Transcriptomic analysis of the adult zebrafish liver in response to exposure to plasticizers and synthetic, steroidal estrogen.

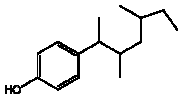
Matthew Huff[1,2], E. Starr Hazard[1,3], Sean M Courtney[1], Willian da Silveira[1], Ludivine Renaud[4], Gary Hardiman[1,4]

[1]MUSC Bioinformatics, Center for Genomics Medicine, [2]MS in Biomedical Sciences Program, [3]Library Science and Informatics,

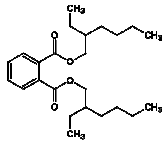
[4]Department of Medicine, Medical University of South Carolina (MUSC), Charleston SC

Background

- Many industrial chemicals are widely dispersed in the environment. In recent years these endocrine disruptors (ED) have been labeled 'contaminants of emerging concern' (CEC), as they have potential to cause adverse effects on wildlife and human health (1-5).
- Two examples of important CECs are nonylphenol (NP), and (2-ethylhexyl) phthalate (DEHP), both of which are termed xenoestrogens (XEs) as they can bind the estrogen receptor (6, 7) and disrupt estrogen physiology in mammals and other vertebrates.



Nonylphenol (NP). A non-ionic surfactant, NP is found in detergents, cosmetics, and PVC pipes. Water that flows through pipes with NP becomes contaminated with the compound, whose lipophilic properties allow it to be stored in the adipose tissue of animals in the food chain (5, 6).



(2-ethylhexyl) phthalate (DEHP). DEHP serves as a chemical building block for the polycarbonate plastic and epoxy resins found in many consumer products (5,8).

- The effects of these xenoestrogens on human health are becoming better characterized, though there remains a gap in knowledge in understanding the exact method by which XE mediate their adverse effects.
- We hypothesize that exposure to low doses of XEs alters key cell signaling pathways, posing potential risks to human health. The long-term goal of our research is to characterize the adverse effects of EDC exposure using a model organism, the zebrafish, as a proxy for human health assessment.

- Zebrafish are a popular model organism for their defined life cycle, and their genome's similarity to humans; about 70% of human genes have a functional zebrafish homolog (9).
- The liver is the main site of metabolism of foreign chemicals, including XEs. Exposure to XEs has been associated with the development of adverse outcomes in the liver.
- XEs act via multiple toxicity pathways to induce adverse health outcomes. The adverse outcome pathways (AOP) framework is a new strategy that organizes mechanistic and/or predictive relationships between initial chemical–biological interactions, pathways and networks, and adverse phenotypic outcomes (10).

Methodology

***In vivo* exposure**

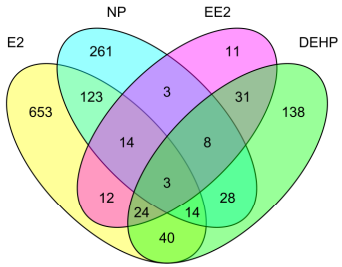
- Male zebrafish were obtained from a commercial fish farm (Euroaquairum Spa Bologna, Italy). The zebrafish were exposed for three weeks in 80 L tanks, housing 40 fish per tank, after being separated into two experimental periods:
  - The first group were exposed to 1000 nM of NP, with 1000 nM of E2 as a positive control.
  - The second group were exposed to 5.8 nM DEHP and 0.65 EE2.
- Following this exposure period, their livers were removed and frozen for molecular biology analysis. RNA was extracted with TRIzol reagent (Invitrogen) and purified on a Qiagen RNeasy column. RNA integrity was verified using RNA 6000 Nano Assay chips run in Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

RNA sequencing (RNA-seq)

- High throughput sequencing (HTS) was performed using an Illumina HiSeq2000 with each sample sequenced to a minimum depth of ~50 million reads.
- RNA sequencing data was analyzed using the OnRamp BioInformatics Genomics Research Platform RNAseq pipeline (11). This 'Big Data' solution utilizes hadoop software with automated data protection to seamlessly scale a 240 TB, 10-node server and storage infrastructure.
- Gene Differential Expression (DE) analysis – and alignment with the zebrafish genome – was carried out via Tuxedo (Bowtie, Tophat, Cufflinks, HTSeq) and DESeq2.

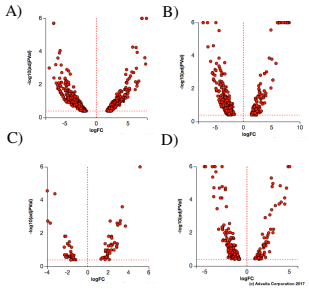
Results

- Transcript count data from DESeq2 analysis were sorted according to their adjusted p-value or q-value, which is the smallest false discovery rate (FDR) at which a transcript is called significant. FDR was calculated using the Benjamini-Hochberg multiple testing adjustment procedure.
- We determined that exposure E2 had the most effect, with 886 genes differentially expressed in total; in comparison, exposure to NP induced differential expression of 454 genes, EE2 induced 106, and DEHP induced 286.

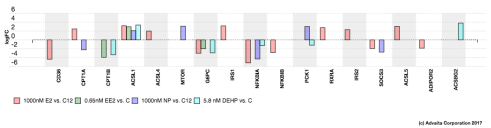


Venn diagram highlighting the overlap of differentially expressed transcripts by XE exposure in zebrafish livers. Generated using Zebrafish gene symbols, using all significant genes q<0.4 for all comparisons.

- Systems analysis was performed utilizing Advaita iPathwayGuide. *Meta Analysis* was carried out between the four experiments, selecting only the transcripts where q<0.4 and an absolute log fold change (0.1). In the context of KEGG pathways, we found a single pathway was significantly impacted between all four exposures: the **Adipocytokine Signaling Pathway**. Individually, we identified pathways of interest unique to specific exposures; these have connections to adverse outcomes, including changes in inflammation (NP), insulin signaling (DEHP), and fatty acid metabolism (all exposures) that may relate to non-alcoholic fatty liver disease (NAFLD).

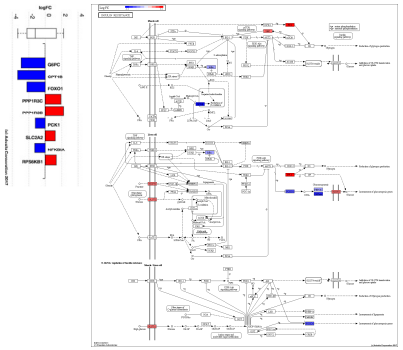


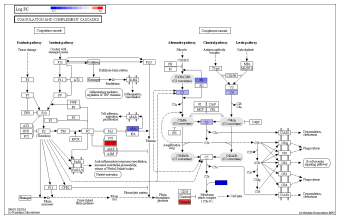
Volcano Plots Depicting Significantly DE Genes after exposure to (A) E2, (B) NP, (C) EE2, and (D) DEHP. Obtained from Advaita iPathwayGuide

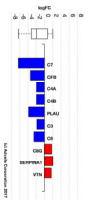


***All XEs studied* – Adipocytokine signaling pathway (KEGG: 04920)**: Increased adipocyte volume and number are positively correlated with leptin production, and negatively correlated with production of adiponectin. Leptin is an important regulator of energy intake and metabolic rate primarily by acting at hypothalamic nuclei. Leptin exerts its anorectic effects by modulating the levels of neuropeptides such as NPY, AGRP, and alpha-MSH. This leptin action is through the JAK kinase, STAT3 phosphorylation, and nuclear transcriptional effect. Adiponectin lowers plasma glucose and FFAs. These effects are partly accounted for by adiponectin-induced AMPK activation, which in turn stimulates skeletal muscle fatty acid oxidation and glucose uptake. Furthermore, activation of AMPK by adiponectin suppresses endogenous glucose production, concomitantly with inhibition of PEPCK and G6Pase expression. The proinflammatory cytokine TNFalpha has been implicated as a link between obesity and insulin resistance. TNFalpha interferes with early steps of insulin signaling. Several data have shown that TNFalpha inhibits IRS1 tyrosine phosphorylation by promoting its serine phosphorylation. Among the serine/threonine kinases activated by TNFalpha, JNK, mTOR and IKK have been shown to be involved in this phosphorylation.

***DEHP* – Insulin resistance (KEGG: 04931):** Insulin resistance is a condition where cells become resistant to the effects of insulin. It is often found in people with health disorders, including obesity, type 2 diabetes mellitus, non-alcoholic fatty liver disease, and cardiovascular diseases. In this diagram multiple mechanisms underlying insulin resistance are shown: (a) increased phosphorylation of IRS (insulin receptor substrate) protein through serine/threonine kinases, such as JNK1 and IKKB, and protein kinase C, (b) increased IRS-1 proteasome degradation via mTOR signaling pathway, (c) decreased activation of signaling molecules including PI3K and AKT, (d) increase in activity of phosphatases including PTPs, PTEN, and PP2A. Regulatory actions such as oxidative stress, mitochondrial dysfunction, accumulation of intracellular lipid derivatives (diacylglycerol and ceramides), and inflammation (via IL-6 and TNFA) contribute to these mechanisms.







***NP* – Complement and coagulation cascades (KEGG: 04610):** The complement system is a proteolytic cascade in blood plasma and a mediator of innate immunity, a nonspecific defense mechanism against pathogens. There are three pathways of complement activation: the classical pathway, the lectin pathway, and the alternative pathway. All of these pathways generate a crucial enzymatic activity that, in turn, generates the effector molecules of complement. The main consequences of complement activation are the opsonization of pathogens, the recruitment of inflammatory and immunocompetent cells, and the direct killing of pathogens. Blood coagulation is another series of proenzyme-to-serine protease conversions, culminating the formation of thrombin, the enzyme responsible for the conversion of soluble fibrinogen to the insoluble fibrin clot. Protease-activated receptors, such as those activated by thrombin, are members of G protein-coupled receptors and function as a mediator of innate immunity. The kallikrein-kinin system is an endogenous metabolic cascade, triggering of which results in the release of vasoactive kinins (bradykinin-related peptides). Kinin peptides are implicated in many physiological and pathological processes including the regulation of blood pressure and sodium homeostasis, inflammatory processes, and the cardioprotective effects of preconditioning.

Conclusions

- Exposure to the Xenoestrogens NP, EE2, and DEHP induces changes in gene expression in the livers of zebrafish.
- Each Xenoestrogen is associated with unique changes in gene expression, while also sharing a pool of differentially regulated transcripts.
- Pathway analysis of significantly DE genes indicate a connection to NAFLD with the plasticizers DEHP and NP.

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