

Report as of FY2007 for 2007MA74B: "Development of a standardized protocol for fish bioassays detecting estrogenic exposure"

Publications

- Dissertations:
 - ◆ Moffatt Lauren, 2008, The Development and Characterization of Fish Gene Expression Bioassays for Detecting Aquatic Endocrine Disruptors and Other Emerging Contaminants , Ph.D. Dissertation, Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA 01003, 261pp.
- Other Publications:
 - ◆ Moffatt Lauren and Kathleen Arcaro, 2007, Quantitative Biomarkers of Estrogenic Exposure in Fish; Gene Expression Bioassays in two Model Species , Massachusetts WRRC 4th Annual Meeting (April 9th, 2007, Amherst, MA) and American Water Resources Association Specialty Conference on Emerging Contaminants (June 25th-27th, 2007, Vail, CO). (Poster)

Report Follows

Problem and Research Objectives:

Endocrine disrupting compounds are of increasing concern in waterways throughout the Northeast. Specifically, estrogenic contaminants have been detected downstream of agricultural operations, industrial discharges, and even municipal wastewater treatment facilities. Concern regarding the feminization of fish and wildlife species has led to the demand for sensitive, accurate, and reliable means to detect estrogenic activity in water samples. Fish models are ideal for examining potentially polluted waterways because fish are directly exposed to, and quickly concentrate aquatic contaminants. In concert with the use of molecular techniques, specifically the analysis of changes in gene expression, fish bioassays can be a sensitive indicator of estrogenic pollution. Although vitellogenin is a well-established biomarker of estrogenic endocrine disruption in male fish models, there is a great deal of variability in the assay design used by investigators measuring its induction. Despite the importance of vitellogenin as a biomarker, there has been no published study in which the parameters that affect vitellogenin expression, including length of exposure, number of fish per tank, volume of water per tank and frequency of water changes have been systematically investigated. Therefore, investigating the effects of experimental variables on bioassay outcome will help to characterize the assay with respect to its utility and limitations, and may aid in establishing a standard protocol resulting in a rapid and sensitive bioassay. The goal of this work was to characterize the sensitivity of the fish vitellogenin bioassay to changes in experimental design.

Methodology:

Male Japanese medaka (*Oryzias latipes*) were exposed in the laboratory to a range of environmentally detected concentrations of 17 β -estradiol (E₂) over a 96 hour time course. Water changes for all exposures occurred every 24 hours along with new chemical (or 1:10,000 or 1:1 million DMSO solvent control, depending on dilutions used to achieve exposure concentrations) addition to ensure constant exposures. At given 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. Tissues removed (brain, liver, and gonad) from each fish (1 fish = 1 sample) were placed immediately into RNAlater solution and stored at 4°C until RNA isolation, or archived at -20°C for later use. High quality RNA was isolated from liver tissue using TRI-Reagent and quantified before being used in real time RT-PCR reactions along with gene-specific primers to quantify expression of vitellogenin and housekeeping gene mRNA. Vitellogenin quantities were normalized to the housekeeping gene and a combination of t-tests (p<0.05) and one way ANOVA (p<0.05) were used to determine statistically significant induction of vitellogenin over controls.

Experimental parameters that were manipulated (independently or in combination) in a series of experiments included the specific estrogenic compound (E₂, 17 α -ethynyl estradiol EE₂, or bisphenol A), the concentration of compound (1, 10, 100, or 1000pM), the length of exposures (24, 48, 72, or 96 hours), the volume of water per 2 fish used in exposure tanks (0.5L, 1.0L, or 2.0L), the lag/depuration time between chemical exposure and dissection/analysis (immediate, 24, 48, or 72 hours), and feeding status of fish (fed during exposure as in colony, versus unfed). These variables were examined with respect to their effects on vitellogenin mRNA induction.

Principal Findings and Significance:

The medaka vitellogenin mRNA bioassay was found to be sensitive and reliable in detecting levels of estrogens as low as 100pM (with environmentally detected levels being around 600pM), with the exception of bisphenol A which was undetectable via vitellogenin induction at the range of concentrations

tested. This is not entirely surprising considering the literature reports of bisphenol A's very weak estrogenic capabilities. Vitellogenin induction typically occurred in as little as 24 hours and was significant over controls at 48 hours. This assay is therefore rapid and sensitive. It was found that changing the volume of water in which the fish were exposed to E₂ did not affect the outcome of the assay in the range of volumes that we tested in this static system. However, it was determined that vitellogenin mRNA levels that have been induced by a short exposure to E₂ will begin to decline after 24 hours of depuration or absence of estrogenic contamination, and will continue to decrease over time. This indicates that samples should be processed in a rapid manner after exposure occurs. It was also found that, although not feeding the animals during exposure did not significantly reduce the vitellogenin response, the expression of some genes involved in energy balance and stress response were altered, indicating longer exposures and therefore deprivation of food could result in a change in vitellogenin response, or reproductive response entirely. Perhaps most importantly, it was found within the first set of experiments performed that the handling and presentation of gene expression data can alter the interpretation of the outcome. Specifically, data presented as fold change (obtained from dividing the relative expression values of treated animals by the expression values in control animals) did not typically resemble the data presented as relative quantities with both treatment and control displayed side by side. The reason for this is that although baseline, control levels of vitellogenin in male fish are all very low (hundreds to thousands fold lower than in animals treated with 100pM or higher of E₂), there is a substantial amount of individual animal variability at those low levels. This variability, which is likely biologically meaningless, can be by tens to almost hundreds fold, thus affecting the fold change data where the denominator becomes highly variable. Therefore, it was demonstrated that in this assay the more appropriate presentation of the data is through showing relative quantities of vitellogenin expression in both treated animals and control rather than fold change.

The work performed here has helped determine the sensitivity of the medaka vitellogenin mRNA assay to variability in experimental parameters. Both the flexibility and limitations of the assay have been characterized. This work has shown that the outcome of the assay is sensitive to some variables and not to others, however careful consideration must still be given to the design of such experiments because of the nature of the assay. In other words, the model is shown to be innately sensitive with respect to the accumulation of pollutants that occur in fish and the sensitivity of real time RT-PCR, making it a good sentinel of low levels of estrogenic contaminants, but a tool that must be handled appropriately to obtain meaningful and reliable results. Furthermore, the handling and interpretation of the data must be managed appropriately to gain the most biologically and environmentally relevant information.

References

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