again later in life (Lange et al., 2009).

In addition to steroid estrogens, nonsteroidal estrogen mimics have also been found in WWTP effluents. Alkylphenols and bisphenols are the most common. Alkylphenols, such as 4-t-octylphenol and 4-nonylphenol, are mainly used to make alkylphenol ethoxylates, a class of synthetic surfactants used in detergents and cleaning products. Bisphenols, such as bisphenol A (BPA), are used in the production of plastics. These estrogen mimics are much less potent than steroid estrogens, but they are more environmentally persistent, tend to bioaccumulate in animals, and have additive effects with each other and with steroid estrogens (Jobling and Tyler, 2003). The concentrations of BPA and nonylphenol measured in streams and wastewater effluent are most frequently reported in the 0.0088 to 12 µg/L range for BPA and 0.110 to 40 µg/L range for nonylphenol (Koplin et al., 2002; Vethaak et al., 2005; Fernandez et al., 2007). Both alkylphenols and bisphenols have been shown to have endocrine disrupting effects in laboratory studies. For example, both nonylphenol and octylphenol induced a significant increase of plasma VTG and a decrease of testis growth in rainbow trout (*Oncorhynchus mykiss*) at environmentally relevant concentrations of 30 µg/L (Jobling et al., 1996). And concentrations of BPA as low as 16 µg/L have been shown to interfere with

spermatogenesis in mature male fathead minnows (*Pimephales promelas*) (Sohoni et al, 2001).

In the United States, the fathead minnow is commonly used as a model organism for aquatic EDC studies. This is the model currently being used by the Environmental Protection Agency (EPA). The fathead minnow is a cyprinid fish that is most commonly distributed from central Canada south along the Rockies to Texas, and east to Virginia and the Northeastern United States. Fathead minnows exposed to WWTP effluent from sites in the United States, including plants in Texas, Colorado, and Minnesota, had elevated VTG levels, inhibited gonadal growth, and suppressed expression of secondary sexual characteristics (Hemming et al., 2001; Martinovic et al, 2006; Vajda et al., 2011). Fathead minnows have also been used as a model for determining the estrogenic effects of WWTP effluent in Canada. In a seven year whole lake study conducted by the Department of Fisheries and Oceans Canada with their EPA partners, chronic exposure of fathead minnows to environmentally relevant concentrations of EE2 (5-6 ng/L) led to feminization of males through production of VTG mRNA and protein, continued production of VTG in females beyond the normal breeding season, impacts on gonadal development, as evidenced by intersex in males and altered oogenesis in females, and a near extinction of this species from the lake (Kidd et al., 2007).

Although many studies have already been conducted using VTG as a biomarker of estrogen exposure, in order to examine the worldwide threat of EDCs, more species need to be studied. The fathead minnow is a popular model in the United States, but its natural range does not include the southeast. The largescale stoneroller (*Campostoma oligolepis*) is an oviparous fish commonly found in the upper Mississippi River and Lake

Michigan drainages of Wisconsin, eastern Minnesota, eastern Iowa, and northern Illinois; Ozarkian streams of central and southern Missouri, and northern Arkansas; Mobile Bay drainage of Georgia, Alabama, and eastern Mississippi; parts of Green, Cumberland, and Tennessee River drainages of Kentucky, Tennessee, Georgia, and Alabama (Burr and Cashner, 1983). It inhabits rocky riffles and runs of clear creeks and small-to-medium rivers. It is considered to be "pollution tolerant" and is able to persist in streams that contaminants have made unsuitable for other species (Burr and Cashner, 1983). Males can be distinguished from females during the breeding season, from early March through April, by the presence of breeding tubercles on the head. One purpose of this study was to establish the largescale stoneroller as a model organism for monitoring estrogenic contamination in the aquatic environment, particularly in the southeastern region of the United States, where this species is native.

One important aspect of a model organism for endocrine-disruption studies is the ability to determine when the VTG gene has been induced. Thus, an assay for VTG gene expression must be developed. We chose to determine when the gene was "on" by measuring the amount of VTG mRNA expression using the RT-PCR technique. This required a VTG-specific primer which did not exist previously since this organism has not been used before. First, a partial sequence of the VTG gene in largescale stonerollers was obtained by amplifying a segment of the gene using primers designed based on conserved regions of the VTG gene in other fish in the Cyprinidae family and then sequencing the amplified DNA. The obtained partial VTG sequence was then used to design primers for quantitative real time RT-PCR analysis. In order to determine the relative rate of expression of the VTG gene, RT-PCR analysis was performed on both the

VTG gene and a housekeeping gene, ribosomal protein L8 (RPL8). The ratio between the mRNA levels of these two genes was used to quantify the VTG gene expression.

The newly developed RT-PCR assay was used to study the effects of EE2 and some WWTP effluents on VTG gene expression in largescale stonerollers. Fish collected upstream of the local WWTPs, and presumably not exposed to environmental estrogens, were exposed to varying concentrations of EE2 in the lab, and RT-PCR analysis was performed in order to determine the dose response of VTG expression. Next, RT-PCR was performed on fish collected from three local WWTPs in order to investigate the effect of WWTP effluent on VTG expression in largescale stonerollers.

The objectives of this study were to (1) develop a quantitative RT-PCR assay for expression of the VTG gene in the largescale stoneroller, (2) characterize the dose-response pattern of VTG gene expression in largescale stonerollers exposed to various known concentrations of EE2, and (3) use the newly developed assay to determine the effects of some WWTP effluents in the vicinity of Birmingham, Alabama on VTG gene expression in the largescale stoneroller.

MATERIALS AND METHODS

Animals

For hormone treatments, juvenile largescale stonerollers (*Campostoma oligolepis*) were collected from Fivemile Creek in Tarrant, Alabama. Since this site is upstream of any WWTPs the fish were presumably not exposed to environmental estrogens. After collection, fish were returned to the University of Alabama at Birmingham and allowed to acclimate for one week in 20 gallon aquaria (10 fish per aquarium) containing carbon-filtered dechlorinated tap water. They were fed Tetrafin goldfish flakes (Tetra Werke, Melle, Germany) *ad libitum* once daily. The acclimated fish were randomly assigned to estrogen exposure treatments (n=10 per treatment).

For the evaluation of the estrogenicity of local WWTP effluent, juvenile largescale stonerollers were collected downstream from three WWTPs in Jefferson County, Alabama (n=10 per WWTP). Fish were returned to the University of Alabama at Birmingham and immediately euthanized with MS-222. Livers were extracted, placed in RNAlater (Ambion, Austin, TX) and stored at -20°C for up to 4 weeks.

Estrogen Treatments

To prepare hormone-treated food, 17α-ethynylestradiol (EE2 ; Steraloids, Inc., Newport, RI) was dissolved in ethanol, and the solution was added to fish food, mixed thoroughly, and allowed to evaporate. Fish were placed in 3.8 liter glass jars (1 fish per jar) containing aerated dechlorinated tap water and fed hormone-treated food *ad libitum* once daily. For a positive control, necessary to obtain a partial VTG sequence, fish were fed 100µg EE2/g food for 7 days. For the dose-response characterization, fish (n=10 per

treatment) were fed 0, 1, 10, and 100µg EE2/g food for 7 days. Water was changed completely on alternate days. After treatment, fish were euthanized with MS-222, and their livers were extracted. Livers were placed in RNAlater (Ambion, Austin, TX) and stored at -20°C.

RNA Isolation and Reverse Transcription

RNA extraction was performed with the RNAqueous-Micro kit (Ambion) following the manufacturer's protocol. Tissues were homogenized in 100 µL of lysis solution using 2.8 mm ceramic bead tubes (Mobio, Carlsbad, CA) in an Eppendorf homogenizer at maximum speed for two 60 sec bursts. RNA was eluted in 20 µL of elution buffer followed by the DNase protocol to remove residual genomic DNA prior to quantification with a Biophotometer Plus with Helma Tray Cell (Eppendorf, Hauppauge, NY). Total RNA had 260/280 absorbance ratios of 1.8–2.0 in elution buffer. Immediately following RNA extraction, 1 µg of total RNA from each liver was reverse transcribed in a 20 µL reaction employing both oligo(dt)15 and random hexamer primers with the 2-step RT-qPCR kit (Promega, Madison, WI).

Identification and Sequencing of *C. oligolepis* VTG

To isolate a VTG cDNA fragment, primers were designed from a 506 base pair (bp) conserved region by alignment of available VTG mRNA sequences from fathead minnow (*Pimephales promelas*, AF130354), common carp (*Cyprinus carpio*, AF414432), and zebrafish (*Danio rerio*, AF406784) using PrimerQuest^{SM} (Integrated DNA Technologies). The primers of VTG cDNA are listed in Table 1.

Primer	Sequence
Forward VTG	5'-AATGCTGGTCACCCTGCTAGTCTT-3'
Reverse VTG	5'-GGCGTCATTGATCATGTAGGCACT-3'

Table 1. PCR primers used for amplifying largescale stoneroller VTG partial sequence.

The cDNA of the positive control group treated with 100 µg EE2/g food was used as a template for the amplification of the VTG gene. The 25 µL PCR reactions were assembled on ice using reagents from the KOD Hot Start DNA Polymerase kit (Novagen, Foster City, CA) as follows: 2.5 µL cDNA, 10 µM forward and reverse primers, 0.5 µL DNA polymerase, 2.5 µL 10X buffer, 2 µL magnesium sulfate, 2.5 µL dNTPs, and sterile water to bring the total reaction volume to 25 µL. The PCR parameters were as follows: initial denaturation at 95°C (2 min) followed by 40 cycles of 95°C (20 sec), 55°C (20 sec), 70°C (1 min), with a final extension of 70°C (10 min). The products were analyzed on a 1.2% agarose gel stained with ethidium bromide. The amplified bands were cut and purified from agarose gel using Wizard® PCR Preps DNA Purification System (Promega). PCR samples were sent to the Heflin Center for Genomic Science at the University of Alabama at Birmingham for sequencing. The sequence was aligned with previously published sequences for VTG of fathead minnow, common carp, and zebrafish.

Quantitative Real-Time PCR

Real-time PCR primers were designed for VTG and RPL8 using Integrated DNA Technologies (Coralville, IA) PrimerQuest^{SM} software for qPCR primers. Real-time PCR primers are listed in Table 2. RPL8 (ribosomal protein L8) was used as a normalizing gene. It has been previously validated as a stable reference gene in studies of estrogen effects in fish (Filby and Tyler, 2007). Both primer pairs were optimized for annealing temperature, and melting curve analyses were performed to ensure amplification of a single product. VTG and RPL8 transcripts were amplified in triplicate from 10 samples from each hormone treatment group and each WWTP group (each sample consisting of liver tissue from one fish) using GoTaq qPCR Master Mix (Promega) on an Eppendorf Mastercycler. Each reaction well contained 7.5 µL GoTaq qPCR Master Mix, 5 µM of each forward and reverse primer, 1 µL synthesized cDNA, and sterile water to bring the total reaction volume to 15 µL. The thermal profile was 95°C for 10 min to activate the DNA polymerase followed by 40 cycles of RT-PCR (95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec) and a dissociation cycle to generate a melt curve. Several controls were included on each plate to control for the specific measurement of cDNA synthesized from the reverse transcription reaction. Two negative controls were included, a no template control in which water was added instead of template, and a no reverse transcriptase control. These controls ensured that the samples were free of genomic and exogenous DNA contamination. Finally, positive controls were run on each plate for an interplate calibrator. Efficiencies of real-time PCR primers were calculated from the slope of standard curves for RPL8 and VTG to validate relative quantification methods.

Primer	Sequence
Forward VTG	5'-TGCCTATTCCCACATCAAGTC-3'
Reverse VTG	5'-CTGTCCAGTTTGCGATTCATG-3'
Forward RPL8	5'-AGGTCATCTCTTCCGCAAAC-3'
Reverse RPL8	5'-TTCCTCTTGGCCTTGTACTTG-3'

Table 2. Primer sequences used for RT-PCR of a *C. oligolepis* VTG gene transcript.

Statistical Analysis

VTG mRNA expression levels were compared between hormone treatment groups and the control using the nonparametric Mann-Whitney U test. Fold changes calculated from ΔΔCt values were used for each group analyzed. The proportion of fish in the WWTP groups with the VTG gene upregulated were compared to the proportion of fish with the VTG gene upregulated in the control group using the Fisher exact test. Upregulation of the VTG gene was defined as a $\Delta\Delta Ct > 28$, which was the median value for the group treated with 1 μg EE2/g food. The cutoff for statistical significance was $p < 0.05$.

RESULTS

Identification and Sequencing of *C. oligolepis* VTG

A fragment of VTG cDNA from largescale stoneroller liver was amplified by PCR. The resulting fragment was purified and sequenced, and a 443 bp sequence was obtained (Fig. 1). The obtained sequence was confirmed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and showed 97% homology to the nucleotide sequence of fathead minnow, 91% homology to common carp, and 89% homology to that of zebrafish VTG. The deduced 147 amino acid sequence for largescale stoneroller VTG showed 95%, 90%, and 84% homology to fathead minnow, common carp, and zebrafish, respectively (Fig. 2).

```
Campostoma oligolepis     CTGAGAACTGCAGCTACTTCTCTGCCTCTTAAAGTCCAGGTTGATGCCGTCTTTGCTCTG  60
Pimephales promelas       ......................A.............................A....G......  1566
Cyprinius carpio          ...............T.A........C....G.................A....G..C...  1580
Danio rerio               ................A.G..T....CA...G.................A....G..C...  1579

Campostoma oligolepis     AGGAACATTGCCAAAAAAGAGCACAAACTGGTTCAGCCAGTGGCCCTGCAGCTTGTATTG  120
Pimephales promelas       ..............G.............................................  1626
Cyprinius carpio          ..............G.....A.CT.....................................  1640
Danio rerio               ..A...........G.....A.C...............T..T..............T...  1639

Campostoma oligolepis     GACAGGGCTCTCCACCCTGAAGTGCGTATGGTTGCTTGTATTGTGTTGTTCGAGGCCAAG  180
Pimephales promelas       ..............T.............................................  1686
Cyprinius carpio          ..T..A...........A..G.....C.......................T...T.....  1700
Danio rerio               .................A..G.....C...................C....T...A.TG..  1699

Campostoma oligolepis     CCCTCAGTGGCTCTCGTCTCCAATCTTGCTGGTGCTTTGAAGACTGAGACTAACATGCAT  240
Pimephales promelas       ...................................................G.........  1746
Cyprinius carpio          ..A.....................G...G..................A..A..C.........  1760
Danio rerio               ..T............A......G.........A.......G.......C.A.........  1759

Campostoma oligolepis     GTTGCGAGCTTTGCCTATTCCCACATCAAGTCCTTGACCAGAATCACTGCACCTGATATG  300
Pimephales promelas       .....A.....................................................  1806
Cyprinius carpio          ....T.........T.....................................T.........  1820
Danio rerio               .....A.............................................T.........  1819

Campostoma oligolepis     GCATCTGTTGCGGGTGCAGCTAATGTTGCCATCAAGCTCATGAATCGCAAACTGGACAGG  360
Pimephales promelas       ...C.......................................G.................  1866
Cyprinius carpio          ...G.......T..............................GC.....G..A.....A  1880
Danio rerio               ....A......T.........................T.....CT....G........A  1879

Campostoma oligolepis     CTTAACTTCCGTTTCAGCAGAGCCATTCAGCTGGACTTCTATCATACTCCTCTTATGATT  420
Pimephales promelas       ....GT.....................................C...............  1926
Cyprinius carpio          ....G...................C.............A.....................  1940
Danio rerio               .......A.....A.........TT..A..A......AT...T.................  1939

Campostoma oligolepis     GGAGCTGCTGGTAGTGCCTACAT  443
Pimephales promelas       ........................  1949
Cyprinius carpio          ...........C............  1963
Danio rerio               ........................  1959
```

Figure 1. Sequence alignment for the largescale stoneroller, fathead minnow (AF130354), common carp (AF414432), and zebrafish (AF406784) VTG. Differences from the largescale stoneroller are shown and identities are indicated with dots. Primer sequences for real time PCR are underlined.

```
Campostoma oligolepis     MRTAATSLPLKVQVDAVFALRNIAKKEHKLVQPVALQLVLDRALHPEVRMVACIVLFEAK  60
Pimephales promelas       L......M........IL.........................................  1686
Cyprinius carpio          L....S....R.....IL.........P..............................S.  1700
Danio rerio               L....NA..IR.....IL.........P..............................TE  1699

Campostoma oligolepis     PSVALVSNLAGALKTETNMHVASFAYSHIKSLTRITAPDMASVAGAANVAIKLMNRKLDR  120
Pimephales promelas       .........................................P............S.....  1866
Cyprinius carpio          .......S.............V...................A............S.....  1880
Danio rerio               .....I.S.....R..P.........................Y.............C....  1879

Campostoma oligolepis     LNFRFSRAIQLDFYHTPLMIGAAGSAY  147
Pimephales promelas       .S.........................  1947
Cyprinius carpio          .S......L...Y..............  1961
Danio rerio               ..Y.Y...FKM.Y.Y............  1960
```

Figure 2. ClustalW alignment of the predicted translations of the largescale stoneroller, fathead minnow, common carp, and zebrafish VTG. Differences from the largescale stoneroller are shown and identities are indicated with dots.

Quantitative Real-Time PCR

Dose Dependency of EE2-Induced VTG Expression in Juvenile C. oligolepis

A dose-dependent increase in VTG mRNA expression was found as shown in Figure 3. VTG mRNA expression levels were compared between hormone treatment groups using the nonparametric Mann-Whitney U test. VTG mRNA expressions at 1, 10, and 100μg EE2/g food were all significantly higher than that of the control ($p<0.05$), and VTG mRNA expression at 100μg EE2/g food was significantly higher than that of 1μg EE2/g food ($p<0.05$).

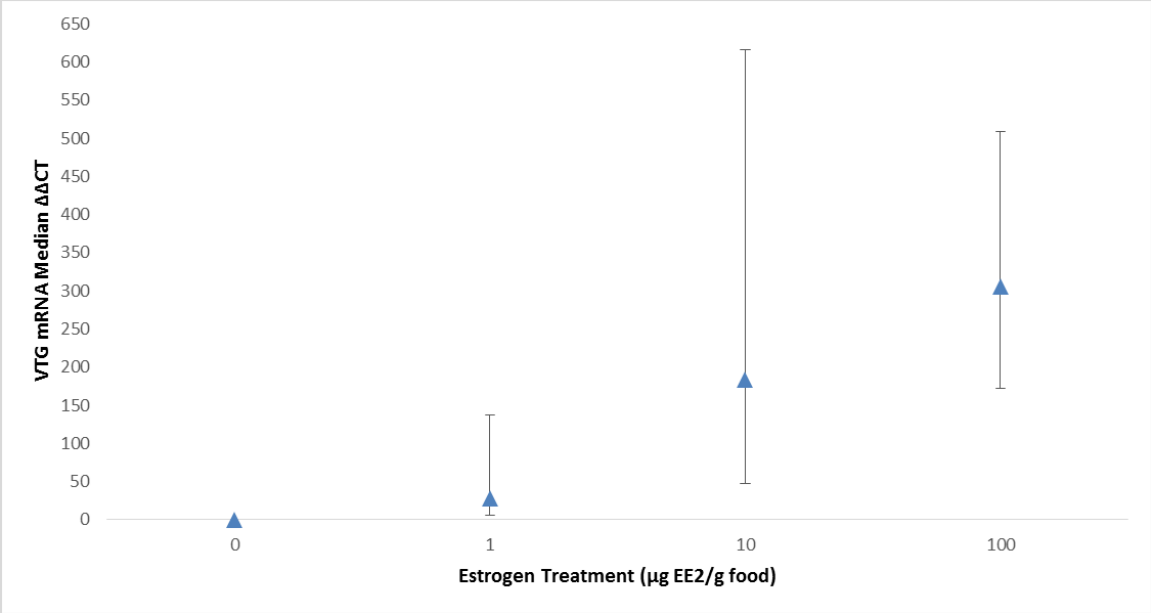

Figure 3. Induction of juvenile largescale stoneroller VTG mRNA by dietary EE2 exposure for 7 days. Data are presented as median ΔΔCt values (±95% confidence intervals).

Effects of WWTP Effluent on VTG Expression in Juvenile C. oligolepis

Real-time PCR was performed on cDNA from the liver tissue of juvenile largescale stonerollers collected downstream from three WWTPs in Jefferson County, Alabama (n=10 per WWTP) (Fig. 4). Significantly elevated VTG expression was not detected in any of the WWTP samples compared to the control using the Mann-Whitney U test. However, in one of the WWTP samples, a proportion of the individuals in the group showed elevated VTG. Using the Fisher exact test, the proportion of individuals in this group with elevated VTG expression was significantly greater than that of the control ($P<0.05$) (Table 3).

Figure 4. Induction of juvenile largescale stoneroller VTG mRNA by WWTP effluent. Data are presented as median ΔΔCt values (±95% confidence intervals).

	Control	WWTP 3
ΔΔCT<28	10	4
ΔΔCT>28	0	6

Table 3. Individual largescale stonerollers in the control and WWTP 3 groups with elevated VTG mRNA expression. Elevated VTG expression was defined as a ΔΔCt > 28, which was the median value for the group treated with 1 µg EE2/g food.

DISCUSSION

Since vitellogenesis is inducible by estrogenic compounds, the presence of VTG in the plasma as well as the presence of VTG mRNA in the liver of male fish is widely accepted as a biomarker of estrogen exposure in the aquatic environment (Sumpter and Jobling, 1995; Heppell et al., 1995). Various teleosts have been studied as potential bioindicator organisms, including the roach (*Rutilus rutilus*) (Nolan et al., 2001), zebrafish (*Danio rerio*) (Brion et al., 2004), rainbow trout (*Orynchus mykiss*) (Jobling et al., 1996), and fathead minnows (*Pimephales promelas*) (Sohoni et al., 2001). While there are valid reasons for studying the above species, their usefulness as bioindicators of the presence of endocrine disruptors in the environment may be limited to certain geographical regions. One purpose of this study was to establish the largescale stoneroller as a model organism for monitoring estrogenic contamination in the aquatic environment, particularly in the southeastern region of the United States, where this species is native. The largescale stoneroller was suitable for this study because it is considered to be "pollution tolerant" and is able to persist in streams that contaminants have made unsuitable for other species (Burr and Cashner, 1983). This was evident in the current study by the presence of largescale stonerollers downstream of some WWTPs where few other species of fish could be found. It is also small enough to be easily maintained in a lab, and the size of the liver is sufficient for division into two or three portions for different analyses. The wild caught fish were easily acclimated to the laboratory and maintained for a period of one week in order to feed them EE2-treated food.

The quantitative RT-PCR assay for the expression of the VTG gene in largescale stonerollers was effective in identifying fish presumably exposed to estrogenic chemicals

in their environment. This RNA-based method has several advantages over protein-based methods. First, it requires considerably less time, and the throughput is much greater than for immunodetection assays. A complete RT-PCR assay can analyze 96 samples in approximately 2h. Second, RT-PCR is more sensitive than immunodetection assays. For example, VTG mRNA was detected by RT-PCR while VTG protein was not detected by Western blotting in mature male mummichog (*Fundulus heteroclitus*) exposed to 4-nonylphenol at a concentration of 65µg/L. RT-PCR was also shown to be more sensitive than an indirect enzyme-linked immunosorbent assay at detecting VTG and zona radiata in rainbow trout after single injections of E2 and α-zearalenol (Celius et al., 2000). And in male common carp exposed to WWTP effluent, VTG mRNA synthesis was detected in the liver, but VTG protein was not detected in the blood plasma (Solé et al., 2001). Third, the assay is readily adapted to a new species by designing the appropriate sets of primers. Even if desired primer sequences are not available, as was the case for largescale stoneroller VTG, it is relatively straightforward to locate evolutionarily conserved sequence regions in the target gene by searching DNA sequences of related fish, amplify these sequences in stoneroller DNA, sequence the amplified band, and synthesize primers with the designer sequence. In this study, a 443 bp sequence was obtained which showed 97% homology to the VTG gene nucleotide sequence of fathead minnow, 91% homology to common carp, and 89% to that of zebrafish. The deduced 147 amino acid sequence for largescale stoneroller VTG showed 95%, 90%, and 84% homology to fathead minnow, common carp, and zebrafish proteins, respectively. Agarose gel electrophoresis confirmed that a single product of the size that the primers were designed to amplify was obtained. These results confirmed that we had successfully isolated VTG cDNA. Using

the obtained VTG sequence, primers were designed for the RT-PCR assay. This assay was tested by performing RT-PCR on fish fed a diet of 0µg EE2/g food (negative control) and 100µg EE2/g food (positive control) for 7 days. There was a highly significant increase in VTG expression ($p<0.00001$) in the group fed 100µg EE2/g food. This result indicates that VTG expression is induced by estrogen exposure in largescale stonerollers, and that the RT-PCR assay designed for this study is effective at detecting this change in VTG expression.

After validating the effectiveness of the RT-PCR assay, the dose-response pattern of VTG gene expression in largescale stonerollers exposed to various known concentrations of EE2 was characterized. In this study, fish were administered EE2 in food. This has been shown to be an effective means of exposure for the orally active EE2 in other species of fish. For example, mosquitofish (*Gambusia affinis*) exposed to dietary EE2 had increased levels of plasma VTG at 10 and 100µg EE2/g food (Tolar et al., 2001). In the present study, increased VTG mRNA expression was detected in fish exposed to 1, 10, and 100µg EE2/g food. Since VTG protein was measured in the mosquitofish study, it is unclear whether the VTG expression detected at a lower dose in this study was due to increased sensitivity of EE2 induction of VTG expression in largescale stonerollers or due to increased sensitivity of the RT-PCR assay used in this study. And not only was there a significant difference between median VTG expression in each EE2 treatment group and the negative control group, but all of the individuals in each EE2 treatment group had elevated VTG expression. In the negative control group, all of the $\Delta\Delta CT$ values were <1, and all of the $\Delta\Delta CT$ values in the EE2 treatment groups

were >4. This again confirmed that EE2 induced VTG expression in largescale stonerollers and showed that it did so in a dose-dependent manner.

Finally, the newly developed assay was used to investigate the effects of effluents from three WWTPs in the vicinity of Birmingham, Alabama on VTG gene expression in the largescale stoneroller. VTG expression was elevated in six of the ten fish collected from the outfall of one WWTP. It is possible that there are slight variations in the amount of estrogen required to illicit a VTG expression response in individuals exposed to concentrations near the "threshold" level. In another study that examined the effect of 11-ketotestosterone on gonopodium development in female mosquitofish, it was shown that doses of 20 and 40µg 11-ketotestosterone/g food caused an androgenic response (abnormal gonopodium development) in a proportion of the exposed individuals while higher doses caused these developments in the all exposed individuals (Angus et al., 2001). In the current study, all of the fish exposed to EE2 had elevated VTG expression, however EE2 is a potent estrogenic chemical. WWTP effluent is a complex mixture of unknown concentrations of various chemicals that often include weaker estrogens such as alkylphenols and bisphenols. A future study exposing fish to weaker estrogenic chemicals as well as smaller dosages could help in characterizing the effects of lower dosages of estrogenic chemicals on VTG expression in largescale stonerollers. VTG was not elevated in any of the fish collected below outfalls of the other two WWTPs. This does not necessarily indicate that these WWTPs are effectively removing EDCs from the wastewater, however. One disadvantage of using RT-PCR is that VTG mRNA can only be detected after recent estrogen exposure. For example, liver VTG mRNA was detected within 4 h, reached a maximum around 48 h, and returned to normal levels in about 6 d in

fathead minnows injected with a single dose of E2 (Korte et al., 2000). Therefore VTG RT-PCR is only useful in determining if the effluents contained estrogenic chemicals at or near the time of sampling, and since the components of the effluent can change regularly, one sample is not enough to rule out the possibility that estrogenic chemicals may be present in the effluent at other times. Future studies should be conducted with more samples over more time periods to investigate possible seasonal variability in the estrogenic activity of treated wastewater. Another potential complication with using VTG gene expression as a biomarker of estrogen exposure in the environment is desensitization. A recent study of Atlantic killifish (*Fundulus heteroclitus*) showed that multiple chemical exposures can desensitize the reproductive biomarkers of estrogen exposure (Bugel et al., 2014). It is possible that, if stonerollers were exposed more or less constantly to estrogens in the environment, their VTG expression system could have become desensitized. In order to investigate this possibility, the VTG expression of largescale stonerollers collected from these WWTPs and then exposed to known concentrations of estrogen in the lab should be measured.

Conclusions

In this study, a quantitative RT-PCR assay for expression of the VTG gene in the largescale stoneroller was developed. This assay was effective in detecting increased VTG gene expression in largescale stonerollers exposed to dietary EE2. It also showed that largescale stoneroller VTG expression was affected by EE2 in a dose-dependent manner. This assay was also effective in detecting increased VTG gene expression in stonerollers collected below the outfall of one of the three WWTP samples. However,

studying the effects of chemicals in the environment is more complicated than studying these effects in a lab. Future studies are needed to determine the effects of weaker estrogenic chemicals on largescale stonerollers, determine seasonal variability of VTG gene expression in largescale stonerollers exposed to WWTP effluent, and to investigate the possibility of desensitization.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: July 29, 2013

TO: ROBERT A ANGUS, Ph.D.
CH -378A
(205) 934-4799

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Investigation of Possible Endocrine-Disrupting Chemicals in Local Waterways
Sponsor: Internal
Animal Project_Number: 130209343

On July 29, 2013, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Numbers- 80A fish, Husbandry- EE2 food. The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary.

The following species and numbers of animals reflect this modification.

Species	Use Category	Number In Category
Fish	A	80

The IACUC is required to conduct continuing review of approved studies. This study is scheduled for annual review on of before February 22, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 130209343 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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