Use of NMR-based Metabolite Profiling to Study Responses of Fathead Minnows Exposed to the Potent Androgen 17β-trenbolone

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Abstract

Exposure of organisms in aquatic ecosystems to chemicals which possess endocrine disrupting properties can produce numerous detrimental effects. Furthermore, due to the potency of these chemicals, even relatively low level exposures can reduce fitness. As a result, classical exposure assays are not always practical (due to low sensitivity, speed, etc.) for conducting assessments. In response, governmental agencies responsible for regulating such compounds are working to establish molecular approaches in order to overcome these limitations. One potential approach is to measure the impact(s) these compounds have on endogenous metabolite levels in various tissues and biofluids. Here we describe the results of using such an approach to elucidate molecular level effects of the potent androgen 17β-trenbolone in small fish (fathead minnow, *Pimephales promelas*). Using nuclear magnetic resonance (NMR) spectroscopy, we are able to discriminate concentration level and gender specific effects of exposures both rapidly and at a low per-sample cost. As a result, large exposure studies using numerous fish per class (defined by sex, exposure level, etc.) and including sampling of fish at several time points during and after the exposure are easily achieved. This is necessary for obtaining a more informed assessment of the effect(s) of these chemicals.

Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Introduction

It is widely known that the release of endocrine disrupting chemicals (EDCs) by sewage treatment plants and various nonpoint sources into aquatic ecosystems can adversely impact fish and other organisms that may become exposed [1]. Although the toxicities of these chemicals are varied, adverse reproductive and developmental effects are generally observed [1]. To more effectively evaluate exposures of ecologically-relevant species to EDCs for regulatory testing, effective methods for determining the specific mode of action (MOA) for a given chemical is being sought. It is believed that the 'omics' technologies are potentially well poised to address this need [2].

With these considerations, we have applied NMR-based metabolomics with the commonly used small fish toxicity model, *Pimephales promelas* (fathead minnow) exposed to the potent androgen 17β-trenbolone (TRB). The experimental approach was designed to allow for the determination of temporal responses both in a gender-specific and concentration-specific manner. In addition, a depuration phase was included in the design to determine the extent to which the fish could recover once the exposure had ended.

Materials and Methods

I. EE2 Exposure

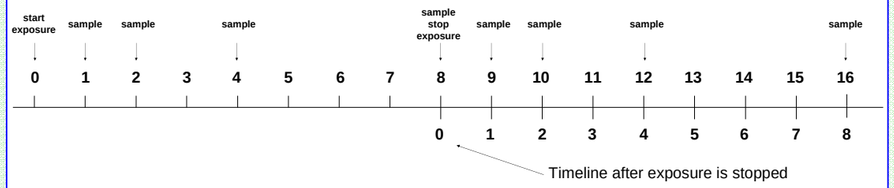
a) Fish Cultures and Exposure - Fish were obtained from mixed populations of sexually mature (5-6 months old) male and female FHM maintained in individual glass aquaria (~200 per tank) at 25ºC with a 16:8 photoperiod. Fish were fed adult brine shrimp (*Artemia*) twice daily and breeding tiles (6 per tank) were provided for cover. Exposures were conducted as follows: sexually mature male and female FHMs were delivered to tanks containing clean water and allowed to acclimate to "exposure-like" conditions. After three days, fish were transferred to tanks (flow-through system) containing 17β-trenbolone (TRB) at concentrations of either 33 ng/L or 470 ng/L. Fish were removed at 1, 2, 4, and 8 days after beginning exposure and then at 1,2, 4, and 8 days (i.e. day 16) after TRB was removed from the system. Just prior to sample collection, fish were individually netted and anesthetized in a solution of buffered tricaine methane sulfonate (MS-222, 100 mg/L with 200 mg/L $NaHCO_3$/L). Livers were harvested and immediately snap-frozen in liquid nitrogen.

b) Preparation of Liver Tissues for NMR Analysis - Tissues were extracted using a methanol chloroform procedure that extracts polar and lipophilic metabolites separately (Viant, 2006). For both fractions, solvents were removed by drying and samples were resuspended in either phosphate buffered (pH 7.4) deuterium oxide (polar) or a 2:1 mixture of deuterated chloroform:methanol (lipophilic). Polar extracts were pipetted into 3 mm NMR tubes and analyzed using a Varian Inova 600 MHz NMR instrument coupled to a triple resonance cryogenic probe (the lipophilic extracts have not been analyzed at this time). Typical acquisition parameters for one dimensional (1D) ^{1}H NMR experiments (shown above) include 2 s presaturation, 12 kHz spectral window, 16 k data points and 128 scans for a total acquisition time of 7 minutes.

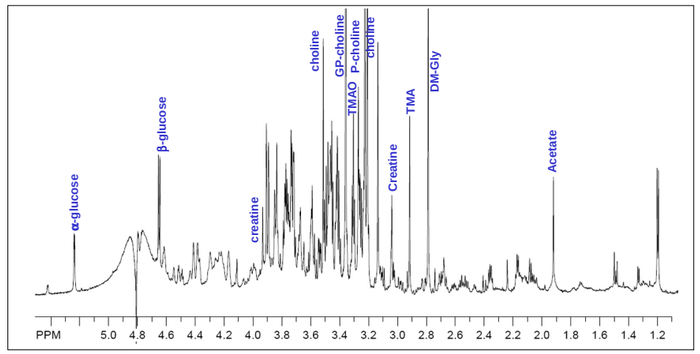
c) NMR Data Analyses – In order to more easily determine changes in the metabolite profiles induced by exposure, the NMR spectra were data reduced by segmenting each spectrum using 0.01 ppm sized bins. All spectra were then normalized by the total spectral intensity and imported into Microsoft® Excel. The Excel spreadsheet of binned spectra was imported into SIMCA-P+ (Umetrics Inc., Umea, Sweden) for multivariate data analysis. For analysis of changes in metabolite profiles using difference spectra, an "average class spectrum" was calculated by averaging the binned spectra across all class members. Note that class was defined by sex, exposure level (including controls), and day sampled. Next, a difference spectrum was generated by subtracting the averaged bins of the relevant control class from those of each exposed class. Then, a t-test (one-tailed, assuming equal variances) was conducted on each bin to see if the average for the exposed class differed significantly from that of the relevant control class, using a p-value < 0.05. If not, the bin value for the difference spectrum was replaced with a zero.

TRB Exposure Study Design

Sampling timeline (in days) for all 3 concentration levels (0, 33, 470 ng/L TRB)



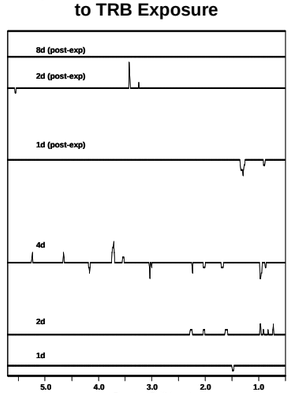
The study design for determining FHM responses to TRB. As shown above, fish (both sexes) were sampled at 4 separate time points (1, 2, 4, and 8 days) after exposure (33 and 470 ng/L), as well as 4 additional collections (9, 10, 12, and 16 days) after the chemical was removed from the water. For a given "class" (where class = sex, exposure level, and sampling-time point), 8 fish were sampled, yielding a total of 384 fish used in the study. Plasma, liver, brain, and gonadal tissues were collected for all fish.



Representative one dimensional ^{1}H NMR spectrum of a control (i.e., unexposed) female fathead minnow liver extract.
Key: DMG, dimethylglycine; TMA, trimethylamine; P-choline, phosphocholine; TMAO, trimethylamine N oxide; GP-choline, glycerophosphorylcholine.

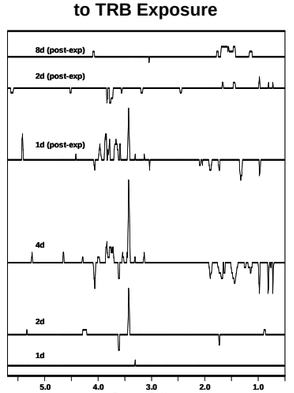
Tracking Sex-specific Responses to TRB Exposure Using Difference Spectra

Temporal Responses of Male Fish to TRB Exposure



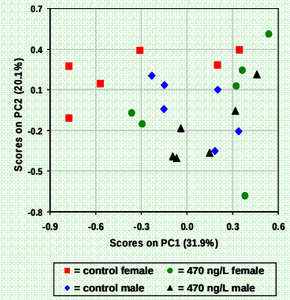
Difference spectra (see methods section) generated from the exposed and control males at days 1, 2, and 4 of the exposure to TRB and days 1, 2, and 8 after the chemical was removed from the water. For each time point, peaks shown above the baseline represent metabolites that have increased in response to exposure, while peaks below the baseline represent metabolites that have decreased.

Temporal Responses of Female Fish to TRB Exposure



Difference spectra (see methods section) generated from the female fish, which correspond to the males shown in the adjacent figure. Note that the magnitude of the responses for the females is generally larger than that shown for the males. This outcome agrees well with what is known about the endocrine disrupting properties of TRB (i.e. it is a potent androgen).

PCA Scores Plot Suggesting Masculinization of Female Fathead Minnows Exposed to TRB

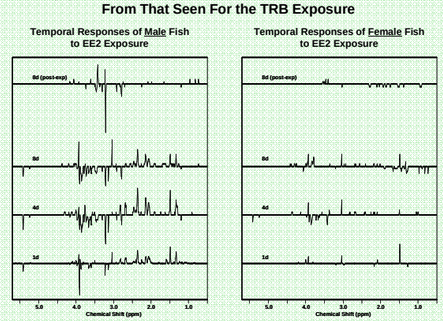


PCA scores plot of the 9 day male and female controls and those exposed to 470 ng/L TRB. From this plot it appears that the liver metabolite profiles of the exposed females have become more like those of both the control and exposed males (i.e. exposure to TRB has masculinized the females).

Metabolite Profiling of Livers from Fish Exposed to EE2 (a potent estrogen) Reveals A Very Different Outcome From That Seen For the TRB Exposure

Temporal Responses of Male Fish to EE2 Exposure

Temporal Responses of Female Fish to EE2 Exposure



Difference spectra generated from a metabolomics study of temporal- and sex- specific responses of fathead minnows to EE2 exposure. From the magnitude of the changes seen in the difference spectra it appears that EE2 has impacted the males more severely than the females. Contrasting this outcome with that seen for the TRB exposure reveals opposite effects in relation to sex (i.e. the females are more severely impacted by exposure to TRB than the males).

Conclusion

Using a metabolomic approach for studying temporal responses of FHM liver metabolite profiles has proven useful for determining the responses of fathead minnows to TRB exposure. It is evident from both the difference spectra and principal components analysis that TRB produced a greater response in the female fish. Moreover, after exposure, the female fish appear to become more like the males (i.e. masculinization) when comparing the metabolite profiles in their livers. This contrasts with the responses observed after exposure to a strong estrogen such as EE2, which appeared to feminize the males while having relatively little effect on the females. These responses concord well with what is known regarding the endocrine disrupting properties of these two chemicals. We are now currently looking into the responses of other organ systems samples from these same fish in order to obtain a more systemic understanding of responses to these chemicals.

References
1. Lal, K. M., Scrimshaw, M.D., Lester, J.N., The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol. 2002, 32, (2), 113-132.
2. Ankley, G. T., Daston, G.P., Degitz, S.J., Denslow, N.D., Hoke, R.A., Kennedy, S.W., Miracle, A.L., Perkins, E.J., Snape, J., Tillitt, D.E., Tyler, C.R., Versteeg, D., Toxicogenomics in regulatory ecotoxicology. Environ. Sci. Technol. A-Pages 2006, 40, (13), 4055-4065.