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Sensitivity of the vitellogenin assay to diagnose exposure of fathead minnows to 17α-ethynylestradiol

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ABSTRACT

Vitellogenin is frequently used as a biomarker of exposure to environmental estrogens due to its specificity and sensitivity. Appropriate incorporation of this biomarker into environmental monitoring and assessment necessitates evaluation of its critical performance parameters. In this study, we characterize the sensitivity of both vitellogenin gene (*vtg*) mRNA transcripts in liver and protein (VTG) in plasma over a range of concentrations and exposure durations. Male fathead minnows were exposed to 17α-ethynylestradiol (EE2) in a flow-through system for 2, 4 and 7 days at multiple EE2 concentrations in order to provide information regarding the sensitivity of each of these biomarkers to diagnose exposure to this representative estrogen. Measurements of the expression of the vitellogenin gene and protein both reliably detected exposures to EE2 at concentrations of 5 ng/l and higher at all time points. *Vtg* mRNA and plasma VTG appear to have similar sensitivities, though the lower variability in VTG in control fish may make it more sensitive to small changes in expression compared to *vtg*. For lower concentrations, sensitivity may be improved by increasing exposure duration. A sample size of ~12 fish was sufficient in many cases to produce a statistically significant increase in vitellogenin. Larger sample sizes may provide more sensitivity at low concentrations, but detecting exposure to estrogens in the lower range of environmentally relevant concentrations may need larger sample sizes. These data will assist in designing experiments that have sufficient statistical power necessary to determine if fish have been exposed to estrogens.

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1. Introduction

Over the past few decades there has been increasing concern that contaminants in the environment may be disrupting the endocrine systems of animals as well as humans. Among the many types of endocrine disrupting compounds, estrogens have received a great deal of attention because they are common contaminants in wastewater effluent (Kostich et al., 2013). Some estrogens are produced naturally by humans and other animals, while others are man-made chemicals that mimic the functions of natural estrogens. Among the most potent of these synthetic estrogens is 17α-ethynylestradiol (EE2), the active ingredient in many types of birth control pills. Because estrogens function as hormones in many animals, estrogens in the environment can interfere with normal hormonal and reproductive functioning in fish and other vertebrates exposed to these compounds.

The negative effects of exposure of fish to estrogenic contaminants are numerous. For many reproductive endpoints, no-observed-effect concentrations (NOECs) for estrogens are typically in the low ng/l range (Caldwell et al., 2012). For EE2, NOECs below 10 ng/l are common, and for some species the NOECs for reproductive endpoints are 1 ng/l or less (Caldwell et al., 2012). In the fathead minnow, *Pimephales promelas*, a species often used in toxicological studies, exposure to concentrations below 1 ng EE2/l skewed sex ratios of offspring toward females and demasculinized adult male fish (Parrott and Blunt, 2005).

One of the functions of endogenous estrogens in females of egg-laying vertebrates is to stimulate production of vitellogenin (vtg), an egg yolk protein precursor (Nicolas, 1999). Although males normally do not express vitellogenin, exposure to an estrogen can induce its expression. For this reason, the presence of vitellogenin protein (VTG) and its messenger RNA (mRNA) precursor (*vtg*)

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Table 1
Concentrations of the two EE2 stocks and four dilutions for each of the two flow-through exposures. The first dilution was produced by diluting the stock solution 1:100; the second dilution was produced by a 1:100 dilution of the first dilution.

Exposure	Stock	Stock concentration (μg EE2/l)	First dilution concentration (ng EE2/l)	Second dilution concentration (ng EE2/l)
1	A	10.0	100	1
	B	1.0	10	0.1
2	A	5.0	50	0.5
	B	0.5	5	0.05

have been extensively used in fish as biomarkers of exposure to estrogenic compounds in the environment (Jones et al., 2000).

Environmental estrogens may exert effects over long exposure periods. The biomarker response, however, is relatively rapid and is generally constant with continued exposure (Schmid et al., 2002). Thus biomarkers can be considered early indicators of exposure, and to some degree, as early indicators of potential longer term effects on higher biological levels (Hutchinson et al., 2006). Although expression-based exposure biomarkers are routinely used to detect estrogenic exposure using both deployed and laboratory-exposed fish, exposure conditions vary widely across studies. In our search of literature, we found deployments lasting from 48 h (McClain et al., 2003) to several months (Liu et al., 2012), with sample sizes ranging from 3 (Yan et al., 2012) to 20 fish (Miller et al., 2012). This variability in experimental design may have important implications because experimental conditions have the potential to alter aspects of exposure, which, in turn, can impact the biomarker response and subsequent interpretation.

The purpose of this work was to characterize the expression of vitellogenin in adult fathead minnows exposed to EE2 for two, four and seven days in the laboratory under controlled conditions. These data will help provide guidance regarding the number of fish to expose and the duration of exposures in order to have the statistical power necessary to determine if fish have been exposed to estrogens. Exposure of too-few individuals, or for too short a time, reduces the likelihood of detecting a positive response to an exposure. Unnecessarily lengthy exposures, both in the field, where mortality becomes an issue with caged fish, or in the laboratory, where the volume of water collected from the environment becomes unmanageable, should also be avoided. Understanding the concentration-response curve for vitellogenin at multiple time points will assist in guiding the design of experiments. In the present work, EE2 was used as a model estrogen because its potency makes it a substantial contributor to the estrogenicity of many surface waters. Both vitellogenin protein and mRNA were analyzed to determine if one biomarker has greater sensitivity at the low concentrations of estrogens often found in surface waters.

2. Materials and methods

2.1. Test chemical and exposure water

A master stock solution of EE2 was prepared in distilled, deionized water without carrier solvent. The solution was stirred for 24 h, filtered, and the concentration quantified according to methods outlined below. All exposures were performed in dechlorinated, charcoal-filtered tap water, supplemented with $CaCl_2$ to a hardness of 180 mg/l (hereinafter referred to as 'lab-line' water). Exposure solutions were prepared by diluting an appropriate volume of the 1.6 mg EE2/l master stock in15 l of lab-line water in stainless steel containers.

2.2. Exposure organisms

Fathead minnows were obtained from the on-site culture at the U.S. EPA Andrew W. Breidenbach Environmental Research Center (AWBERC) in Cincinnati, OH. Adult males ranging from 6 to 12 months of age were isolated from females for two weeks prior to the start of the experiment. Fish were randomly assigned to exposure aquaria with four fish in each 10 l glass aquarium. Fish were fed a combination of newly-hatched and frozen adult brine shrimp daily. Debris was siphoned from the tanks each day. Temperature of the tanks was maintained via water bath at $25 \pm 1\,^{\circ}C$ throughout the exposure. Temperature, dissolved oxygen, conductivity, and pH were monitored daily. Animal use was approved by the AWBERC Animal Use and Care Committee.

2.3. Experimental design

Exposures were performed in a flow-through diluter system housing 30 aquaria. In order to produce a total of eight concentrations of EE2, two separate exposures were conducted. For each exposure the flow-through system was configured to produce four concentrations of EE2 via dilution of two separate EE2 stock solutions (Table 1; Supplemental Figure S1). Each stock EE2 solution was delivered at a rate of 3.0 ml/min to a mixing chamber where it was diluted with 297 ml/min lab-line water. This 100-fold dilution produced the first concentration of EE2 that was delivered to six aquaria. Additionally, 3.0 ml/min of this first concentration from each mixing tank was further diluted with 297 ml/min lab-line water to produce a 10,000-fold dilution of the original stock concentration, and this second concentration was delivered to six aquaria. This configuration produced eight EE2 concentrations over the two flow-through exposures: 100, 50, 10, 5, 1, 0.5, 0.1 and 0.05 ng/l (340, 170, 34, 17, 3.4, 1.7, 0.34 and 0.17 pM). Diluted EE2 solutions were gravity-fed to exposure tanks at a target rate of 40 ml/min. Flow rates were checked and adjusted daily to maintain flow rates between 36 and 44 ml/min. In addition, six tanks of control fish received only lab-line water during each of the two exposures.

For each exposure the system was equilibrated with EE2 solutions for 72 h prior to the addition of fish. Stock solutions were replaced and fish were added to initiate exposures. Subsequently, stock solutions were replaced every two days. On days 0, 2, 4, 6 and 7 an aliquot of each stock solution was removed for chemical analysis to confirm EE2 concentration. On these days water was also collected from the overflow tubes of each tank and composited within identical treatments and analyzed (Supplemental Table S1). A 1-l field blank was processed on each day that water was collected for chemical analysis.

To minimize the length of the experiment and maximize the use of tanks, during each of the two exposures the 2 d and 4 d time-points were run back-to-back, and these ran concurrently with the 7 d time-point (Supplemental Table S1). After 96 h of exposure, fish from three tanks at each concentration were removed for necropsy. New fish were added to these tanks and exposed for 48 h prior to sacrifice. After 7 d the last set of fish was sacrificed and tissues collected. For each of the two exposures, 180 fish were exposed, for a total of 360 fish for the entire experiment. Each time point comprised 60 fish, with 12 fish per concentration of EE2.