Characterizing Metabolomic Responses of Zebrafish Following Exposure to Ethinyl Estradiol and Bisphenol A

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Introduction

Background and Objectives

•Ethinyl estradiol (EE2) is used in some female contraceptive pills, while bisphenol A (BPA) is an industrial chemical widely used in the production of plastics, epoxy resins, coatings, and papers.

•EE2 and BPA are common contaminants of receiving waters around waste water treatment plant (WWTP) outfalls and pose a risk to certain aquatic organisms at low (ng/L concentrations).

•In this work, the metabolomic response of entire zebrafish embryos 96 hour post-fertilisation (hpf) was characterized after sub-lethal exposure for 24 h to two estrogenic agonists EE2 or BPA.

Objective: To investigate the metabolomic response of zebrafish embryos to EE2 and BPA.

Dosing

•Each experiment consisted of 5 replicates of 60 strain AB zebrafish embryos (96 hpf) per dose.

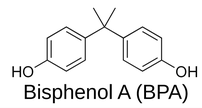
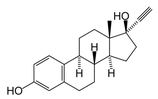
•Dosing was carried out for 24hrs in 6-well cell culture dishes with low-evaporation lids.

•2 dosing concentrations were used for each test substance (Table 1), in addition to ethanol (EtOH) and non-vehicle controls.

•At 120 hpf, embryos were placed in a 1.5mL microcentrifuge tube and flash frozen in liquid nitrogen.

•Samples were shipped to AXYS on dry ice and then stored at -80°C prior to analysis.

Table 1. Dosing levels for each experiment. BPA doses were set 1000-fold higher than EE2 due to the weaker potency of the former substance.

Test Substances	Dosing Level
 Bisphenol A (BPA)	10 ug/L
	1000 ug/L
 Ethinyl estradiol (EE2)	10ng/L
	1000 ng/L
EtOH	0.1%
Control	-

Targeted Metabolomic Analysis

Extraction and Plate Preparation

•~100mg of ZrO_2 beads and 250uL of MeOH were added to each 1.5mL centrifuge tube containing 60 embryos.

•The tubes were transferred to a bullet blender blue and blended for 1 minute, and then centrifuged for 30 seconds.

•The supernatant was transferred to a clean 1.5mL centrifuge tube. The extraction procedure was repeated with 250uL chloroform, the two extracts combined and centrifuged.

•200uL of extract was added to the plate, which contained internal and native standards.

•Amino acids and biogenic amines were derivatized with phenylisothiocyanate and the plate was eluted with 200uL of 5mM ammonium acetate in MeOH.

• Final extracts were diluted with 200uL water and analyzed immediately using 4 different LC-MS/MS methods and an FIA-MS/MS method for 8 target classes.

•Data processing was carried out using MetaboAnalyst 2.0. (Xia et al. 2012)

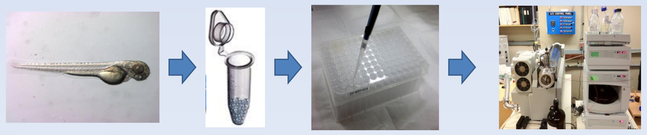
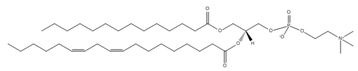
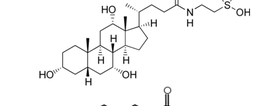
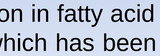
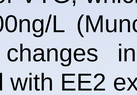
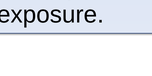
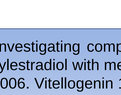


Figure 1. Flow chart of experimental workflow.

Table 1. Structure and function of metabolites quantified in the present work.

Structure	Target	# of Analytes
	Glycero-phospho lipids (PCs)	92
	Sphingo-myelines (SMs)	15
	Acylcarnitines (carnitine shown)	41
	Amino acids (alanine shown)	14
	Biogenic amines (taurine shown)	19
	∑Hexose (glucose shown)	1
	Bile acids (taurocholic acid shown)	13
	Fatty acids (arachidonic acid shown)	17

Results

Bisphenol A (BPA)

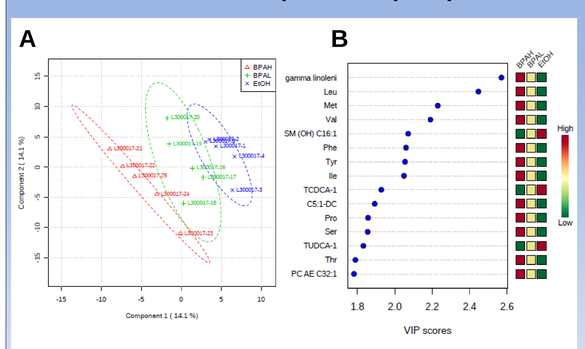


Figure 2. A) Partial least squares discriminant analysis (PLS-DA) scores plot of metabolite profiles observed in zebrafish exposed to 0.1% EtOH vehicle control (EtOH), 10ug/L BPA (BPAL), or 1000ug/L BPA (BPAH) and **B)** the ranking of metabolites contributing to the observed variability.

Ethinyl estradiol (EE2)

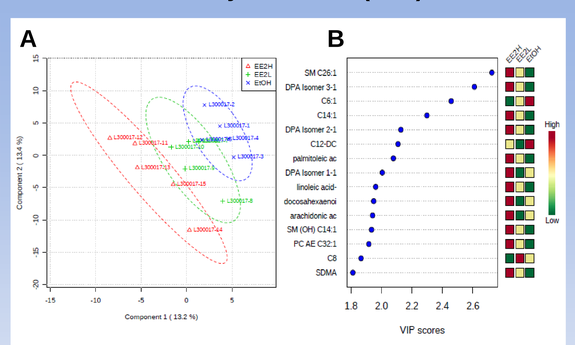


Figure 3. A) PLS-DA of metabolite profiles in zebrafish exposed to 0.1% EtOH vehicle control (EtOH), 10ng/L EE2 (EE2L), or 1000ng/L EE2 (EE2H) and **B)** the ranking of metabolites contributing to the observed variability.

EE2 vs BPA

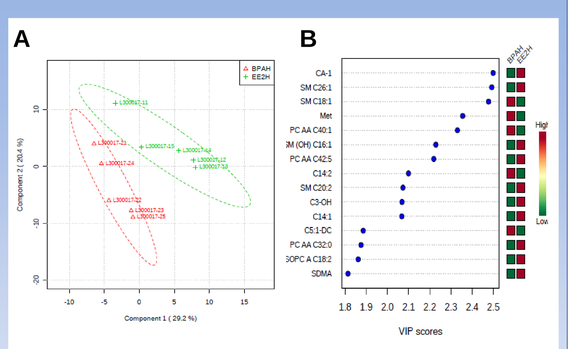


Figure 4. A) PLS-DA of metabolite profiles in zebrafish exposed to 1000ng/L EE2 (EE2H) vs 1000ug/L BPA (BPAH) and **B)** the ranking of metabolites contributing to the observed variability.

Summary

•In BPA exposures, metabolites associated with the observed PLS-DA groupings included amino acids and bile acids. Consistent with this result, Villeneuve et al. (2011) demonstrated exposure to waterborne BPA (4 d, 10ug/L) resulted in VTG induction along with changes in hepatic gene expression and steroid production, both of which are related to changes in amino acid and bile acid levels.

•In EE2 exposures, metabolites associated with observed PLS-DA groupings included acylcarnitines and fatty acids, possibly indicating a disruption in fatty acid oxidation. This may be related to induction of VTG, which has been reported in zebrafish exposed to EE2 at 100ng/L (Muncke et al. 2006). Ekman et al. (2008) also observed changes in hepatic fatty acids in fathead minnow associated with EE2 exposure.

References

Ekman et al. 2008. Investigating compensation and recovery of fathead minnow (Pimephales promelas) exposed to 17α-ethinylestradiol with metabolite profiling. *Environ. Sci. Technol.* 42, 4188-4194.
Muncke and Eggen 2006. Vitellogenin 1 mRNA as an early molecular biomarker for endocrine disruption in developing zebrafish (Danio Rerio). *Environ. Toxicol. Chem.* 25, 2734-2741.
Villeneuve et al. 2011. Ecotixcogenomics to support ecological risk assessment: A case study with bisphenol A in fish. *Environ. Sci. Technol.* 46, 51-59.
Xia et al. 2012. MetaboAnalyst 2.0 - a comprehensive server for metabolomic data analysis. *Nucl. Acids Res.* 40, W127-133.