Ecotoxicology and Environmental Safety 73 (2010) 1835–1841

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Effects of exposure water volume, depuration time, and feeding status on vitellogenin mRNA induction in male medaka (*Oryzias latipes*) exposed to17 β-estradiol

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ARTICLE INFO

Article history:
Received 13 April 2010
Received in revised form
15 August 2010
Accepted 18 August 2010
Available online 9 September 2010

Keywords:
Depuration
Estrogen
Oryzias latipes
Real time RT-PCR
Vitellogenin
Water volume

ABSTRACT

Bioassays measuring the induction of vitellogenin gene expression in male fish are widely used for revealing estrogenic activity in water samples. Measuring induction of vitellogenin mRNA in males by means of RT-PCR analysis is a sensitive way to detect exposure to estrogenic chemicals. To date, little work has been done to examine variations in exposure conditions for assessing estrogenic activity in water samples using this model system. Here we report the results of experiments investigating the effects of volume of treatment water, time since removal from treatment water (depuration), and short-term food deprivation on vitellogenin mRNA induction in male Japanese medaka (*Oryzias latipes*). Fish exposed to a single concentration of E_2 while volume was manipulated were found to have similar levels of vitellogenin mRNA, though more E_2 was present at larger volumes. Removal of fish from E_2-treated-water to clean water after exposures reduced vitellogenin levels in as little 24 h, however, the vitellogenin levels of the fish transferred to the clean water remained above those of the control fish for at least 72 h. Depriving fish of food for up to 72 h during exposure to E_2 did not significantly reduce vitellogenin induction. Together these results support the conclusion that real time RT-PCR measurement of vitellogenin in male fish can be used as a robust indicator of exposure to estrogenic contaminants in water.

1. Introduction

In recent years it has been shown that contamination of natural water resources is widespread (Kolpin et al., 2002) due to input from a myriad of industrial, agricultural, and municipal sources. A specific class of pollutants, those demonstrated to have estrogenic activity, is of notable concern because of their potential for disrupting normal endocrine function, especially in male non-target organisms (Vajda et al., 2008). Sensitively and accurately detecting these estrogenic contaminants and their biological activity is therefore of great interest. Gene induction of vitellogenin in male fish, an egg yolk precursor normally only expressed in female fish, has been widely demonstrated to be a reliable indicator of aquatic estrogenic exposure (Islinger et al., 2002; Larkin et al., 2003; Scholz et al., 2004; Yamaguchi et al., 2005). Furthermore, real time RT-PCR has allowed highly sensitive measurement of changes in vitellogenin mRNA (Biales et al., 2007; Tong et al., 2004). However, small variations in experimental design are likely to affect the measurement of such a sensitive bioassay. It has not been determined how variability in volume of treatment water, depuration time, and feeding status might affect vitellogenin mRNA induction in fish exposed to estrogens.

In the present study, it was hypothesized that if fish were exposed to identical concentrations of the endogenous estrogen 17β-estradiol (E_2) while the volume of the exposure water was manipulated, the availability of more (or less) molecules of E_2 would impact the amount the fish concentrated during the short-term exposure. Therefore we predicted that we would see volume-dependent changes in the timing and/or robustness of vitellogenin mRNA induction in fish exposed to the same concentration of E_2.

Several studies have examined vitellogenin mRNA or protein kinetics in fathead minnows (Korte et al., 2000), rainbow trout (Arukwe et al., 2001), and sheepshead minnows (Denslow et al., 2001; Hemmer et al., 2002) during exposure to an estrogen that was either injected or added to the water, or after the exposure ceased. To our knowledge there is no published report on the

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0147-6513/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2010.08.024

effects of short-term depuration on quantitative measures of vitellogenin mRNA in medaka exposed to an estrogen. Because we are interested in bringing fish from natural waters into the laboratory for measurement of vitellogenin and transporting caged fish between the laboratory and natural waters, during which time fish may be removed from the contaminated water for short periods, we wanted to determine if short-term depuration resulted in a significant reduction in vitellogenin mRNA. Additionally, field experiments in which laboratory fish are placed in cages in streams, lakes, reservoirs, etc. may result in short-term food deprivation. Reduction of nutrient intake by withholding food may impact the energy balance and metabolic processes of fish resulting in changes in gene expression (Pierce et al., 2005; Salem et al., 2007). We hypothesized that the short-term food deprivation during exposure to E_2 would refocus metabolic energies towards maintaining homeostasis resulting in a reduced vitellogenin response.

2. Materials and methods

2.1. Animal rearing and housing

Japanese medaka (*Oryzias latipes*) were reared and housed in a recirculating flow-through system (Aquatic Habitats, Apopka, FL) in which water temperature was maintained between 21 and 25 °C, pH 6.8–8.0, and conductivity 500–700 μS, with a 16:8 light:dark photoperiod. Fish were fed twice daily with ground Deli Flake dry food and once every other day with live brine shrimp hatched from cysts (flake and cysts from Brine Shrimp Direct; Ogden, UT, USA) unless otherwise noted. Rearing and experimental protocols were approved by the University of Massachusetts Institutional Animal Care and Use Committee (IACUC).

2.2. Exposure and dissections

Male Japanese medaka were exposed in glass tanks with 10 L capacity to 17β estradiol (E_2) (Sigma-Aldrich, Inc.; St. Louis, MO, USA) or the solvent control (0.001 or 0.0001% dimethyl sulfoxide, DMSO) for each of the times indicated, with 100% static water changes and new E_2 or DMSO every 24 h. For all treatment groups in all experiments $N=4$ fish, except where otherwise noted. All fish per treatment group (4) were housed together for the duration of the experiment. Standard exposures were conducted with 4 fish in 2 L of water (1 fish per 0.5 L of water) except where noted otherwise. Small sample sizes were selected based on the desire to restrict the number of fish sacrificed, while accommodating a broad study. Further, preliminary results showed significant vitellogenin induction and little variability in male fish exposed to low levels of E_2. At indicated time points, fish were anesthetized in buffered 0.5% tricaine methanesulfonate (MS-222) until immobile, blotted dry and weighed, and then decapitated immediately prior to dissection. All fish were of similar size (approximately 0.5–0.8 g) and age (8–9 months old) and were randomly assigned to experimental and control groups. Brain, liver, and gonads were excised from each fish and were placed immediately into RNAlater solution (Ambion, Austin, TX) and stored at 4 °C until RNA isolation which typically did not exceed 2 weeks. Samples not immediately processed for this study were stored at −20 °C for later use.

2.2.1. Experiment one: analysis of available E_2 in treatment water and vitellogenin expression

To determine the amount of available E_2 remaining in the treatment water after 24 h of exposure, male medaka were exposed for 24 or 48 h to 0 (solvent control), 1 pM, 10 pM, 100 pM, or 1 nM E_2 and extracts of water samples were analyzed for E_2 by liquid chromatography-mass spectrometry (LC-MS). One liter water samples were collected from each tank for E_2 measurement at the following four time points: $t=0$ (just after addition of fish and E_2), $t=24^1$ (just prior to disposal of the first 24 h exposure water), $t=24^2$ (just after the addition of clean water and new E_2), and $t=48$ (at the end of the second 24 h exposure). Additionally, water samples were collected at $t=0$ and $t=24^1$ from control tanks containing 10 pM and 1 nM concentrations of E_2, but without fish to provide an indication of the amount of E_2 that was neither taken up by the fish nor remaining in the water. Duplicate tanks were set up and sampled for each treatment ($N=2$). Fish were sacrificed after 24 and 48 h of exposure and dissected as described.

2.2.2. Experiment two: effect of volume of treatment water on vitellogenin expression

Male medaka were exposed to 0 or 100 pM E_2 for 24, 48, or 72 h in one of three volumes of treatment water: 0.25 L/fish, 0.5 L/fish (the standard used in all other experiments), or 1 L/fish. The concentration of 100 pM was chosen because previous data indicated that it induced significant vitellogenin expression over controls at 72 h (Moffatt et al., in press). Keeping all other conditions the same, we could determine if a larger volume of treatment water allowed detection at an earlier time point, or conversely, if a smaller volume reduced the induction of vitellogenin. Fish were sacrificed at 24, 48 and 72 h to determine effects of volume on vitellogenin induction. Dissections were carried out as described.

2.2.3. Experiment three: effect of depuration time on vitellogenin expression

Male medaka were exposed for either 24 or 48 h to 0 or 1 nM E_2, after which they were transferred to uncontaminated conditioned water where they were held for 24, 48, or 72 h, during which time the water was exchanged with new uncontaminated conditioned water every 24 h. Dissections were performed after 0, 24, 48, or 72 h of depuration.

2.2.4. Experiment four: effect of food deprivation on vitellogenin expression

Male medaka were placed in field exposure apparatus (4 fish per unit) within the standard laboratory glass exposure tanks and exposed to 0 or 1 nM E_2 for 24, 48, or 72 h without food. Field exposure units were constructed according to guidelines provided by the American Society for Testing and Materials In Situ Assessment Task Group (ASTM, 2002). The capped PVC tubes with two fine mesh screen-covered windows to allow water flow-through were set up with one end capped and the other end uncapped and sealed to the front side of the tank so that fish could be observed during the exposure. Fish were sacrificed after 24, 48, or 72 h of exposure and dissections were performed as described.

2.3. RNA isolation and preparation

Tissues were removed from RNAlater, placed in 1 mL of TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH) and homogenized using a stainless steel hand-held homogenizer (Tissuemiser; Fisher Scientific, Pittsburgh, PA, USA). RNA was isolated from tissues using TRI Reagent protocol (Chomczynski and Sacchi, 1987) and quantified spectrophotometrically in the Genequant (Amersham, Piscataway, NJ). RNA quality as indicated by 260/280 ratio was measured and recorded, and no sample was used for gene expression analysis that had a ratio outside the range 1.7–1.9. RNA was then diluted to 0.1 μg/μl in RNase-free water and stored at −20 °C until real time RT-PCR analysis. Samples were then moved to −80 °C for storage.

2.4. Real time reverse transcriptase polymerase chain reaction (real time RT-PCR)

Vitellogenin mRNA and the housekeeping gene ribosomal protein L7 (L7) were quantified in each liver RNA sample with gene specific primers (Table 1) designed to span introns. Primers were designed with the assistance of Primer3 software (Rozen, 2000). Gene sequences and location of introns and exons were obtained from NCBI's Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide&itool=toolbar) and the Ensembl Genome Browser (Hubbard et al., 2007). Primers were ordered and synthesized as DNA oligos from Integrated DNA Technologies (Coralsville, IA). Real time RT-PCR reactions were run in a Roche Lightcycler 1.2 (Roche, Indianapolis, IN) in capillary format. For each 15 μl total volume reaction, 0.75 μl of sample RNA (at 0.1 μg/μl) was added to master mix prepared with the Qiagen One-Step RT-PCR kit (Qiagen, Inc, Valencia, CA), with SYBR green dye for fluorescent detection (SYBR green, Molecular Probes Inc, Carlsbad, CA), and primers. Program settings included reverse transcription for 30 min at 50–60 °C, denaturation for 15 min at 95 °C, amplification (denaturation at 94 °C for 30 s, annealing at a temperature set 3 °C below primer melting temperature for 30 s, and extension for 1 min at 72 °C) cycling 30–40 times, and the melting curve analysis for 15 min.

Primer tests were performed to confirm product size (cDNA products were run on a 1% agarose electrophoresis gel with a 100 bp ladder) and melting temperatures. Standard curves were constructed using a tenfold serial dilution of total RNA isolated from reproductively active adult female medaka liver. Standard curves were subsequently imported into each sample-screening real time RT-PCR run and used in quantification, performed by the Lightcycler software using the second derivative maximum method. Each RNA sample was screened for vitellogenin, as well as the housekeeping gene, L7, which was selected for its uniform expression throughout tissue and cell types and its unresponsiveness to treatment with E_2 (Filby and Tyler, 2007; Zhang and Hu, 2007).

Table 1
Primer sequences used in gene expression analysis using real time RT-PCR.

Gen bank accession #	Gene name	Primer sequences
AB074891	Vitellogenin (II)	F: gacagttcgtccgcttcatc R: gagcaaaggaatggtttcca
DQ118296	Ribosomal protein L7	F: gagaaaaaggcccgtaaggt R: cctgatgacaaaggccagtt