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Genetic and behavioral responses in two aquatic species
(*Daphnia magna, Danio rerio*) exposed to endocrine disrupting compounds and candidate
biofuels: Toward development of new Adverse Outcome Pathways

By

Marianna Augustine

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular Toxicology

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Graduate Division

of the

University of California, Berkeley

Committee in charge
Dr. Christopher D. Vulpe, Co-Chair
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Dr. Daniel K. Nomura
Dr. John Arnold

Summer 2018

Abstract

Genetic and behavioral responses in two aquatic species
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biofuels: Toward development of new Adverse Outcome Pathways

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Doctor of Philosophy in Molecular Toxicology

University of California, Berkeley

Professor Christopher Vulpe, Co-Chair
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The adverse outcome pathway (AOP) approach combines patterns of molecular events across levels of biological organization and complex endpoints such as reproduction, behavior, and toxicity, and provides regulatory decision criteria for Environmental Protection Agency (EPA). Useful empirical data for AOPs is derived from key indicators of toxicant exposure such as conserved transcriptional factors, nuclear receptors and their targets. One approach to establish changes in toxicant response is gene expression profiling, or 'omics' tools, which can lead to improved mechanistic models of toxicity in closely related species (fathead minnow and zebrafish), and divergent species (fish and crustaceans). Gene expression patterns have been used to characterize toxicant-dependent signaling for crustaceans exposed to metals, flame retardants, and narcotic toxicants. Gene expression studies can identify new assay development needs, and generate new hypotheses about conserved molecular pathways leading to direct evidence from knockout models.

In the first study, we focus on conserved nuclear receptors in the model crustacean *Daphnia magna* exposed to endocrine disrupting chemicals (EDCs). The Daphnia reproductive X-Y axis model involves key orthologous genes which drive metabolism and endocrine-toxicity. Previous work in Daphnia hypothesized a unified mechanism of endocrine disruption that leads to inappropriate production of male offspring through effects on a sex-differentiation gene (dsx-1) in the methyl farnesoate (MF) pathway. We carried out a microarray gene expression study with eight different EDCs in female Daphnia and found two distinct patterns of transcriptional response induced by male-inducing chemicals. Pyriproxifen and methoxychlor produced similar gene expression patterns overall and in key endocrine response genes, while methyl farnesoate and arochlor exposure shared a distinct response pattern. In particular, there were inverse responses at the ecdysteroid receptor (EcR), a PAS-binding protein that regulates key steroid

biosynthesis metabolism between the two sets. We also noted differences in key molting genes (CYP450s) from the ecdysteroid pathway. Together our study demonstrates a dsx-1 dependent mechanism for male-differentiation (dsx-1) in the ovary, and a dsx-1 independent EDC mechanism for altered reproductive mode.

In the next study, we assessed *Daphnia magna* exposure to polycyclic aromatic hydrocarbons (PAHs) and pyrethroids, which may lead to neurotoxicity. Since PAH exposure activates the conserved AHR pathway in vertebrates, we tested the hypothesis that PAH exposure in Daphnia would induce AHR-related gene expression endpoints (cytochrome P450s, oxidative stress, metabolism). Despite the lack of a clear orthologous *AhR* gene, we observed induction of antioxidant pathways and CYP450-related oxidoreductase enzymes that suggest similar crustacean and vertebrate mechanisms of PAH toxicity.

To link molecular endpoints to an ecological phenotype, we used an automated video-tracking system to assess Daphnia neuro-behavioral endpoints including swimming speed and tank location preference. Altered *Daphnia magna* swim behaviors were upward (top of tank) and downward (bottom of the tank), similar to phototactic behaviors recognized in zebrafish anxiety-related research. Pyrethroids reduced swim speed and induced a bottom swimming phenotype at doses well below acute toxicity. Swim responses differed within the PAH chemical class. Daphnia behaviors in light and dark environments are dependent upon circadian clock mechanisms, which may be sensitive to toxicant exposure. Thus, we hypothesized that altered circadian clock (CLK) mechanisms may be a nexus between toxicant exposure and altered behavior. We observed that exposure to phenanthrene (PHE), pyrene, and cyfluthrin influenced cryptochrome-2 expression, an upstream regulator of Daphnia circadian clock genes. We also identified a broad range of functional genes involved in PHE-altered neurological pathways, involving dopamine and the Mbln12 gene involved in photo-receptor cell growth. The behavioral and molecular studies suggest that these chemicals disrupt neurologic function which results in altered behavioral outcomes.

In the final study, we used a zebrafish developmental and behavioral chemical toxicity screening assay to assess the toxicity of a candidate biofuel (2,5-DMF) and related furan compounds. Because of structural similarity to aromatic hydrocarbons, we hypothesized that 2,5-DMF targets the AhR signaling pathway and produces characteristic zebrafish developmental toxicity. AhR agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and beta-napthoflavone (BNF) cause characteristic pericardial edema and Cyp1a1 activation in zebrafish. However, we found that 2,5-DMF toxicity, as well as some polyaromatic hydrocarbons, did not produce morphological defects. Non-AhR mechanisms that include hypoxia and neuro-behavioral effects were observed. We assessed AHR-dependent mechanisms using morphological endpoints, AHR-XRE cell-based reporter assays, and *in silico* AhR-LBD binding models. Zebrafish were also assessed for embryonic and larval photomotor responses. We evaluated several furan derivatives and found that 2-ethyl furan and 2,3-dihydrofuran had the least biological effect in our assays. The highest aquatic risk to developing fish was from 2,5-dimethyl furan, and 2-methyl furan. Overall, we determined that zebrafish exposure to biofuel

candidates did not induce AhR mediated toxicity, but produced significant behavioral toxicity from non-AhR mechanisms.

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CHAPTER 1

**GENOMICS AND BEHAVIORAL APPROACHES IN CROSS-SPECIES TOXICOLOGY
USING DAPHNIA MAGNA AND ZEBRAFISH**

Introduction

The translation of 'omics' technologies to mechanistic biology is now merging with EPA's adverse outcome pathway (AOP) framework. Because a core component of AOP development is translation of molecular interaction-based endpoints (such as predictive, computational relationships or activation of conserved molecular pathways) to phenotypic responses, there is a growing interest in determining underlying mechanisms of toxicity that may allow for more relevant testing endpoints. Molecular tools of the 21rst Century now supersede traditional toxicity testing where adverse effects are often far below EC_{50} values.

In this introduction, I review the use of molecular endpoints as key events in adverse outcome pathways and focus on conserved gene expression differences between fish and crustaceans linked to reproductive and behavioral endpoints. The zebrafish mechanistic model is considered complementary to fathead minnow, making them pair together for similar functions in biology; however, *Danio rerio* and *Daphnia magna* are two model aquatic species that occupy diverse ecosystem roles, and exhibit completely separate biology. Transcriptomic profiling of genes promises to become an integral part of the AOP framework in aquatic species,[1,2] and overcomes the difficulties in cross-species extrapolation from a single model organism.

The Environmental Protection Agency's framework for validating new assays is the adverse outcome pathway (AOP), which links molecular events to endpoints of reproduction, survival, and fitness.[3-6] AOP elements require a mechanistic basis for determining toxicant pathways that connect effects at the genetic level to higher level effects of altered phenotypes, i.e., organs, individuals, and populations. leading to the progression to an adverse outcome (AOP) considered relevant to regulatory decision making.[7] AOPs require both direct and indirect evidence produced from plausible key molecular events. Direct evidence is obtained through empirical data, data obtained from mutant strains, reversibility studies, that conclusively prove by interdependence of key events.[4] Therefore important AOP endpoints include molecular initiating events (MIE) captured from transcriptome data,[1] mitochondrial responses,[8,9] in vitro receptor binding assays,[5,10] and predictive computational models.[10,11]

Transcriptomic Profiling Approach: Molecular mechanisms contribute to Adverse Outcome Pathway (AOP) framework

A heavy dependence upon transcriptomic profiling approaches arose from limited genomic resources for non-model aquatic organisms such as fish and crustacean species. Since the completion and release of the *Daphnia pulex* genome, there are increased possibilities for developing genomic tools for aquatic crustaceans.[12] Genetic profiling can translate the needed aspect of chemical bioavailability for a toxicant, incorporating absorption, disposition, metabolism, and elimination (ADME) into relevant responses for chemical stressors in *D.magna* and *D. rerio,* as well as reduce data gaps in adverse outcome studies. Poynton et al. utilized Daphnia transcriptomic responses to estimate metal stress. Gene expression demonstrates chemical modes of action when coupled with validation by known biomarkers. [13,14] In addition to determining chemical mode of action, fish transcription responses have been utilized as 'biosensors' for comparing exposure sites,[15,16] and a basis for quantifying a no-effect levels of transcription (NOTEL) for Daphnia.[17] The toxicogenomics approach has been used extensively to highlight similarity in biological structure and function across taxa,[18] and has resulted in defining mechanistic drivers for flame retardants, metals, and narcotic exposure in *Daphnia magna*.[11,13,19-21] By emphasizing key biochemical pathways, mechanistic diversity can be detected. Ecological risk assessments seek sensitive endpoints for aquatic species that are predictive, inclusive of cause and effect,[22,23] and links multiple levels of biological organization.[3] reason for using molecular approaches is assessing bioavailability, which relates to the action of chemicals on receptors, producing a quantitative biochemical response, i.e., CYP1A induction.[3,24,25]

Transcriptomic approaches combined with phenotypic endpoints have successfully demonstrated functional differences of orthologous genes in fish exposed to toxicants.[1,26-28] Common gene orthologs may present a novel approach in the efforts to profile the effects of toxicants. An estimated number of 17 conserved signaling pathways in developmental processes have been described.[6] Transcriptomics can assess species sensitivity without a priori knowledge of chemical mode of action at conserved gene targets. As a fundamental aspect of species sensitivity, the chemical must interact with a target molecule, and subsequent absorption, disposition, metabolism, and elimination (ADME).[29] Authors have used innovative ADME approaches to generate AOP hypotheses, combining transcriptional response with predictive approaches to identify properties of narcotic chemicals,[11] where bioavailability of a toxicant is uniquely related to physical- chemical features of a toxicant.

The conserved Aryl hydrocarbons receptor pathway is a model adverse outcome pathway

Transcriptomic profiling is a useful approach to identify common pathways of toxicity from conserved biological pathways. Comparative transcriptional approaches across chemicals has shown that PAH induction of CYP1A is tissue and compound specific,[30] and many environmental PAHs are AHR-independent.[31-33] Activation of the aryl hydrocarbon nuclear

receptor (AhR) nuclear receptor pathway is adopted by REACH, [34] and is a hazard endpoint for many carcinogens. AHR signaling pathway is an important AOP because there are several key events that are measureable: ligand binding, activation, transcription of genes, abundance of CYP family enzymes, and downstream detoxification and/or toxification which may lead to neurotoxicity, genotoxicity, carcinogenicity in many species. The ligand-binding aspect of this pathway is simple to identify using *in silico* homology models (i.e., AHR-LBD homology). Computational modeling of AHR interactions is cost effective for large numbers of compounds and phylogenetically distinct species.[26,28,35-38] Another single-endpoint that has been published in support of AOP development for fish is mitochondrial toxicity,[8,9,39] where mitochondrial genes and bioenergetics are dysregulated and contribute to oxidative stress.

The conserved AHR pathway is not a universal across species.[40] In *Daphnia magna*, there is no equivalent of toxic effects AhR orthologs because AhR agonists are not detected[41]. Instead, toxic effects in Daphnia may arise from interaction with other PAS family of nuclear receptors; many Daphnia PAS transcription factors are more promiscuous in ligand binding and co-activation of these transcriptional complexes may increase in crustaceans. For example, the vertebrate NR class 1C and NR3A are nuclear receptor families containing estrogen receptor, is absent in invertebrates. Across species, conserved nuclear receptors are often PAS binding proteins.

There are 48 human transcription factor families and 26 nuclear receptor subfamilies in *Daphnia magna*. The theoretical explanation for having fewer nuclear receptors is additional cross-functionality of each receptor.[41] Toxicants affecting Daphnia growth and development agonize the ecdysone receptor (EcR), which is similar to human FXR.[42] Daphnia RXR homologs are 60% similar to Drosophila. RXR activates multiple outcomes in response to EDCs, [43] which may be profiled through transcriptomic experiments. Daphnia EcR forms a heterodimer with ultraspiracle protein (UsP), which is similar to human RXRs. A major challenge for ecotoxicity testing is the lack of orthologous receptors represented; although 40% of pharmaceuticals target nuclear receptors;[44] nuclear receptors are the least conserved drug target type (compared to ion channels, enzymes, transporters).

There are limitations for using single-target endpoints in an AOP framework. The high-throughput ToxCast/Tox21 bioactivity endpoints have potentially misleading data[45] and undetected effects of both chemicals and metabolic biotransformation.[45-49] Underestimation of toxicity can arise from compounds that test negative in targeted assays (AHR), when non-AHR modes of action or interaction with other AHR paralogs are overlooked .[31,33] Members of the same paralogous gene family often have distinct functions.[50,51]

For the cytochrome P450 gene family, enzyme function differs across species. In vertebrates, P450 enzymes have functional roles in steroid biosynthesis and the conversion of testosterone to 17β-Estradiol.[52] Daphnia CYP450 gene families have not been fully annotated. Although these genes are phylogenetically different, functional redundancy ensures that critical mechanisms continue in the absence of a common gene.

Adverse outcome pathways for EDC endpoints in aquatic vertebrates and crustaceans

The common structure and function of the hypothalamus-pituitary-gonadal axis (HPG) reproductive axis across vertebrates is an ideal basis for AOP-based predictions across species. [53,54] HPG signaling can be interrupted by EDCs at numerous molecular endpoints, i.e., estrogen, thyroid, PPAR, or AR.[55] PAHs have been known to produce antiestrogenic effects in females. Fish collected from oils spills have shown low E_2, and vitellogenins, compared to fish from pristine sites.[56] Specifically, estrogenic compounds mimic the natural 17β-estradiol- binding or antagonizing estrogen receptors in the nucleus, forming an ER-ligand complex to promote a multi-component ER-transcription complex with co-regulator proteins required to activate or suppress transcription of ER-responsive genes.

The zebrafish genome shares over 60 percent of genes with humans; and the zebrafish reproductive model predicts mammalian EDC outcomes. Therefore zebrafish models can be used as alternatives to expensive mammalian testing.[57] There are commonalities between vertebrate species in embryonic life stages.[6] The transgenerational life stage which affects both maternal and offspring at the same time, is a unique aspect of EDC toxicity. In early life stage exposures, disruption of maternally deposited genes can lead to developmental toxicity.[58] [59,60] Effects at "critical windows of development" may not be accurately assessed from high-throughput screening approaches, such as EPA's ToxCast/Tox21. These studies utilize in vitro cell testing from 331 enzymatic and receptor signaling, but may lack ability to assess transgenerational, behavioral, or effects at critical life stages. Depending on the testing approach or the life stage, reproductive endpoints may underestimate toxicity. Interestingly, at common developmental processes, there are many conserved signaling and regulatory circuits, but timing and gene targets differ across species.

Need for Endocrine disruption assays for Daphnia X-Y Reproductive Axis

To fully describe adverse outcome endocrine disruption endpoints for Daphnia, additional pathway-based data for Daphnia X-Y reproductive axis should be considered. Molecular signaling in the reproductive axis occurs between a brain-neurological X-organ and a negative regulating Y-organ. Like the EDC targets on the HPG axis, toxicants may affect signaling at multiple endpoints for crustaceans. The crustacean X-organ, a brain-like gland near the eyestalk, stimulates the Y-organ to secrete ecdysone, which is hydroxylated in peripheral tissues to form 20-hydroxyecdysone (20-HE), the primary hormone for molting. The molting hormone 20-HE is a ligand of the ecdysteroid receptor (EcR). EcR is phylogenetically related to human LXR and FXR (Class II nuclear receptors). [42] Authors have posited mechanisms of action of ecdysone in Drosophila may be to modify DNA via EcR, and that 20-HE regulates the 'early genes' for in morphogenesis in larval tissues.[61,62] A unique feature of EDC chemicals is that toxicity at low levels of exposure can perturb hormone production.[63] Daphnia studies have shown that altered ecdysteroid ratios of ecdysone and ponasterone A can affect the molt cycle. [64,65] The molting hormone in insects and crustaceans are considered identical, [66] which may have implications for non-target toxicity to crustaceans. Although not yet assessed as an AOP endpoint, disrupted molting is a lethal event for insects. RXR-dependent molting cycles[67] suggest the sensitivity of Daphnia and insects to ligands of PAS binding proteins. Since an RXR-binding

element is absent from vitellogenins, this suggests other co-regulatory pathways for EDCs affecting ovarian maturation.

Daphnia reproductive endpoints are a basis for new Daphnia EDC assays and AOP endpoints

The most well-recognized Daphnia EDC endpoint involves the production of male offspring, a process regulated by methyl farnesoate (MF) hormone and molecular signaling from methyl farnesoate receptor (MfR), a heterodimer of the PAS protein Met and SRC.[68] Maternal exposure to MF and juvenile hormone agonists like pyriproxifen[69-72] results in male offspring. [62,73-77] This 'male-production' pathway involves conserved genes for sexual determination that are expressed in the ovary. Although not a recognized EDC endpoint, Daphnia alter their reproductive mode,[78,79] triggering sexual reproduction and transforming parthenogenetic females into males. The *D.magna* transformer gene is neither a sex-determinant gene nor in the male-production pathway, but may be a key molecular event for altered sexual reproduction[79] Under certain chemical exposures, Daphnia exhibit a gynadomorphism, or intersex phenotype.[71] Male production is regulated by MF receptor complex and undetermined doublesex (dsx-1) gene mechanisms.[68,69,71,76,80-83] Although not a recognized EDC endpoint, Daphnia alter their reproductive mode,[78,79] triggering sexual reproduction and transforming parthenogenetic females into males. In manipulative experiments using methyl farnesoate agonists, authors report that parthenogenetic Daphnia and sexually reproducing Daphnia utilize different gene mechanisms.[78,79,84]

Daphnia Reproductive X-Y axis regulates vitellogenin, a common reproductive marker

Vitellogenin is a common cellular marker for exposure to estrogenic compounds in fish.[85] In vertebrates, E_2 is transported to the liver and binds to the estrogen receptor (ER) in the cytosol. The E_2-ER complex binds to the estrogen response element on the vitellogenin gene and initiates transcription. Although Daphnia do not possess an estrogen receptor, EDCs that target vitellogenins can disrupt egg maturation in the ovary,[86,87] and neonate growth.[88] Daphnia vitellogenin (VTG) is co-regulated with methyl farnesoate,[89] leading to downstream pleiotropic effects: adverse ovarian development,[87] regulation of embryonic hematopoiesis,[90] hemoglobin transport via the hemoglobin gene battery,[91] and induction of superoxide dismutase (SOD) subunits involved in reduction of oxidative stress.[92,93] Daphnia vitellogenins are transported into circulation to reach ligand-activated Vtg receptors on oocytes.[94,95] Therefore vitellogenin expression may be a marker for multiple stressors in Daphnia.

Cross-communication within the X-Y axis produces complex signaling of VTG by ecdysteroids at a critical reproductive time point.[86] Signaling across overlapping generations in Daphnia has shown that regulation of vitellogenin affects maternal and embryonic processes, [95] a potential challenge when distinguishing life-stage effects using a transgenerational model. Studies have also found that increased vitellogenin is a maternal signal marking a shift in female reproduction from parthenogenetic to asexual mode preceding production of the diapause eggs [92,96] and biotic stress (unfavorable food or population density).[97]

Based upon a review of available literature, new Daphnia EDC assays may be necessary that involve genes that regulate sex determination, reproductive mode, and ecdysteroid pathways. The USEPA endocrine disruption screening program relies on a unified endpoints associated with male production;[71,74,76,77,98-100] however, questions remain about additional endocrine signaling pathways.[70,91,101-106] The transition from parthenogenetic to ephippial females may be an additional endpoint for a new EDC assay for *Daphnia magna*. Parthenogenetic and sexual females exhibit different gene profiles,[78,107] and genes involved in the switch of reproductive mode have not yet been linked to EDC exposure. In Chapter one of this thesis, we use a transcriptomic approach to identify candidate genes for EDC effects in *Daphnia magna*. Transcriptomic tools are a first step to determining candidate genes needed for production of genetic knockdown strains to functionally characterize and link these genes to a biological phenotype.

Adverse outcome pathways for behavior are relevant to neurotoxicity: Commonalities between zebrafish and Daphnia

Behavioral assays are considered an early warning signal for toxicity, and are used for identification of neurotoxicants. The behavioral adverse outcome endpoint has a measureable relationship to biochemical, genetic, cellular and molecular responses relevant to predicting neurotoxicity.[108] Zebrafish and rats have parallel dose-dependent behavior responses when exposed to dopamine agonists[109]; behavioral assays in fish are internationally recognized tools for screening anxiolytic substances. Common anxiety-related behaviors between fish and mammals serve as assays for effects of neuro-active substances.[110-112]

There are broad categories of endpoints included for altered adverse outcome behavior responses; these include reduced intracellular calcium levels, effects at voltage gated channels, increased neuronal apoptosis, and events leading to disrupted motor responses.[108] The repertoire of zebrafish behaviors include upward or downward swimming, escape, and light-dark responses.[109] Swim behaviors of Daphnia may be similar to that of zebrafish exposed to anxiolytic chemicals. Daphnia exposed to neurobehavioral compounds share behavioral phenotypes similar to other species, i.e., phototaxis, and altered swim behavior.[113-116]

Despite the high conservation of the genetic components of the neural synapse and neurotransmission machinery in zebrafish, Daphnia, and humans,[40] studies are limited on functional identification of orthologous Daphnia genes. Pharmaceutical drugs interact with common neurotransmitters in zebrafish, daphnia, and humans.[40,117] The unique biology of the daphnia naupliar eye neurons show that synapsin is a primary marker for neurons in the brain.[118] Recent exploration of Daphnia behavioral responses to serotonin and dopamine demonstrates a forthcoming effort to assess anxiological chemicals in this species.[117,119-122]

Behavioral barcoding in *Danio rerio* and *Daphnia magna* use photomotor responses

Zebrafish PMR assay for behavior

Although the battery of behavioral endpoints for AOPs can include elaborate designs, a simple approach of modifying light conditions is extremely effective for assessing light-driven

behaviors. In aquatic organisms, light is the main stimulus for determining daily migrations in order to feed and escape predators.[123] The zebrafish photomotor response (PMR) is a behavioral assay at the embryonic stage before vision has developed. This assay measures changes in photo-responsive hindbrain neurons in the eye after a pulse of light. During three intervals (basal, excitation, and refractory) zebrafish spontaneous movement is measured using an automated camera system.[124] Photo-taxis regulates zebrafish turning behaviors, also known as "behavioral barcodes."[109,125-129] Numerous barcodes are used to describe behaviors such as dark-flash induced, spontaneous turns, and other motor pathways.[130,131] PMR assays are thought to be a more sensitive endpoint of toxicity than traditional toxicity tests.[132-136] Although not yet recognized as part of an AOP for zebrafish, neurotoxicity is the primary mode of action for effects at these endpoints.

Daphnia use light-driven "Behavioral barcodes"

Light is a primary driver of Daphnia behavior.[137] Daphnia photo-motor behavioral responses rely upon highly conserved neuronal mechanisms and circadian clock genes.[40]   Exposure to neurotoxins alter phototactic behavior and reproduction in *Daphnia magna*,[117,119] therefore many toxicants may alter circadian genes. Recent transcriptional profiling showed that a key circadian gene (takeout) was among 21 genes essential to increasing lifespan[138]. Daphnia and Drosophila proteins involved in circadian signaling have conserved amino acid composition,[139] however, the circadian clock components in Drosophila differ from Daphnia,[140] and there are different numbers of clocks. [139]

AOP behavioral endpoints require validation of genes that regulates locomotion

Studies linking transcriptomic profiles to Daphnia behavioral endpoints are increasing, as is the needed validation using knockdown mutant strains[81] or RNA interference technologies.[141,142] However, studies are still needed to identify candidate genes for knockdown experiments. Particularly, AOP development links patterns from key events established from dose-response studies as evidence of causality. The baseline expression profiles for Daphnia movement in the water by circadian genes[137] should be validated through additional studies that assess regulation of circadian genes. Daphnia and zebrafish both depend upon a conserved circadian clock gene mechanism for survival-based feeding, movements, and reproduction.[143] Daphnia have a unique physiology involving photo-dependent genes[144] and a "central complex" in the brain [118]involved in circadian adaptations and locomotion that is essential to life. Daphnia express 135 circadian clock genes [145] that synchronize daily behavioral movements and escape from predators. [145] Both light and light independent mechanisms act on a 'negative transcription-translation feedback loop (TTFLs)[146] regulated by bHLH-PAS transcription factors, CLOCK (CLK) and CYCLE (CYC)[140,144], to regulate period (per) and timeless (tim) transcription[137]. TIM and PER proteins accumulate and translocate to the nucleus to inhibit CLK-CYC. Cryptochrome is a photoreceptor that resets the circadian clock, synchronizing light:dark cycles.[140] Light and dark environments are differently regulated,[139,147] and sudden darkness may induce anxiety behaviors in fish[148].

Disruption of circadian clock signaling has challenged assumptions that these genes are not affected by external influences,[149] but there are studies that indicate the environmental toxicants can alter circadian gene expression through interference with PAS domain-containing protein regulation.[149,150] Toxicants that disrupt this clock gene expression may potentially adversely affect aquatic species. A baseline understanding of clock mechanisms in light and dark environments may be needed to interpret behavioral responses in phototactic species. In addition to circadian genes, muscles controlling the naupliar eye are also required for prey-avoidance.[151-153] Therefore, circadian genes as well as genes for neurotransmitter release may be dysregulated by neuro-behavioral toxicants.

Behavioral toxicity assays developed for alternative species may need to consider the role of circadian clock genes, which may be more sensitive than survival endpoints.[124,135,136,154-157] Because of the importance of the evolutionarily conserved molecular clock across species[158-162], it is unusual that there are few studies that investigate effects of toxicants on circadian regulation. Toxicants that disrupt circadian rhythm can produce starvation and reproductive peril. To distinguish effects of circadian genes from other mechanisms, genes in multiple pathways can be estimated using a transcriptomic approach for crustaceans.[13,19,20,163]

Research Study Objectives

In these studies, we measured genetic and behavioral responses in two aquatic species, *Daphnia magna* and *Danio rerio* exposed to PAHs, EDCs, and candidate biofuels. In the first study, we used endocrine disrupting chemicals to assess genomic responses and mechanisms of toxicity in the X-Y reproductive axis of the model aquatic crustacean *Daphnia magna.* We hypothesized that EDC exposures producing male-offspring, characterized by the MfR and dsx1 male differentiation pathway, may involve additional non-male producing mechanisms and other EDC endpoints. Using maternal exposures in the parthenogenetic life stage, we estimate abundance of nuclear receptor gene targets in the X-Y reproductive axis, such as ecdysteroid inducible proteins, CYP450s, dsx-2, and vitellogenin receptor.

Using cluster analysis of microarray expression profiles, we identified three gene signatures among compounds and two distinct signatures between male-producing compounds. We quantified expression of candidate genes involved in these pathways, and used annotation tools to produce a new hypothetical gene pathway for EDCs. We measured the contribution of Daphnia cytochrome P450 genes involved in steroidogenesis, vitellogenin superfamily paralogs, transformer genes signaling the switch from parthenogenetic to sexual reproduction. Particularly, we identify gene profiles that differ between male-producing compounds, and describe a dsx-1 independent EDC model.

In Chapter 2 we further evaluated conserved nuclear receptor signaling in aquatic species, using a published zebrafish AHR developmental toxicity model. Aromatic hydrocarbons such as petroleum fuels and furans are known to activate the conserved aryl hydrocarbon receptor (AHR) in both fish and humans. Exposure to PAHs can produce oxidative stress through increased ROS production,[164,165] reduced antioxidant capability, or inhibition of CYP enzymes.[166] Aromatic compounds cause chemical-mediated ROS through the metabolism of parent compound (by

CYP4501A1) to a free radical, or mitochondrial toxicity resulting in impaired removal of ROS via antioxidant enzymes.[167] Using the 21-endpoint AHR developmental assay, candidate biofuels did not cause significant mortality or morphological defects associated with typical AHR agonists or oxidative stress.

We ranked the relative toxicity of a candidate biofuel, 2,5- dimethyl furan (DMF) and analogs for ligand-activation of zfAHR2 compared to potent AHR agonists beta-napthoflavone and benzo(a)pyrene using multiple criteria. We used an AHR homology model for multiple zebrafish isoforms to assess zfAHR1, zfAHR1B, zfAHR2 binding affinity, as well as a reporter cell assays containing XRE/AHR. We found that furan analogs do not activate zebrafish AHR (zfAHR2). Due to the potential for non-AHR toxicity, we also used zebrafish assay to assess the behavioral toxicity of DMF and its analogs. We determined that biofuels produced behavioral defects and ranked their relative potency to identify the least toxic alternative.

In Chapter 3, we link molecular mechanisms with behavioral phenotypes of *Daphnia magna* exposed to chemicals from the PAH and pyrethroid chemical class. We hypothesized that behavioral responses from toxicants may differentially alter Daphnia circadian genes and neurological endpoints, and that crustacean-specific gene expression from exposure to PAHs will differ from that of vertebrates. *Daphnia magna* photo-tactic behavioral responses were recorded without light, to measure the unique property of circadian locomotion in dark environments. We used microarray experiments to assess gene profiles from benzo(a)pyrene, fluoranthene, phenanthrene, pyrene, and cyfluthrin. In Daphnia, there are no known inducible AhR ligands due to loss of function of AhR after divergence from vertebrates.[168] The ligand-binding properties of PAHs to Daphnia PAS-bHLH proteins has not yet been demonstrated. Although AHR mechanisms were not involved, PAS-bHLH proteins and transcription factors in Daphnia regulate xenobiotic metabolism, growth, reproduction, and circadian rhythms.[169-173] Daphnia exposed to PAHs resulted in upward swimming behaviors as well as differentially expressed genes involved in neurological, circadian, and oxidative stress mechanisms. Cyfluthrin exposure produced a bottom swimming profile and reduced dopamine activity.

CHAPTER 2

Differences between juvenile hormone agonists on EDC endpoints in the Daphnia X-Y Reproductive axis

Abstract

The endocrine disruption assay for *Daphnia magna* is identified by the undesirable production of males, a complex mechanism regulated by the methyl farnesoate (MF) nuclear receptor complex, transcription of dsx-1, and many undefined genes. Additional mechanistic data is needed to develop adverse outcome pathways to estimate endocrine disrupting chemical (EDC) hazards in *Daphnia magna*. To obtain this data, we proposed to assess underlying mechanisms of EDC toxicity regulated by gene targets of key nuclear receptors (MfR and EcR) in *Daphnia magna* exposed to male-producing compounds. We conducted microarray analysis which led to identification of three chemical-gene clusters. Exposure to compounds in cluster 1 (methoxychlor and pyriproxifen) produced a complex signature including induction of vitellogenin-related genes involved in heme-transport, hemolymph coagulation, and lipid regulation. Vitellogenin expression has been associated with switching from parthenogenetic female reproduction to production of diapause eggs in arthropods. In cluster 2, methyl farnesoate and arochlor numerous downregulated mechanisms point to separate mechanisms of action within this chemical class. In particular, we found distinct regulation of steroidogenic enzymes (Shd, Phm) that are key markers for molting between cluster 1 (induction) and cluster 2 (repression). Cluster 3, exposure to 20-hydroxyecdysone, produced a unique profile that differed from other male compounds.

We estimated reproductive rates associated of Daphnia reproduction following exposure to 2.5% LC_{50} and 5% LC_{50} benzo(a)pyrene (BAP), a juvenile hormone agonist independent compound, and a known ligand of PAS-binding protein. Reproductive rates increased in Daphnia exposed to BAP. The lowest level of BAP had the highest reproduction and was correlated with altered gene expression of Jhe, Arnt, and UsP.

We report chemical-specific impacts on distinct sets of nuclear receptors in the Daphnia endocrine disruption pathway, raising the possibility of a non-dsx-1 pathway for an EDC toxicity AOP in Daphnia. As additional evidence of an alternative AOP for male production, we report increased reproductive rates correlate with exposure to known PAS-binding proteins, and reproductive gene markers juvenile hormone esterase and ultraspiracle.

Introduction

Endocrine-disrupting compounds (EDCs) have unique hazard properties for both vertebrates and invertebrates which requires a non-traditional toxicity approach. [63,174] The

adverse outcome pathway framework is a regulatory instrument to identify potential reproductive toxicants based upon key molecular events. The EDC model for vertebrates identifies effects at key nuclear receptors in the HPG axis,[5,53] harmonized with effects at estrogenic, anti-estrogenic, thyroid, and androgenic receptor mechanisms.[175-178]

In the model crustacean *Daphnia magna*, close orthologs to the vertebrate nuclear receptors specific for estrogen, thyroid, and androgen do not share mutual biology. The central hypothesis of this chapter is that molecular mechanisms for reproductive toxicity in *Daphnia magna* are established through methyl farnesoate and ecdysteroid receptor pathways. In this chapter, I use high throughput genomic technologies to determine the gene targets of known PAS-family nuclear receptors that likely play a role in response to crustacean EDCs can be determined. As a corollary to this hypothesis, I test whether different juvenile hormone agonists produce differential gene expression signatures. Finally, I use an independent chronic reproductive output assay to directly assess toxicant effects from benzo(a) pyrene, a reproductive toxicant involved in altered transcription of PAS-family regulated genes. The gene responses reported from multiple male producing chemicals provide gene targets for developing new Daphnia EDC toxicity endpoints.

Daphnia male production is the primary EDC adverse outcome pathway

Alternative test systems such as toxicogenomics have been promoted by OECD risk guidelines for the potential for elucidation of AOPs, and sufficient information to develop new assays.[3,179] Proving more sensitive than traditional toxicity testing approaches (mortality and viability), system-wide genomic profiling provides mechanistic information for extrapolation to nonmodel species, and reduces the numbers of animals required for testing. In previous toxicogenomic work, Antczak, Scanlan, and Vulpe (2013) applied the transcriptional approach in *Daphnia magna* to rank the most significant genes that are predictive of exposure to a variety of chemical classes.[21] A large number of transcriptomic studies have been used to estimate sensitivity of fish to endocrine disruptors,[53,180-184] permitting the study of tissue-specific regulation of molecular initiating events, leading to abnormal intersex phenotypes in fathead minnow,[185] or a meta-analysis to identify significant toxicant biomarkers.[186]

Under ideal conditions, Daphnia utilize a parthenogenetic reproductive mode which produces all female offspring. But under conditions of stress or toxicant exposures, females alter their reproductive mode and produce male offspring. Studies have shown that exposure to methyl farnesoate (MF), a juvenile hormone analog is the primary male-producing hormone in Daphnia.[72,74,75] Therefore, the consensus endpoint for Daphnia reproductive toxicity is a physiological assay for male production, [77 70,73,76,99,170,187] while other hormone-dependent EDC endpoints have not been described.

However, compounds which agonize methyl farnesoate receptors can also influence reproduction [188,189] sex ratios, [75,190] ovarian development, vitellogenesis,[191] and metamorphosis.[192] Therefore the Daphnia EDC disruption hypothesis fails to explain the range of developmental effects produced between juvenile hormone agonists.[70] Daphnia may not have

adequate protection from chemical toxicants affecting reproductive status or reproductive rates[193] because the primary adverse outcome is based upon one mechanism of action (male production).

AOP endpoints in Daphnia X-Y reproductive axis

In the current model for Daphnia EDC toxicity, exposure to MF causes repressed ecdysone pathways of development (Figure 3), [105,188,194] and induction of the dsx1 gene in male embryos.[76,81,82] Previous studies determined the role of dsx-1 and dsx-2 in male differentiation using knock-down approaches.[81] The methyl farnesoate receptor complex (MfR) upregulates doublesex genes,[76] and increased levels of the male determining gene, *dsx1,* is required to produce males.[81] The sex determination genes in Daphnia differ from that of insects; [79] subsequently, expression of the transformer gene indicates a shift from maternal reproductive mode from asexual to sexual.[78]

There are a number of insect growth regulators that may trigger different endocrine disrupting effects in the Daphnia reproductive X-Y axis (Table 1).[64] The X-organ is analogous to a brain-like neuronal control center such as the corpora allata in insects (Figure 1).[192] The X-organ releases neuropeptides to regulate ecdysone titers. Juvenile hormone production is negatively regulated by neuropeptides released by the X-organ.[76] Juvenile hormone (MF) and ecdysone regulate growth, development, and reproduction. The induction of vitellogenin, a common marker for reproductive toxicity, is induced by both methyl farnesoate (regulated by the X-organ) and ecdysone in the Y-organ. These two hormonal axes communicate with each other; methyl farnesoate may negatively regulate ecdysone through co-repression of the EcR/USP heterodimer.[195,196] The juvenile hormone receptor is a basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) receptor which dimerizes with a steroid receptor coactivator (SRC).[68] While some key receptors in the X-Y axis are known, there are additional transcriptional regulators and nuclear receptors in Daphnia [102,168,197-200] which have unknown function and may play a role in response to endocrine disrupting chemicals.

Based on the multiple modes of action of juvenile hormone agonists (JHAs), unidentified mechanisms for development and sex ratios may contribute to AOP endpoints not involved in male production. An alternate hypothesis for juvenile hormone agonists may have a significant impact on current thinking on the basic process of Daphnia reproductive toxicity.[68,76] EDCs are believed to primarily affect the non-estrogenic X-Y hormonal axis underlying sex differentiation and development in crustaceans. Changes in methyl farnesoate levels and temperature can induce male sex-determination pathways in parthenogenetic Daphnia eggs.[71] Transcriptomic profiling offers a tool to identify new molecular endpoints for Daphnia EDC assays and AOP endpoints. Our study links the modes of action of previously known toxicants to identify upstream molecular endpoints.

Conserved nuclear receptors and nontarget toxicity

Nuclear receptors likely play an important role in response to EDCs in Daphnia;[102,174,199,201-208] however the responses of Daphnia nuclear receptors to many compounds is incomplete. Benzo(a)pyrene is considered a reproductive toxicant in vertebrates, and a ligand

of members of the PAS-binding protein family. Methyl farnesoate, which produces males in Daphnia, regulates several pathways including the eponymous methyl farnesoate complex (MFC), a PAS-binding protein. Another key regulator for endocrine responses involving growth is the ecdysteroid receptor (EcR), an active heterodimer with the ultraspiracle protein (UsP), a RXR homolog.   Arthropod nuclear receptors (EcR) belong to the NR1H1 family which includes E75 and E78,[199] which are involved in oxidative stress, metabolism, and hormone production. Because of the promiscuity of Daphnia nuclear receptors, effects from PAHs and estrogenic compounds may disrupt biological signaling in crustaceans.[86,209,210]

In the present study, we use gene expression responses to male-producing chemicals to assess conserved nuclear receptor activation, an approach for linking significantly regulated genes to the male-producing endocrine endpoint in the X-Y reproductive axis. As a basis for observing reproductive phenotypes from a JH-independent agonist, we conducted a test of reproductive rates following PAH exposure.

Our results identified distinct gene expression patterns of key genes involved in molting and sexual programming after exposure to different male-producing chemicals.[193] We propose a novel dsx1-independent pathway responsible for a reproductive switch in maternal females to produce males in addition to a dsx-1 dependent mechanism for male-differentiation (dsx1) in the ovary. The observed changes in gene expression are a basis for further validation through knock-down mutants of these genes, increasing the direct evidence for a pathway for sex determination in developing oocyte that has been proposed.[63,76,211]

Results

Gene expression patterns differ within chemical class of juvenile hormone agonists

To identify gene expression patterns from male producing compounds in parthenogenetic Daphnia, we selected compounds previously known to affect reproduction; particularly pyriproxifen, methyl farnesoate, and methoxychlor.[75,77,170,191] Phenotypic results from these exposures were determined in other experiments comparing several chemicals, pyriproxyfen and methyl farnesoate induced males and an intersex reproductive state in some exposures.[71]

I showed that gene expression patterns in Daphnia exposed to two male producing compounds (pyriproxifen and methoxychlor) produced a shared gene expression profile, gene cluster 1 (Figure 1); while methyl farnesoate and arochlor exposure clustered independently suggesting distinct mechanisms of action (gene cluster 2). Exposure to 20-hydroxyecdysone produced a third unique gene profile. Common genes and developmental pathways for embryonic chitin binding were observed between clusters (Table 4).

Differential regulation of CYP450 steroidogenic genes in methoxychlor (MX) and pyriproxyfen (Cluster 1)

In analysis of significant genes from cluster 1, we observed 42 upregulated and 10 downregulated genes responsive to methoxychlor and pyriproxyfen [103] (Table 2, Table 2A). Exposure profiles in this cluster had significant gene effects from ecdysteroid receptor pathway.

Gene paralogs of ecdysteroid receptor (EcR-B, EcR-A1) were upregulated on the array, as well as its heterodimer, ultraspiracle (UsP). These effects were confirmed by altered regulation of EcR targets CYP306A1 (Phm) and CYP314A1 (Shd), two genes required for the last steps of 20-hydroxyecdysone synthesis (Figure 2). Shd and neverland (Nvd) are known to regulate Daphnia molting,[212] similar to their control of the precise timing in Drosophila metamorphosis.[213] Together these results are consistent with increased ecdysteroid regulated responses.

Within this cluster we also observed differential regulation of other endocrine endpoints: hormone biosynthesis, neurotransmitter signaling, development, yolk protein transport, heme assembly and binding, respiration. (Table 2).

In this analysis, four vitellogenin transport proteins were markedly upregulated, however a different Vg protein (DM01546) required for vitellogenin synthesis not strongly upregulated (Table 2). Methoxychlor and pyriproxyfen increased expression of Daphnia hemolectin-related genes (DM01546, DM11085), involved in coagulation and wound response (Table 2). Hemolectin proteins actively defend embryos from bleeding injury. [90,214,215] Also downregulated was a gene involved in hemoglobin production (DM11495), although which hemoglobin genes are transcribed is not always clear (Table 2A).

Methyl farnesoate and arochlor gene expression (Cluster 2)

Methyl farnesoate and arochlor exposure resulted in a distinct pattern of gene expression with 35 upregulated and 119 downregulated genes (Table 5). An array of genes with similar function for molting and development (cuticle and laminin) were substantially reduced. Organogenesis pathway signaling for trachea formation (DM11934) and nerve elongation (DM04779) were also reduced.

We observed downregulation of USP by this cluster (methyl farnesoate), while pyriproxyfen exposure increased transcription of CYP450 enzymes (regulated by EcR/USP). Pyriproxifen and methoxychlor show increased gene expression of the ecdysone biosynthesis genes (phantom, shade). USP is a cross-regulator of MF signaling; and MF depresses phantom and shade, which would lower ecdysone.

Inverse effects by different compounds on EcR and MfR pathways

We carried out additional focused analysis of known sex determining transcriptional factors in each cluster (Figure 3). We detected upregulation of EcR-B, EcR-A1, and USP receptor isoform in cluster 1, consistent with an increases ecdysone signaling in contrast to the inverse effect produced by methyl farnesoate (cluster 2) suggesting a down regulation of ecdysone signaling. Methyl farnesoate (MF) is a ligand of MET receptor complex, inducing transcription of doublesex (DSX) genes, therefore increased expression of DSX markers can be considered estimates of MET receptor activity. Two transcripts, DSX2_DM06301 and DSX2_DM14083, are both annotated as DSX2 genes in this array. Curiously, they showed different expression patterns between chemicals in cluster 1 which suggests mis-annotation or alternative transcripts with independent regulation. We observed upregulation of a Daphnia Tra

gene (DM00998), a key reproductive gene for sex determination in insects and a marker of altered reproductive mode in Daphnia[78,79,84,107]

Male production and intersex gene profiles differ between Juvenile hormone agonists

Pyriproxifen is more potent at producing gynadotrophic phenotypes, or intersex progeny[71] while there is a sex-determining specific concentration required in the exposure by methyl farnesoate. The different gene profiles between methyl farnesoate (cluster 2) and pyriproxifen (cluster 2) are consistent with the previous studies which considered in more detail the physiological differences in response to these chemicals. Based on the differential induction of EcR and Shd genes, we produced a hypothetical model to describe differential molecular pathways between methoxychlor/pyriproxifen and the methyl farnesoate chemicals (Figure 3).

Previous work found that methyl farnesoate induces a complex of sex-determination genes, including dsx-1,[76] with DapmaDsx1 being necessary for a phenotypic change. [81] Our profile of different JH agonists (methyl farnesoate and pyriproxifen) suggests a possible role for dsx-2 in reproductive toxicity. The function of dsx-2 in determination of sex is not known,[76,216] but it is expressed in the period of susceptibility for male sex determination. We saw inverse signatures of expression between these compounds: pyriproxifen reduced expression of dsx2_DM14083 while methyl farnesoate increased expression. We hypothesize that these difference could underlie the differences in Daphnia intersex frequency determined by Olmstead, LeBlanc.[71]

Vitellogenin is a pleiotropic marker for Daphnia EDC toxicity

Vitellogenin responses are well known vertebrate reproduction biomarkers, they are transported into circulation to reach VgR ligand-activated receptors on oocytes. There are many different vitellogenin genes that we could not fully characterize in this study. However, future testing may permit the use of Daphnia Vg mutants to show identify the role of candidate vitellogenin genes which may have a role in male production, or altered sexual modes are both induced by stress. [217-223] VTG also transport hydrophobic ligands[224] and may be part of reducing oxidative stress.[225] [94,95] Mutant rescue experiments coupled with transcriptomic profiling may show if vitellogenin-regulated heme genes[76] provide protection from a general stress response. We observed differential regulation of a vitellogenin-lipid domain involved in wound response and bleeding (von Willebrand factor type D domain (DM01546)), also reported to be involved in embryonic development.[92]

Endocrine disrupting compounds can affect both developing maternal and ovarian-embryonic stages which have MF-dependent X-Y axis regulation. Thus vitellogenins are involved in this complex signaling of overlapping generations, and vitellogenin-dependent growth.[95] Chemical-induced vitellogenin downregulation has been demonstrated for neonates [88] and is suppressed by ecdysteroids in adults at the reproductive time point in development.[86] EDCs that target vitellogenins can disrupt embryonic and egg maturation in the ovary. [86] [87] Multiple Vgs were induced by Male producing chemicals. In future studies, we propose to develop knockdown strains to conclusively determine the role of vitellogenin in Daphnia. Multiple endpoints including oxidative stress, immune responses, altered lipid transport, and

ovarian egg production could each induce vitellogenins. Vitellogenin (VTG) is co-regulated with methyl farnesoate,[89] leading to downstream pleiotrophic effects: adverse ovarian development,[87] regulation of embryonic hematopoiesis[90] and hemoglobin transport via the hemoglobin gene battery,[91] and induction of superoxide dismutase (SOD) subunits involved in reduction of oxidative stress.[92,93]

Methyl farnesoate significantly regulates Daphnia transformer homolog

A homolog of the Drosophila transformer gene (DmagTra) was previously identified,[79] and was not implicated as part of the male production response. However, we found that this gene was upregulated by methyl farnesoate/arochlor, but not pyriproxifen/methoxychlor. Because chemicals that alter reproductive mode also produce male-determination pathways in oocytes, this is an important marker for EDC toxicity. Altered reproductive mode is also a marker for changes in energy allocation and resistance to parasites. [217,226-229] In separate investigations, alternative mechanisms for sex-determination responses have been proposed,[68,71,76] which may be useful for defining a cross-generational endpoint (Figure 7). Other suggested mechanisms for endocrine disruption is a shift in female reproduction from parthenogenetic to asexual mode preceding production of the diapause eggs in arthropods[92,96] that most notably occurs in Daphnia exposed to biotic stress (unfavorable food or population density).[97]

Cytochrome-genes are key endocrine markers that are critical to molting

Both adult and embryonic life stages require CYP-dependent steroidogenic genes, [213,230,231] and are adversely affected by CYP inhibitors, i.e., anti-ecdysteroids.[62] We determined that juvenile hormone upregulated key molting regulators: CYP306A1 (Phm) and CYP314A1 (Shd) (Figure 2). In a previous report using RNAi to knock down *Daphnia magna* Shd (shade), it was shown that the key steroidogenic enzymes for molting are shade and neverland, produced in the gut of *D.magna* epithelial cells. [212] We observed the inverse response from methyl farnesoate in cluster 2. Downregulation of ecdysone from the Y-organ may lower levels of hormones for metamorphosis in larvae and induce transport proteins needed to deliver lipids to follicle cells. Arthropod nuclear receptors (EcR) belong to the NR1H1 family which includes E75 and E78.[199] In Drosophila, E75 is activated by juvenile hormone and induces the expression of embryonic metamorphic commitment genes (the Broad-complex). [188] The endogenous ligand for EcR in Daphnia is 20-hydroxyecdysone, or ecdysone, produced from cholesterol.[232] Daphnia CYPs regulate biosynthesis of 20-HE as well as decrease toxicity through biotransformation of hydrophobic toxicants.[233,234] Measured levels of juvenile hormones may be dependent on available CYPs. Based on the candidate genes from our findings, we recommend that cytochrome p450 genes be included as key gene markers for Daphnia EDC toxicity (Figure 7).

20-hydroxyecdysone exposure is not protective of adverse EDC effects

Anti-ecdysteroids perturb normal ecdysone levels, but sensitivity to 20-HE may also have anti-ecdysteroidal effects through altered endocrine homeostasis. Anti-ecdysteroids can delay molting, through increasing or decreasing the required levels of 20-HE. The comparison of JH agonists with 20-hydroxyecdysone resulted in a novel expression profile which may be similar to the anti-ecdysteroid chemicals, which lower the endogenous ecdysone levels[100], and least similar

to the juvenile hormone agonists. 20-hydroxyecdysone is the ligand of EcR however exogenous exposure does not result in male production or altered molting, as it can have a protective effect against anti-ecdysteroids. Exposure to 20-HE had a unique profile that differed from Clusters 1, and 2 for overall gene expression. We noted that the ecdysteroid genes ECR_DM01649 (EcR-B) was upregulated but EcR_DM00110 (EcRA1) was largely downregulated. 20HE also downregulated cytochrome biosynthesis genes Shd, Phm, consistent with repressed EcRA1 (Figure 2).

Chronic Reproduction assay

To demonstrate the reproductive effects of a non-juvenile hormone analog that is also a PAS-binding protein ligand, we used benzo(a)pyrene (BAP) exposures to estimate changes in reproduction rate. After 15 days of testing, the number of neonates were counted after each brood, and compounded to show to overall result of the 15-day test after exposure to acetone (control) and Benzo(a)pyrene (Figure 5). We determined 620 neonates in control tests, 1323 neonates at the 2.5% LC_{50} BAP exposure, and 1130 neonates at 5% LC_{50} BAP. This experiment demonstrates that non-AHR mechanisms may produce reproductive changes in crustaceans.

Gene transcription of reproductive biomarkers

PAH exposure increased mRNA expression for toxicant metabolism while gene expression at a hormonal biomarker was reduced in Daphnia adults. We found that toxicant-response pathways (ARNT) were more responsive than juvenile hormone (JHE) responses for both Benzo(a)pyrene and phenanthrene exposures (Figure 2). Benzo(a)pyrene significantly induced more transcriptional response at all genes tested. Benzo(a) pyrene exposure increased mRNA levels in the following order from highest to lowest: ARNT, RXR, JHE, and COX.

Discussion and Conclusion

We investigated several chemicals known to lead to endocrine disruption in Daphnia. The common endpoint of endocrine disruption is inappropriate male production in the usually parthenogenetic Daphnia. In the current model for Daphnia endocrine toxicity, exposure to male inducing chemicals, such as methyl farnesoate causes repressed ecdysone pathways of development,[105,188,194] and induction of the Dsx1 gene in male embryos.[76,81,82] Our gene expression shows a more complex reality with at least two distinct modes of action.

Alternative model for JH agonists: EDC chemicals suggest a non-Dsx1 dependent pathway

In a current model for male-production by juvenile hormone agonists and reproductive stressors (Figure 4), exposure to methyl farnesoate activates the methyl farnesoate receptor complex, producing transcription of Dsx1 the sex determination gene. Methyl farnesoate may induce a complex of sex-determination genes, including dsx-2,[76] whereby the function of its paralog dsx-1 is not clear. [81]

Dsx-2 is not thought to have a role in endocrine function, and no role in sex-determination in the critical period of male-production in female Daphnia. The EcR/UsP signaling pathway has not previously associated with male production, but the UsP binding partner can also bind MfR, and ecdysone levels negatively regulate production of MF from the Y-organ. The functional role of many Daphnia nuclear receptor transcription factors is still undefined.[207] Although components for EDC mechanisms are not yet produced, Dudycha, Shaw, and Kato have developed knock-down mutants to perform loss-of-function studies for new genes of interest.[141,142]

We propose a model based on our results for EDC target genes from each cluster to identify potential pathways of juvenile hormone agonist signaling (Figure 6). In pathway 1, methoxychlor-pyriproxifen related compounds are potent inducers of the EcR-UsP signaling pathway. EcR and ecdysone are regulators of molting genes, Shd and Phm, which were also upregulated. We observed upregulation of Dsx2 and reduced Dsx1 downstream of the MfR pathway. Pyriproxifen was in this cluster but was dissimilar to methoxychlor in the downregulation of Dsx2 by pyriproxifen. Interestingly, pyriproxifen was more potent at producing altered sex-ratios than methyl farnesoate, which produced an inverse signature for EcR regulation.

A separate gene cluster responds to methyl farnesoate and arochlor exposure. These compounds downregulated EcR, which may be negatively regulated by increased methyl farnesoate. Low activation of the EcR/UsP receptor resulted in lower levels of Shd and Nvd genes. These compounds upregulated Dsx1 and downregulated Dsx2 in the MfR pathway. Vitellogenin genes for ovarian development and Hsp70 were upregulated. We observed a 7-fold upregulation of DmagTra gene, involved in alternating reproductive modes in parthenogenetic daphnia.

We found an inverse-gene regulation of EcR isoforms between methoxychlor and pyriproxifen chemicals and methyl farnesoate clusters. Differential gene induction may support findings that pyriproxifen may be a potent inducer of gynadomorphism, a phenotype regulated by specific titer of maternal hormone. [71] Toxicological responses shared between JH-agonists suggests an additional mechanism for EDC chemicals in Daphnia. Commonly shared nuclear receptor mechanisms across species are a first step toward determining species-specific endpoints that translate into better identification of EDC hazards for aquatic organisms.

Our chronic reproductive assay using benzo(a)pyrene, a model reproductive toxicant for vertebrates resulted in increased reproductive rates and altered gene expression at Daphnia PAS gene markers. This suggests BAP may interact with Daphnia PAS receptor orthologs using alternate MfR/EcR mechanisms that influence reproduction. We anticipate that using Daphnia RNAi strategies will further define the effects of juvenile agonist chemicals for EDC adverse outcome pathways.

One factor in our investigation that could not be overcome was the recognition of nuclear receptors which were not present on our microarray. A second limitation was the inability to separate the independent effects from maternal and embryonic exposure in overlapping

development windows. While problematic to discern individual life stage effects in the published studies, this work did find that maternal genes may be important reproductive markers for male-development and neonate development.[68,75,76,87,89,99,191,235] Effects of chemicals that target Daphnia endocrine homeostasis likely differ depending on life stage.[98] Daphnia bear parthenogenetic live young which develop internally after egg release from the ovary into the brood pouch. It is unlikely that neonate gene profiles can be distinguished from maternal gene responses in overlapping exposure window, and the scientific literature contains inconsistent exposure regimes for Daphnia neonates.[70,89,187,236,237]

Previous work on EDCs [70,89,187,236,237] generally encompass the complex time period including egg maturation within the ovary, brood pouch development and the immediate neonatal perinatal period. Maternal genes may be important reproductive markers for male-development and neonate development.[68,75,76,87,89,99,191,235] Prenatal exposure to the pesticide methoxychlor (MXC) impacted multiple targets in the female HPG axis (steroidogenesis and ovarian development);[238] as well as adverse epigenetic effects throughout the multiple life stages.[239]

In concordance with previous work, we find expression patterns consistent with downregulation of ecdysone biosynthesis by methyl farnesoate and arochlor (cluster 2), but not with pyriproxyfen and methoxychlor (cluster 1) where upregulation of ecdysone biosynthesis genes (Phantom,shade) is observed. In addition to Dsx1, differential effects on Dsx2 genes suggests a possible role for these gene products in reproduction. We also noted distinct effects on USP (the RXR homolog which complexes with MfR, SrC) in that 20HE and the pyriproxyfen cluster 1 shows upregulation of USP while cluster 2 does not. We observed that benzo(a)pyrene altered reproductive rate and altered gene markers for endocrine endpoints (Jhe, UsP). Although not considered a reproductive toxicant for Daphnia, Benzo(a)pyrene alters DNA methylation and behavior over successive zebrafish generations.[27,240] Effects on multiple-generations of Daphnia EDC exposure have not yet been performed.

A recent screening assay developed by Kakaley and LeBlanc (2017), utilizes a MfR bioluminescence resonance energy transfer assay for ligand-mediated responses;[106] however, this is only one target for an adverse outcome therefore, accompanying validation of other candidate genes are needed. In the Daphnia EDC toxicity model used in this chapter, commonalities with vertebrate EDC biomarkers, such as vitellogenin and steroidogenic enzymes were found. Our work supports other findings that vitellogenin responses are involved in the critical reproductive test window.[186] We observed that maternal exposure to synthetic juvenile hormones affects transcriptional regulation of vitellogenin, ecdysteroid production, and hormone levels required in the developing oocyte, as well as genes involved in the maternal reproductive switch. We report at least two distinct mechanisms which result in a common endpoint of male production. The candidate genes provided increase the endpoints for EDC determination by an adverse outcome pathway; and lend support for performing computational and transgenic experiments to identify the precise nuclear receptor mechanisms of the Daphnia endocrine system.

Methods

Culture of *Daphnia magna*

Daphnia magna culture and maintenance protocols were obtained from USEPA methods for aquatic toxicity testing. [241] Culture medium was produced from stock solutions of macronutrients and micronutrients according to standard protocols.[242] Ages of clutches and adults were tracked by date. Standardized feeding regime and media conditions were followed. [241] A non-stressed environment of 16h light/8h dark was maintained.

Chemicals and microarray hybridization

EDC chemicals evaluated for this study were beta-estradiol, nonyphenol, ponasterone A, methoxychlor, pyriproxifen, arochlor, methyl farnesoate, and 20-hydroxyecdysone. Control Daphnia were exposed to COMBO medium (pH7.4) and DMSO; Treatment exposure dose was $1/10$ LC$_{50}$ concentration of each chemical, according to protocols from previous report. [21] Daphnia exposure occurred at the stage of sensitization to MF in the ovary, 14-day old.[81,87] Treatment and control beakers/tests contained 20 adult Daphnia each (Figure 1).

Following homogenization in Trizol reagent (Invitrogen, CA) at 4°C, RNA was extracted using methods for RNA Purification mini-columns (Qiagen) and assessed by spectrophotometer and agarose gels. Methods were obtained from the 'One-color Microarray Labeling Protocol' for transcription of cDNA, and amplification of cyanine 3-labeled RNA for hybridization.

Gene expression and cluster analysis

The custom Agilent 14K microarray was design AMAID: 023710). Scanning and feature extraction were performed using GenePix4000B and GenepixPro 6.0 software.[21] Raw microarray expression values for EDCs were analyzed separately from a large chemical screening dataset.[21] A loess normalization was used on the filtered dataset. Hierarchical clustering was performed with Euclidean distance parameters; SAM (statistical analysis of microarray) compared gene expression between chemicals within the male-producing phenotype and the other chemicals. SAM analysis was performed using a software tool for genomic data exploration, TMeV (TIGR multiple experiment viewer microarray analysis). HOPACH plots and gene heatmaps were used on data to compute ordered distance matrices using the cosangle metric as a measure of similarity using packages from the R-environment. Enzyme code annotation was performed using the web-based InterPro functional analysis tool,[243] *D. Pulex* genomic database (wFleaBase),[244] and Drosophila (Flybase)[245] ontological database.

Chronic Daphnia Reproduction test

14-21 Day Chronic Exposure

The 14-21 Day chronic reproductive test was used to test the physical reproductive effects of a reproductive toxicant, benzo(a)pyrene. Environmentally relevant doses were used for exposures (2.5 % and 5% of the LC$_{50)}$; number of *Daphnia* alive and the number of neonates in each brood were counted.

24 Hour Acute Exposure and qPCR gene expression of biomarkers

A 24-hour acute exposure of Daphnia adults conducted at 10% of the LC_{50} of benzo(a)pyrene and phenanthrene with a no-chemical control. Reproductive and toxicant gene markers (Arnt, Jhe, Usp, Cox) were obtained from published literature.[88] After a 24-hour exposure, RNA was extracted from the *Daphnia* to measure changes in gene expression using qPCR. We utilized standard protocols for DNase digestion (TURBO DNase DNA-free kit), iScript reverse transcription protocol amplify cDNA from the RNA template (BioRad iScript RT-qPCR kit #170-8840), followed by labeling reactions with SYBR Green quantitative PCR amplification kit (BioRad: SsoFast EvaGreen Supermix, cat#172-5200) using the supplied user protocol for a thermal cycler reaction with 15ul of amplification mix to 5ul RNA for reverse transcription. A PCR thermocycler was set for 5 min @25˚C, 30 min at 42˚C, and 5 min at 85˚C. Raw expression values were obtained using BioRad's CFX-96 real-time PCR detection system, CFX Manager software and analysis.

CHAPTER 3

Anxiety-like behavioral patterns in *Daphnia magna* exposed to PAHs and pyrethroids:

Toxicant-modulation of Daphnia circadian and neuro-responsive genes

Abstract

Behavioral endpoints are gaining acceptance as a sensitive toxicological endpoint for vertebrates and invertebrates. We assessed the utility of several behavioral endpoints in Daphnia exposed to two chemical toxicant classes, polyaromatic hydrocarbons (PAHs) and pyrethroid insecticides. We found characteristic changes in swimming speed and behavior in response to these distinct chemical classes. Daphnia exposed to toxicants were moved to a dark-exposure chamber and recorded using an automated camera video over 1-hour and 24-hour intervals. Similar to zebrafish, we found that Daphnia exhibit anxiety-like behaviors such as preferential swimming at the bottom or upper surfaces of testing tanks in response to some toxicants. Interestingly, PAHs generally increased swimming speed and increased upper surface swimming while pyrethroids generally decreased swimming speed and led to bottom swimming behavior. In parallel to these studies, we assessed the genomic response of the Daphnia to each stressor to determine if we could correlate particular behavioral responses with transcriptional responses. All compounds affected the swimming speed of <24-hr-old daphnia consistent with a neurological effect. We identified multiple genes whose differential expression correlated with increased swim speed and surface swimming. Of particular interest was an effect on a circadian rhythm gene, cryptochrome-2, which suggested a possible disruption of the biological clock in these organisms. We conclude that Daphnia behavioral changes that may be important in assessing biological effects of exposure of neurotoxic chemicals to aquatic crustaceans.

Introduction

Daphnia anxiety-related behavior may be a new adverse outcome for neurotoxicants

There is a need to promote new ecological assays that detect sublethal physiological endpoints that relate to survival and growth[112] that can be assessed more rapidly.[246] *Daphnia magna* is an ideal model for multiple-endpoint studies needed for developing adverse outcome pathways (AOPs). Because of the small size and rapid development, Daphnia behavioral, developmental, and cellular endpoints can be performed in a 96 well plate using whole organisms at different life stages. In this chapter we propose to utilize the AOP framework for linking molecular and behavioral endpoints from exposure to two chemical classes with neurotoxic potential. Using this approach, we hypothesize that neurological endpoints for PAHs and pyrethroids may be similar between crustaceans and zebrafish, and are causative agents for behavioral changes. In many species, pyrethroids and other pesticides are known to pass the blood-brain barrier [247-250] (multiple entry points for insects[251]), and interfere with neurological signaling and synapse machinery.[252,253] PAHs are characterized by two or more aromatic rings, which have differential binding affinity to the aryl hydrocarbon receptor (AHR) and induction of the cytochrome P450 gene family.[254-257] According to the general pathway for AHR toxicants, common constituents of crude oil mixtures such as benzo(a) pyrene, phenanthrene, and pyrene produce a toxicant response through tissue-specific induction of AHR-genes, particularly CYP1a1 and CYP1b.[258] In addition to these molecular events, key PAH-related behavioral effects have also been reported.[259,260] Behavioral abnormalities are proposed to be a more sensitive predictor of PAH toxicity even when traditional CYP1A markers are absent.[30,259] There is no single behavioral test for vertebrate species, but commonalities in behavior endpoints between rats and fish are reported.[148,261,262] Behavioral endpoints have been shown to be more sensitive endpoints for fish species,[30,112,133,136,263-265] and more protective for filter feeding organisms.[266]

Molecular basis for PAH-induced Behavioral endpoints using Transcriptomic approach

As demonstrated by Chapter 1, Daphnia do not share a similar biology with fish, although many conserved nuclear receptors have been shown. A new behavioral AOP for PAH exposure in Daphnia may utilize other non-vertebrate molecular endpoints. Since aquatic crustaceans do not possess a AHR receptor that is sensitive to vertebrate AHR ligands,[168] many toxicants induce other related proteins in the Per-Arnt-Sim (PAS) family of ligand-binding nuclear receptors.[102,198,199,213,267] PAS-bHLH proteins and transcription factors regulate growth, reproduction, and circadian rhythms.[169-173] Activation of orthologous PAS-bHLH toxicant receptors are a mechanism for conserved cross-species toxicity.

To adequately identify large number of changes in the molecular response of Daphnia, we utilized the transcriptional profiling method for PAH-exposed Daphnia. We hypothesize global gene responses will involve toxicant mechanisms that arise from non-AHR mechanisms such as increased ROS production,[164,165] reduced antioxidant capability, or inhibition of CYP

enzymes.[166] This data will be bridged with standard behavioral endpoints observed among mammals and fish, particularly for common anxiety-related endpoints such as swimming along the edge or bottom of a tank.[262,268,269] In this chapter, we observe that *Daphnia magna* may similarly demonstrate preferential surface or bottom swimming in response to chemical toxicants. While a wide range of anxiety-like behaviors is reported in literature,[110,136,155,270] similar neuro-behavioral endpoints have not been yet been proposed for crustaceans.

Zebrafish 'behavioral barcodes'[271] have been proposed as a new tool for identification of new pharmaceutical drugs and for linking chemicals to neurotoxicity.[124] Recent investigations have shown that zebrafish exposure to benzo(a)pyrene in a light/dark test (larval zebrafish prefer the dark) and swimming at the bottom of the tank was an anxiety-like behaviors.[272] This repertoire of behaviors includes patterns for escape,[269,273] geotaxis,[269] time spent at the top or bottom of the tank, light/dark preference, or avoidance.[110] These behaviors involve photo-responses in embryonic zebrafish [124,154,271,274] guiding swim behavior, phototaxis, and spontaneous turning.[131,275] Photomotor behavior assays in fish and mammals often utilize similar endpoints[127,157,276-278] that are recognized as anxiety-like behaviors for defense, [279] stress,[269,280,281] and exposure to pharmaceutical drugs.[269,282] Anxiety phenotypes are useful in stress research to assess potency of anxiolytic drugs[262] and understand models of brain disorders such involving stress-induced motor and learning functions.[280,281]

Molecular basis for Pyrethroid-induced Behavioral endpoints using Transcriptomic approach

A large precedent has been established for the nexus between gene expression patterns and important ecological phenotypes.[283] Therefore identification of key orthologous genes that play a role in toxicant responses contribute to development of more sensitive assays for monitoring recreational waters and fishery habitats. Microarray experiments have measured exposure concentrations in field exposed fish and invertebrates using comparisons to known concentrations of laboratory exposure fish to endocrine disrupting compounds;[15,16] this approach is found to be consistent between laboratories using the same protocols.[15]

As an exposure 'barcode' in field samples, gene expression profiling was used to identify mechanisms of toxicity for *Daphnia magna* exposed to flame retardants,[19,21] metals,[13,14] and endocrine disrupting compounds.[163] For the present study, utilization of transcriptional profiling approaches on Daphnia exposed to pyrethroids, a known neurotoxicant and sodium channel agonist, is a first step toward needed comparisons for neurotoxicant pathways between fish and crustaceans. Pyrethroids target the conserved gene family of voltage-dependent ion channels involved in communication between neurons at the neuronal synapse.[284,285] Prolonged opening of sodium channels can induce hyperactivity,[286-288] and neurotoxic symptoms.[289] However, reported non-significant motor changes may result from using exposure regimes at a later life stage for zebrafish exposed to bifenthrin.[290] Pyrethroids decreased dopamine receptor 2A (DR2A) expression in rainbow trout [291] and mice [253] which could represent a compensatory response. Dopamine neurotransmission is produced from binding to dopamine 1 or 2 receptors (D-1 and DR-2), and reabsorption into the cell by dopamine transporters.[253] Pyrethroid could target these processes through altered release of dopamine released from synaptic vesicles,[250,284]

a process controlled by calcium-dependent phosphorylation by calcium/calmodulin kinase II (CAMKII). In addition, exposure to another pyrethroid, cyfluthrin. can lead to oxidative stress and protection from cyfluthrin-related oxidative stress was shown by co-administration of antioxidants.[292]

Using transcriptional profiling tools, we determined sensitive neurobehavioral endpoints for development of Daphnia AOPs for exposure to pyrethroids and PAHs that are coupled with Daphnia anxiety-like behavioral responses. The behavioral phenotypes used for an adverse outcome pathway approach involve conserved neurological or biological mechanisms. [293,294] Further studies will be required to test effects of known neurological agents (neurotransmitter agonists, antagonists,) to delineate specific effects of pyrethroids and other potential neurotoxicants as part of a predictive Daphnia neurotoxicant AOP.[108]. Studies involving brain activities in Daphnia are rare; however, Daphnia possess conserved neuronal mechanisms and retinal circuits underlying behaviors.[162,295,296] Behavioral responses that are coordinated with other endpoints from similar exposures (i.e., PAHs, mitochondrial toxicants) are suitable for producing an AOP linkage.[8,9,39]

Other Behavioral Endpoints may involve circadian interactions

Biologists recently identified a new behavioral endpoint for macroinvertebrates: 'behavioral drift.'[297] *Daphnia magna* and other similar macroinvertebrates depend on nightly locomotion to the water surface for survival, which is an endogenous swim behavior in dark environments. Disruption of swim behavior to the surface of a tank increases the risk for predation and thus represents a useful phenotype to assess effects on circadian rhythm functioning. The potential for a disruption in the circadian rhythm may occur from environmental temperature, predation, light-dark exposure. A supporting study demonstrated that exposure from a pyrethroid altered behaviors in dark environments.[298] Based on this unique biological system, our next hypothesis involved whether conserved circadian genes were involved in toxicant-exposed Daphnia. We expected that toxicants that caused altered behavior would also affect conserved mechanisms for locomotor activity, driven by circadian clock control genes. As a baseline for circadian gene phenomena in dark environments, we utilized an automated video camera recording system of toxicant-exposed Daphnia during a dark exposure.

Movement in response to light involves the circadian "clock genes," a conserved gene network that is shared across species. [137,144,151,295,299,300] Circadian rhythm genes coordinate behavior of aquatic species to migrate in the water column and synchronize with a 24-hour external environment. Circadian genes are evolutionarily conserved regulators of light-sensitive CLOCK transcription factors and cytochrome proteins.[144,162,301] The clock-gene mechanism involves 134 genes involved in photo-sensitive locomotor activity; circadian clock genes are regulated by their own expression.[169] Circadian outputs are regulated by a transcriptional feedback loop that is driven by bHLH-PAS transcription factors, CLOCK(CLK) and CYCLE (CYC);[144] mechanisms of circadian regulation are negatively regulated by cryptochrome proteins. Daphnia circadian clock is more similar to mosquito than Drosophila -which has a peripheral clock and a core clock. Based upon homology of cryptochromes, ligand binding carriers, PAS domain proteins, the LDLR scavenger receptor, Daphnia-specific clock

mechanisms have been identified.[144] The 'core clock' regulates the endogenous biorhythm of Daphnia orientation and locomotion.[145,300,302296] Interestingly, recent work suggest that external influences, such as salt exposure, can affect the Daphnia clock-gene network.[149]

Photo-sensitive motor responses are used in aquatic toxicology testing as a sensitive ecological endpoint in embryonic fish,[133,156] with numerous studies in Daphnia that indicate diel locomotion is a photo-tactic response.[137,295,300 145] Recently, a group of cryptobiologists have identified *Daphnia magna* circadian genes and co-factors.[139] Based on this shared physiology that may be a useful tool for Daphnia assays, we hypothesize that the transcriptional program for clock genes and swim behavior may be toxicant-responsive.

Increased potential for pyrethroid toxicity to affect vertebrate and invertebrates

Pyrethroids have been a recommended replacement for organophosphates to control agricultural pests,[303-305] and thus has increased aquatic contamination with these compounds. Widespread use on strawberries, lettuce, and orange crops has created occupational hazards for California workers.[306] Organophosphate exposure reduces neurotransporter function and contributes to neurologic disease and behavioral effects.[307] Pyrethroids are suggested to be less toxic in people than organophosphates,[308] however this contention is under evaluation by EPA[306] and are not currently listed on the 2018 REACH Restricted Substance List. Permethrin and cyfluthrin are type II pyrethroid insecticides that are not bioaccumulative,[309] but have caused toxicity to non-target organisms[310] and have shown endocrine disruptive and neurotoxicant effects in humans. [287,305,311,312 313] Endocrine disrupting effects of cyfluthrin are exerted by antagonistic action on androgen and thyroid receptors,[311] and antagonism of estrogen receptors.[291]

To determine *Daphnia magna* neuro-behavioral phenotypes, we used a high resolution video-tracking system to precisely follow individual daphnia neonate movements on very short time scales. To identify phenotypes, we compared swim phenotypes (speed, positional placement, locomotor patterns) of *Daphna magna* neonates exposed to toxicants with that of unexposed Daphnia. To better understand the molecular basis of toxicant-induced behavior, we measured transcriptional responses of *Daphnia magna* to each chemical. We further evaluated heartbeat as an additional physiological indicators of stress.[314,315]

Significance of study

There is a need for more sensitive ecological endpoints in hazard assessments for aquatic invertebrates, that are integrated with molecular responses and phenotypic traits.[136] Behavioral toxicity is a change that exceeds the normal range of motor activity (swim performance, speed) that may involve learned or innate responses.[316,317] Anxiety-like behaviors are reported from behavioral studies in zebrafish exposed to toxicants.[155,262,268,269,273] Recent investigations have shown that zebrafish anxiety-like behaviors are elicited from exposure to benzo(a)pyrene in a light/dark test (larval zebrafish prefer the dark) and a swimming test assessing time spent at the bottom of the tank[272]. Similarly, altered behavior was noted in exposure from a pyrethroid in dark environments.[298] Although many PAHs cause neurobehavioral responses in fish, similar

anxiety like behaviors in *Daphnia magna* have not yet been reported. Behavioral phenotypes in daphnia include swim speed and rotation angles, [122] CO_2-dependent anti-predatory response,[318] and pharmaceutical-induced phototactic behaviors.[117] As also described in fish, photomotor behavior in Daphnia is a factor in reproduction, feeding,[123,129,319,320] escape responses, and photo-dependent diel migration. Methods for assessing fish anxiety-behaviors include video-tracking and real-time monitoring in 2D and 3D,[269] and light/dark conditions.[321] Upward and downward geotaxis in fish has also been described in Drosophila, [322,323] and similar mechanisms for anxiety-like behaviors in Daphnia and other taxa has been suggested.[120] Evaluations of neurobehavioral toxicant effects in Daphnia are not abundant in literature, and available information regarding nervous system signaling pathways are slow to emerge.[324]

Behavioral phenotypes are considered a complex endpoint that is an 'early warning signal,[321] that integrates biochemical and metabolic processes and results in changes at higher ecological levels of organization. The benefits of animal models are to show efficacy of neurological compounds, and unravel unknown aspects of mechanisms of action. A potential confounding factor, or more interestingly a useful neurobehavioral endpoint, in Daphnia models may be the regulation of the endogenous system of circadian clock driven behavior used by insects and nektonic crustaceans. [162] Identifying effects on the circadian clock mechanisms may be achieved using the transcriptomic approach. Recent findings in other organisms have demonstrated effects on clock gene expression from exposure to common pollutants. [150,325]

The role of circadian interactions in Daphnia response to toxicants is not well described. We utilized a toxicogenomic approach for identification of candidate genes that correspond with anxiety-like behavioral phenotypes observed in *Daphnia magna.[326]* We hypothesize that Daphnia anxiety-like behaviors may involve genes that are known to have a role in swim orientation, and genes regulating neurotransmitter synthesis and storage. In this current study, we use the established approach of transcriptional profiling that has a identified a wide range of diverse toxicant mechanisms under transcriptional regulation.[13,14,327]

Research Approach

We used a dark exposure system for behavior to identify anxiety-like behaviors, which have not yet been described in Daphnia magna. In the absence of light, the infrared light in the assay detects motion of daphnia. Daphnia use phototactic movements toward the dark, a characteristic of diel vertical migration that prevents predation.[117,297] A behavioral assay in the dark has multiple advantages for assessing sensitivity of Daphnia to toxicants. The darkened chamber simulates the ability for Daphnia to escape predation, similar to "behavioral drift", a daphnia movement toward the surface.[297] A dark chamber prevents exposure to blue light wavelengths which may affect phototransduction pathways in the eye which are required for visual-mediated responses (therefore measures nonvisual responses). The lack of a light source may permit the assessment of the specialized trait of "feeding at the surface" to be assessed.

Results

Daphnia swimming behavior in PAH exposures

We identified two behavioral patterns in Daphnia exposed to PAHs, which is similar to time spent at the top of the tank or tank diving in PAH-exposed zebrafish.[272] PAH-exposed Daphnia either swam near the surface or the bottom of the aquaria. Exposure to pyrene, phenanthrene, and benzo(a) pyrene resulted in a surface-swimming behavior. Fluoranthene, cyfluthrin, and permethrin exposure resulted in more swimming at the bottom of the tank (Figure 4C). Control Daphnia did not seek the edges of the tanks.

Swim speed did not significantly differ between control groups (DMSO, Acetone and COMBO) 1 (Figure 2). Cyfluthrin, permethrin and fluoranthene significantly decreased the average swimming speed both at 1-hr and 24-hr of exposure (Figure 3). A significant decrease in swimming speed following benzo-a-pyrene and pyrene exposure was noted at higher concentrations only. Phenanthrene was the only test agent which increased the swimming speed of the Daphnids especially at higher concentrations (Figure 3).

Gene profiles from Toxicant exposures

Phenanthrene

We estimate more than 17 significantly affected GO processes involved with increased metabolism in response to phenanthrene (Figure 6). PHE decreased the expression of two genes involved with photoreceptor functions: Mbn12 and Cry-2 (Table 4A). These genes are members of a regulation pathway (GO:0065007) which includes at least 9 other genes ((Table 4B), Figure 5). Genes critical for neuronal signaling (dopamine neurotransmitter) and integrity of blood brain barrier (neurexin-4, and merry-go-round (Mgr/ Pfdn3)) were deregulated by PHE exposure. (Table 4A). These findings were consistent with the hypothesis that PAHs may affect neuronal mechanisms; many neurotoxicants are characterized by neurotransmitter toxicity and interference with synaptic machinery. Although there is recent investigation that Daphnia circadian clock pathways can be modulated by serotonin and anxiety drugs,[117] we determined that exposure to PAHs may also lead to disrupted circadian pathway (Cry-2) outcomes.

Pyrene

Daphnia pyrene exposure reduced expression of 32 genes, and increased expression for 59 genes. We observed a 15-fold increased expression of neurexin-4, a protein for protection of the blood-brain barrier (Table 4). Induction of *NRX* genes are linked to abnormal presynaptic signaling.[248,284] Other neurodegenerative gene markers that were affected were spin and Anx-B9 (Table 6). The sensitivity of the brain to oxidative stress is a common feature of neurotoxicants, and can induce neurobehavioral toxicity in zebrafish.[272] Other gene products observed from pyrene exposure were also apoptosis genes and laminin adhesive proteins (Table 6), which may suggest that pyrene opposes fortification of membrane structures which would amplify cellular damage.

Fluoranthene

We observed downregulation of 171 genes following fluoranthene exposure (Table 7). Although Daphnia xenobiotic metabolism of PAHs may utilize many unknown molecular receptors, we identified GO pathways for heterocyclic binding, transporter activity, ion binding (Figure 9). Genes involved in dopamine receptor function, [328] ion transport, oxygen binding, oxidoreductase activity, and cation binding were affected by fluoranthene.

Benzo(a)pyrene

BAP exposure in adult Daphnia was associated with GO pathways for peroxidase activity (GO:0004601), and antioxidant activity (Figure 10), indications of increased oxidative stress. We estimated 49 significantly upregulated gene transcripts, and 109 downregulated genes. Consistent with effects of neuronal excitability were increased expression of dopamine, oxygen transport, and ligand-gated ion channel genes (Table 8). Benzo(a)pyrene exposure induced increased expression of organic cation transporter, brain specific serine protease, and sulfotransferase (SULT2A), known markers of toxicant stress (Table 8).

Cyfluthrin

Cyfluthrin exposure resulted in 39 significant GO terms representing protein regulation and mitochondrial function. Cyfluthrin exposure reduced expression of calcium/calmodulin genes, essential for nerve transmission and regulation of heart rate.[11] (Table 9). Genes observed with involvement in molecular damage or inflammation were heat shock protein, Eph receptor, and Cathepsin D. Our hypothesis that circadian rhythm genes may coincide with altered behavioral profiles in Daphnia was supported; we observed toxicant-effects on cryptochrome-2 expression and altered regulation of genes in this pathway.

Heart Rate Parameters

A common physiological endpoint for linking neurobehavioral responses to environmental stress is heart rate.[281] All the test agents except for pyrene significantly increased the heart rate at 1-hr of the exposure as compared to the control (Fig.12). Increased heart rate was observed at 24-hr following BAP and PHE exposure. In contrast, at 24-hr after exposure, both the pyrethroid compounds significantly decreased the heart rate. Potential biomarkers of hypoxia (hemoglobin (*Dhb)*) and neuro-toxicity (dopamine receptor 1(*D1R)*) were assessed following 24-hr exposure to the test agents. Phenanthrene, benzo-a-pyrene and pyrene significantly increased the expression levels of *Dhb,* while the other test agents down regulated expression. In contrast, all the test agents, except for pyrene, significantly decreased the expression level of *D1R* (Figure11).

Discussion

We probed a unique ecological phenotype (swimming in dark conditions) that is part of the endogenous regulation of the conserved circadian clock system, and used transcriptional

profiling to estimate molecular responses from two chemical classes. Our video-tracking system subsequently measures baseline regulation of dark-swimming environments, and simultaneous adverse effects of toxicants on natural escape behaviors (upward and downward swimming) that occur in the dark. The escape behaviors of Daphnia induced by predators and exposure to fish kairomones [151] have not yet translated into anxiety-related responses that are key endpoints in fish exposed to neuro-toxicants. We also observed increased heart rates, which are increased under anxiety and altered respiration rates.[329] We support our hypothesis that Daphnia anxiety-related behaviors involve circadian genes and unique swim profiles.

From our study, we show that PAHs alter Daphnia swim behavior. Other work corroborates that neurotoxicants disturb top swimming i.e., "behavioral drift," and can cause adverse effects such as starvation.[297] Increased upward swimming responses from BAP, PYR, and PHE require additional energy and oxygen, which was supported by our findings of increased vitellogenin precursors, oxygen transport and binding genes. Constant swimming is required for essential functions in aquatic species. This behavior may mean higher respiratory rates, and increased need for food and oxygen.[39] When food is scarce, pelagic organisms must expend more energy for obtaining food, and increase swim speed. Daphnia that exhibited an upward swimming toward top of the tank that has been previously described by zebrafish and rats following benzo(a)pyrene exposure.[272,330]

We hypothesized that *Daphnia magna* exposed to PAHs would not be similar to known mechanisms for vertebrates; we do find that Daphnia increase expression of peroxidases and antioxidant enzymes to protect from reactive oxygen species (ROS). Cytochrome P450 dehydrogenase and phase II enzymes such as epoxide hydrolase, and conjugation enzymes were induced.

Our results show that neurobehavioral mechanisms involve obstructed communication from genes at synaptic terminals. Dopamine is critical to neurological function [137,144,160] and motor activity.[331][332] Changes in dopamine neurotransmitter concentrations can produce neurodegeneration.[250] We observed that dopamine receptor 1(*D1R)* was significantly decreased for all the test agents, except for pyrene. (Figure 11). Dopamine is also involved in the functioning of photoreceptors and retinal neurons, [301] however heart rate differences may not be a result of dopamine signaling.[122] There is an absence of studies that test compounds that affect Daphnia nerve synapse machinery or daphnia heart rate; insensitivity to some vertebrate β-adrenergic receptor antagonists has been shown.[333] We conclude that Daphnia heart rate may not be a useful marker for dopamine agonists. The group of genes involved in neurological disorders were induced from PAHs in this study, which are not similar to mechanisms producing PAH-induced behavioral changes in fish.[259]

Daphnia and insects may exhibit unique exposure susceptibility to pyrethoids because of chitin exoskeleton permeability,[251,334] an open circulatory system, and vulnerability of sodium channels to hyper-stimulation through a pyrethroid-binding mechanism[297,334,335]; therefore oxidative stress would not be a primary mechanism for this chemical class. Associated cyfluthrin effects involved in neurological disorders included calcium/calmodulin-dependent kinase II.

Protection of the CNS from cyfluthrin toxicity would requires induction of antioxidants and regulation of cellular damage of proteins.

Like many environmental neurotoxicants, pyrethroids and PAHs may readily permeate the blood-brain barrier, whose permeability is under clock gene control.[247] Circadian genes influence fluctuations in heart rate,[329] macroinvertebrate position in the water column,[146,297] photo-receptor responses.[139,140,154,159,299,336-341] Daphnia utilize light-induced swim behavior to escape predators and feed at the water's surface ,[116,117,297] which may be irreversibly altered by toxicant exposure[113] or adaptations.[149] Our experiment was useful to assess circadian-driven phenotypes identified using transcriptomic profiling. Daphnia homologs for cry-2, scavenger, spin, and mbnl2 genes involved in circadian signaling were observed to be affected by cyfluthrin, phenanthrene, and pyrene exposures. A future investigation may involve further detection of toxicant-modulation of crustacean circadian clock systems to expand on ongoing studies of salt-disrupted circadian gene regulation of the per gene (period).[149]

Our work may provide needed mechanistic endpoints for developing a proposed adverse outcome pathway for circadian-regulated swim behavior in Daphnia. Swim behavior is a survival endpoint required for pelagic aquatic species to escape predators, obtain food, regulate reproductive cycles, and respond to light.[123,342] Our study may be the first to describe Daphnia toxicant-induced behavioral responses that involve cryptochrome signaling. Cryptochrome-2 is a negative regulator of clock-genes, and has a positive role in signaling EcR/E75-dependent genes such as melanopsin[169] which is required for vision.[343] Daphnia exhibit phototactic behaviors,[115,117] which may be used to measure low levels of neuro-active pharmaceuticals and assess water quality.[121] [113,114]   This work represents a proof of principle and additional research is needed to standardize the behavioral profiles for neurotoxicants, as translated for anxiety-related behaviors in zebrafish and mammals.

Conclusions

Our overall objective was to identify toxicant-related behavior endpoints for *Daphnia magna* AOPs that may provide a basis for improved hazard assessments. Our study of swim behavior in a dark environment provided a test of behavior responses which may be a coordinated marker for an evolutionarily conserved pathway involving CLK signaling. Daphnia rely on endogenous CLK signaling to migrate to deeper positions in the water column for predator avoidance; [123,344] but this requires a trade-off of lower reproductive output.[319] Our initial work identified toxicant-induced effects on the circadian gene pathway, however validation of CLK gene effects would require knockdowns in this pathways to confirm toxicant effects on this mechanism.

 In our examination we determined that exposure to cyfluthrin, permethrin, and fluoranthene produce a bottom-position behavioral change that may negatively impact reproduction, and limit feeding and thus increase risk of starvation. Compounds producing the surface swimming behavior also involved circadian rhythm activity associated with Daphnia scavenger genes. [144] Exposures to benzo(a)pyrene, phenanthrene, pyrene were associated with upward surface swimming which would increase susceptibility to predation. Upward surface

swimming is also a anxiety-related and "antioxidant defense" phenotype observed from zebrafish exposed to BAP.[272]   Although characterized as a carcinogen, benzo(a)pyrene is a behavioral neurotoxicant that may result in inhibition of brain antioxidant enzymes.[345], Altered antioxidant defense systems is an identifiable anxiolytic effect.[330,346] Behavioral assays do not often consider endogenous circadian clock regulated oscillations that may influence behavior. Daphnia swim behavior phenotypes that correspond to anxiety-related behaviors in zebrafish, may provide an entry point for understanding the neurotoxicity in crustaceans.

Materials and methods

Daphnia magna culture

Daphnia magna were cultured in an environmental chamber according to standard guidelines.[347] Before the behavioral test, the daphnia are maintained in 16-hours of light and 8-hours of dark (LD conditions), shown by other studies as a normalized baseline of a circadian clock oscillation used in the 24-hr diel vertical migration interval.[137]

The 24-hr acute toxicity assays for 14-day old Daphnids was conducting using protocols in literature. Test compounds were obtained from Sigma Aldrich. Phenanthrene, benzo(a)pyrene, pyrene, fluoranthene, cyfluthrin, and permethrin were solubilized according to recommended solubility in DMSO or acetone or media control. (Figure 1). LC_{50} was calculated according to a PROBIT analysis package (Risk Assessment Tools, SETAC, 2009).

Toxicity testing (PAH and pyrethroid) and Agilent Microarray Hybridization

Adult 14-day old daphnia (N=20) were exposed to 10% LC_{50} for each all PAHs and pyrethroid compounds, sacrificed on ice, and subjected to RNA extraction and microarray hybridization. RNA extractions were performed using Trizol Reagent RNA Isolation protocols. 20 whole Daphnia (pooled) extracted for RNA, and quantified using ND-1000 Nano Drop Spectrophotometer (Thermo Scientific). RNA integrity was checked in Bioanalyzer and samples with a RIN value above 9 were used for amplification.

The microarray hybridization was conducted using the One Color microarray hybridization protocol and Agilent microarray hybridization kit.   The RNA was reverse transcribed into cDNA using oligo-dT-promoter primer, then Cy5 labeled. We conducted hybridizations at 65C overnight on the Agilent slides (AMAID:012719,GPL15139)(Table 1).

Daphnia magna swimming behavior assay

The Daphnia swimming behavior test is performed in a dark chamber. A baseline of the Daphnia circadian rhythm was achieved by maintaining daphnia in 16-hours of light and 8-hours of dark (LD conditions) before testing and chemical exposure.[137] An automated infrared light camera detected Daphnia swimming behaviors.

We quantified a swim speed, distance moved, and 3D video behavioral patterns using the 'Viewpoint Video track for DaphniaLab™' (Viewpoint Life Sciences, Montreal, Quebec, Canada). Recording and setup for Viewpoint Life Science Behavior technology was adapted

from Zebrafish Viewpoint tracking system; specialized algorithms for recording in daphnia are not well- developed to detect patterns of movement in the z-axis; however, users can identify patterns through the recordings. The camera recorded 30 images per second, computed from subtracting the reference image each time. The live-video tracking records (x) active swimmers and their speed in 12 separate aquaria (recorded simultaneously). Twelve individual glass aquaria tanks (5 x 5 x 1cm) were used in the Viewpoint Behavior Video tracking system.

The 'Viewpoint Video track for Daphnia Lab™' (Viewpoint Life Sciences, Montreal, Quebec, Canada) is a high throughput video tracking equipment to measure swimming activity in *Daphnia*. The equipment allows simultaneous quantitative assessment of multiple swimming parameters like individual and group swimming velocity tracking, activity quantization, space use pattern (distribution in within the measurement cell), and participation (average number of individuals participating in swimming activity at a given period of time). The Viewpoint software determined ten daphnia speed classes (C1-C10), which were simultaneously monitored for all daphnia. The speed classes were $C_1 < 0.5 < C_2 \leq 1 < C_3 \leq 2 < C_4 \leq 4 < C_5 \leq 8 < C_6 \leq 16 < C_7 \leq 32 < C_8 \leq 64 < C_9 \leq 100 \leq C_{10}$ in mms.$^{-1}$

Aquaria tanks for controls and treatments were run in the same day, to compare control Daphnids and exposed (1-hr or 24-hr) Daphnids for each chemical. Measurement of speed distribution and average speed was recorded every 5 minutes for 60 minutes. Behavior for 3 biological replicates and 4 technical replicates was assessed for each treatment.

Exposures for Daphnia Swim test

Prior to the behavior assay, < 24-hr old Daphnids were exposed to 1.25, 2.5, 5, 10 and 20% of the respective LC_{50} concentrations of the test agents in a COMBO media at 21 ± 0.5 ºC. After 1 and 24-hr of exposure the Daphnids were transferred to control media for measurement of swimming activity. At each concentration, 24 replicates of 5 Daphnids each were analyzed (total 120 Daphnids per treatment). The total swimming activity was recorded for a period of 1 hr.

Behavioral patterns determined from Video Recording

Daphnia behavior patterns were determined from 10-minute tracking videos from daphnia in dark environment, exposed to 10% of LC_{50} concentration of the toxicant. Video was replayed at slow motion using the Windows media player. Swim pattern in the tank were observed from video tracking, and shown to be either a control (unexposed pattern), 'swimming toward surface' or 'swimming toward bottom.'

Swim statistical analysis

Behavior in control aquaria was monitored at all speed classes, and was critical to determining change in behavior. Control treatments were DMSO, Acetone, and COMBO. Between group comparisons made using ANOVA (SPSS) for within and between groups; for between groups a coefficient of variation was used for <10%, 10- 20% and >20% difference in swimming. A 10% change in swim speed from controls was used a baseline for the concentration for a behavior

effective dose. Average swim speed was recorded for each chemical from the viewpoint tracking algorithm. Dose-response effects from swim speed and average speed per compound was assessed using SPSS.

Identification of differentially expressed genes

Samples were analyzed using a "Treatment vs. Control" design. Foreground intensities in each array were subtracted with local background. All negatives or flagged spots (using GenePix QC flag system) were labeled as "NA", i.e. treated as missing values. All positive values were log (base-2)-transformed. Relative intensity ratios were calculated (ratio= treatment sample /control) for the log-transformed values for each gene (cDNA). Relative intensity values (log2 ratios known as "M-values") were corrected for non-linear trends (if any) with loess global normalization.[348] A local variance estimator based on loess is used to take heteroscedasticity (if any) into consideration. [349]

As a result, each gene in a given "Treatment vs. Control" pair was characterized by two values: the normalized log-transformed ratio (fold change value) and the corresponding q-value (derived from p-values, which were adjusted for multiplicity of comparisons). [350] The Fisher's method of meta-analysis was applied to combine p-values (this approach is scale-free and, as a result, it does not require the use of between-array normalization). The multiple slide procedure method was used to detect candidate genes (this means that the same techniques was applied to all microarrays in the study based on all possible combinations of a given treatment and the corresponding control biological replicates). This technique was based on the number of biological replicates and treated the gene expression outcomes as Bernoulli trials (independent binary outcomes), and the Fisher's method-based p-values were adjusted with Bonferroni correction. Using these procedures, we created a list of candidate genes per treatment, which was used to compose a summary for all treatments (a gene was placed in the summary if it was detected as differentially expressed in at least one treatment). Candidate genes from the table were annotated using Blast2Go service. [351] Blast2GO used *Daphnia pulex* alignments to identify hits from the *D. magna* transcriptome, create directed acylic graphs (DAG) to represent a network of relationships between GO terms. Significant GO terms identified using annotation tools in Blast2GO. Lists of upregulated and downregulated genes were run through Gene set enrichment (GSEA) using the online Gene Ontology Consortium (Panther web engine).

Quantitative PCR (qRT-PCR) at toxicant gene markers

We analyzed the expression level of two genes, *Daphnia* hemoglobin (*Dhb)* and dopamine receptor 1(*D1R)* as potential biomarkers of hypoxia and neuroendocrine processes involved in locomotion respectively (Table 3). Total RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The protocol followed was 95 $^\circ$C for 30 s followed by 40 cycles of 95 $^\circ$C for 5s and 60 $^\circ$C for 5 s. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that specific product was amplified and detected. Three technical replicates

from three biological replicate were performed at each treatment level to amplify target genes normalized with actin as endogenous control.

Chapter 4

Behavioral responses as key endpoints in exposure to greener biofuel candidates: Effects of AhR-independent compounds in zebrafish

Abstract

To assess the toxic effects of candidate biofuels, 2,5-dimethylfuran (2,5-DMF) and furan derivatives, we screened these compounds for activation at the aryl hydrocarbon receptor (AhR), embryonic development, and altered neurobehavioral endpoints in zebrafish. Cell-based assays for AhR activation were negative for the biofuel derivatives; combustion products furfural and indene weakly elevated AhR activation. We conclude there is low risk of zfAhR2-dependent developmental defects from biofuels. We also utilized an embryo and larval photo-motor response (PMR) assay to profile any neurobehavioral effects. 2,5-dimethylfuran (DMF), 2-methylfuran (2-MF), and 2-pentylfuran altered photo-motor responses significantly while 2,3-dihydrofuran, and 2,3-dimethylfuran did not produce embryonic neurobehavioral deficits. Furan and DMF produced similar excitatory responses which are representative of neuroactive compounds. Increased larval movement in the PMR assay dark interval was observed in 2-methyl furan and DMF; furan produced hypoactivity. Benzo(a)pyrene, 2,3-dimethylfuran, indene, 2-ethylfuran, and 2-methylfuran produced hypoactivity in the PMR light responsive interval. Our combined analysis of AhR mediated and non-AhR mediated behavioral responses suggests that 2,3-dihydrofuran and 2,3-dimethylfuran are less likely to result in ecological harm in early-life stage aquatic species.

Introduction

In this chapter, I utilize the well-established zebrafish model to rank the hazards of candidate greener biofuels at cellular, developmental, and behavioral endpoints. In this study, I determined AHR activation using multiple approaches: 1) exposure of zebrafish at early life stages with candidate biofuels and AHR agonists, 2) screening exposed zebrafish embryos at 21 developmental endpoints, 3) a cell-based reporter assay with humAHR, and subsequent validation of ligand-binding using a computational homology model for zfAHR1,1A and zfAHR2. To account for effects of non-AHR toxicity, I conducted behavioral assays used in identification of neurotoxic exposures. Collectively, these different endpoints may show a causal, mechanistic response that can be integrated into an adverse outcome pathway framework.

The zebrafish multi-endpoint screening model has been extensively used to validate EPA's ToxCast/Tox21 bioactivity endpoints,[133] evaluate concordance between mammalian and fish behavior[127,263,277,352-354] and the basis for comparative models of AHR ligand homology.[36,37] The zebrafish developmental assay for aryl hydrocarbon receptor agonists (AHR) has been

utilized to differentiation between AHR and AHR-independent toxicity.[30,32,355] The appearance of false negatives in high-throughput screening at AhR ligands has been reported,[45] a negative interaction with AhR in a single assay should not lead to a conclusion that a compound does not interact directly or indirectly with AhR.[30]

Potential replacements for PAHs are Monoaromatic hydrocarbons

There is a push to replace petroleum product with renewable resources that contain safer materials, have a short environmental life cycle, and produce no toxicity. Greener biofuels are one alternative to petroleum fuels. Compared to ethanol and gasoline, the biofuel 2,5-dimethyl furan (DMF) has many advantages: improved environmental fate, [356] superior engine performance (octane number), and efficient production. [357,358] Green chemistry principles aim to reduce human and environmental toxicity in production, use, and product life cycle. [63] Greener chemical design may reduce some forms of toxicity; however, safety of chemical alternatives should not be assumed. Products that biodegrade and have lower environmental risk, may produce more toxicity than the parent compound. [359-361] Therefore, greener chemical design should assess both short-term exposure and long-term effects.

Gasoline, diesel fuel, and petroleum products degrade into poly-aromatic hydrocarbons (PAHs) - a class of aromatic ring compounds that have a long environmental life cycle, [362] correlate with higher risks of cancer [363] and reproductive toxicity. PAHs are considered a priority pollutant by the U.S. EPA and European Environment Agency (EEA). Biodegradation of petroleum polymers in the aquatic environment has not lessened their hazardous properties,[364] but instead result in long-term hazards for ecosystems. [365-367] Degradation products are more toxic than parent compounds .[360,366,367] PAHs contaminate aquatic food webs, have low-mobility in sediments, and exacerbate the emerging global issue of microplastics. [368] PAHs are hydrophobic chemicals that sorb to plastics, then re-enter the food chain for another life cycle-[368-370] a detrimental problem that has been recently reported.

2,5-dimethylfuran (DMF) is a member of the monocyclic aromatic hydrocarbon (MAH) family that includes benzene and furan; furan derivatives were identified in foods [371] and burning of hydrocarbons. Furan and benzene are associated with development of diseases in humans [372] and liver cancer. [361] Computational models suggest a low-hazard profile for 2,5-DMF, but genotoxicity was observed in the Chinese hamster ovary micronucleus assay, [373] as well as DNA damage. [374] Furan and furfural elicited significant organ toxicity. [361] Biofuels with similar structure to 2,5-DMF (2-methylfuran, 2-methyltetrahydrofuran, and ethyl levulinate) show low cytotoxicity in fish cell lines. [375] Despite the multiple reports on biofuels, few have linked single-endpoints with a phenotypic response, which is a common criticism of targeted-based studies .[376]

AHR ligands promote toxicity in zebrafish: AHR-dependent and independent effects

Zebrafish possess two forms of AhR, zfAHR1 and zfAHR2. zfAHR1 is inactive, and zfAHR2 is strongly activated by 2,3,7,8-Tetrachlorodibenzodioxin (TCDD). Toxicity from activation of zfAhR2- dependent PAHs are hypothesized to produce cardiac abnormalities, pericardial edema, and developmental deformities.[377] AhR ligands are potent toxicants and may

be resistant to metabolic detoxification, and transcription of AhR-dependent genes can lead to developmental toxicity in fish. [260,355,378,379] There is potential for AhR-ligands to induce genes from both xenobiotic binding element (XRE) and antioxidant response elements (ARE),[380-384] however the precise mechanisms producing cellular stress from 2,5-dimethylfuran is unclear. [385,386] AhR-dependent toxicity has been confirmed through using AhR-null organisms. [384,387]

AhR-independence may occur from weaker agonization of some AhR isoforms.[33] A number of PAHs can exhibit AhR-independent toxic mechanisms [32][31,33] affecting behavior,[355] learning ,[27] response to hypoxia. [388] However, phenanthrene and anthracene produce molecular responses (at zfAHR2),while pyrene does not;[32] chemicals expected to act through AhR may exhibit AhR-independent mechanisms. [31-33] Increased risk for oxidative damage and injury to neuronal tissue may result from differences in distribution and disposition of lipophilic compounds. [389]

The current hazard profile for 2,5-DMF and other candidate biofuels omits cellular and behavioral responses that are linked to key events in zebrafish development. New key endpoints for hazard assessments in aquatic contaminants[135,136] may include spontaneous movement, and links to excitation of photoreceptor neurons in the developing hindbrain- [132,154,271,390] have been proposed as a suitable for detecting neuro-active chemicals.[271,274]

As a first step to understanding effects of greener biofuel candidates, we conducted a multiple –endpoint zebrafish screening assay. We assessed zebrafish AhR-dependent developmental phenotypes using a 21- endpoint developmental assay. To account for non-AhR mechanisms, we measured behavioral embryonic PMR and larval swim assays. We hypothesized that greener biofuel compounds may produce structure-based toxicity at key behavioral endpoints. AhR independence is a mechanism for some aromatic chemicals in the PAH class which is not well understood. [30-33,391] This present study may provide new information to select increasingly safer candidate furans for the production of biofuels.

Results

Biofuel Candidates exhibit impaired binding of AhR in reporter assays or virtual ligand screening

We tested biofuels, AhR agonists, and combustion products for AhR dependence using in vitro XRE- luciferase reporter assays. While all AhR agonists significantly activated the human AhRE, biofuel candidates were negative in this assay. We observed mild induction of combustion products (furfural and indene) at the 100uM. (Figure 2).

We utilized bound-docking protocols, optimized for LBD-ligand configuration, to assess binding of each of the candidate biofuels to the three zebrafish AhR isoforms,. We found no clear pattern of zfAhR2 interactions with furan compounds; and conclude that these compounds are unlikely to mediate zfAhR2 dependent response in fish. We observed differential activation between control AHR agonists (BNF, BAP) at the zfAHR2, where BAP was not as potent as

BNF. The lower activation of BAP with zfAHR2 may explain the differences between BAP and BNF developmental toxicity in our study. 2,5-dimethylfuran, 2-pentyl furan and 2-methylfuran weakly activated zfAHR1A (Table 1).

AhR-dependent developmental toxicity defects not induced by furan analogs

We collected responses from toxicant exposures at 16 morphological endpoints. [133,390] Evidence of pericardial edema and mortality from AhR2-induced toxicity was significant for **β**-napthoflavone and furfural (Figure 3), but not benzo(a)pyrene (Figure 4). Toxicity from the combustion product (furfural) was higher than furan, the parent compound (Figure 3). Other furan biofuels produced no significant developmental toxicity (Figure 4)**.** Increased mortality was present from furan but not candidate biofuels (Figure 5). Our results suggest that developmental toxicity through conserved AhR toxicity mechanisms was not present in candidate biofuels. Furan, furfural, and BNF produced effects for CFIN (caudal fin deformity) and TRUN (trunk deformity). There is high association of these endpoints with xenobiotic metabolism from CYP2B6 .[133]

Embryonic fish exhibit neuro-behavioral endpoints exposed to greener biofuels

We measured responses in embryonic fish of activation of hindbrain neurons in the embryonic photomotor assay (EPR). Non-AhR activated furans produced both hypoactive and hyperactive neurobehavioral effects (Figure 6) in the excitation interval. We report hypoactive responses from 2-ethylfuran, 2-methylfuran, and 2-pentylfuran (Figure 6). Biofuels and AHR agonists producing hyperactive effects were DMF, benzo(a)pyrene, furfural, **β**-napthoflavone. (Figure 6).

Larval zebrafish exhibit hypoactivity and hyperactivity exposed to greener biofuels

We tested photo-locomotor behavior in 5-day old zebrafish exposed dark and light intervals and biofuel candidates at six doses (0-50uM). Fish exposed to 2,3-dimethylfuran, 2,3-dihydrofuran, furan, benzo(a)pyrene, and 2-methylfuran exhibited hyperactive responses in dark test intervals; while controls were less active (Figure 7) . Total movement calculated was significantly higher than controls ($p<.05$) for 2,3-dimethyl furan, 2,3-dihydrofuran, 2-methylfuran, and 2,5-dimethyl furan (Figure 7, 8). Significant hypoactivity was measured from 2-ethylfuran, 2-methylfuran, and indene exposure during light-testing intervals.

Ranking hazards to developing zebrafish exposed to greener biofuels

Using the criteria for developmental toxicity, zfAhR2 activation, and neurobehavioral responses used in this study, we conclude that the minimal hazard from these candidate biofuels was presented by 2 ethyl furan and 2,3-dihydrofuran. The highest aquatic risk to developing fish was from 2,5-dimethyl furan, and 2-methyl furan.

Discussion

Despite greener chemical design, toxicant effects from PAHs (from petroleum fuels) that cause hyperactivity and neurotoxicity in embryos may be similar to that of many greener biofuels. High-throughput screening programs must utilize more complex endpoints for aquatic species. We recommend the use of sensitive, early life stage screening in zebrafish as a regulatory endpoint for detection of ecological hazards at sublethal concentrations.

In our report, neurotoxic behavioral changes in embryonic and larval fish exposed to 2-methyl furan, DMF, and 2-pentyl furan produced an AhR-independent effect. We did not assess if hypo- or hyperactivity in embryonic fish produced differential survival; however early exposure to neurotoxic chemicals may produce long-term effects.[136,264,265] Compounds that cross the brain-barrier can induce neurological damage.[392] Cytotoxicity of 2-methylfuran [375] is thought to be caused by a narcotic effect. The 'narcotic' effect describes over stimulation followed by hypoactivity- a profile for neuroactive compounds such as nicotine.[393] Increased neurotoxicity[30] or cellular damage in mammalian cells[394] has been reported from metabolism of aromatic hydrocarbons. Although not tested in this study, an inducible CYP2E1 has been reported for aromatic exposures,[395] and CYP2E1 metabolism produces stress and behavioral changes;[392] knockdown of this gene reduces reactive oxidative stress.[352]

Metabolites of aromatic hydrocarbons produce a number of cellular effects, including oxidative stress. The metabolite produced from 2,5-dimethylfuran is 3-hexene-2,5-dione (3-HD), a compound without biological data. Oxidative metabolites of other biofuel candidates (2-methyl furan, 2-ethyl furan) suggest activation of the antioxidant response element (ARE). The compounds most hazardous in our behavioral assays were 2-methyl furan, 2-ethyl furan, and 2,5-dimethyl furan. The modification of 2,4 DMF to a 2,5 DMF results in toxic metabolic products.[373,396] Oxidative stress can sequester levels of ARNT from activated AhR.[381,382,391,397]

Our furan docking results support that of other databases that furan is a non-AHR activating compound. We also report that the AHR agonist BAP showed a lower affinity in zebrafish ligand models, which may explain how BAP produces atypical responses compared to other PAHs in zebrafish.[391] Rather than a control for a potent AHR agonist such as TCDD, BAP may provide more information for assessing downstream AHR-independent responses.

As we seek to end reliance on petroleum-based fuels, the emergence of greener biofuels is a seemingly ideal alternative; however beneficial properties of biodegradation and greener chemical design does not ensure green chemicals are safe. We determined that biofuels and similar furan structural analogs exerted toxicity through AHR-independent pathways. The zebrafish embryonic model has previously identified metabolism-based structural toxicity within the PAH chemical class.[30,156] Due to similar structural relationships with monoaromatic compounds and PAHs, we first measured AHR-dependent ligand activation in a cell-based reporter assay; weak activation of AhR and induction of Cyp1a has been reported for 2-and-3-ring PAHs.[398] Biofuels tested using the AHR-luciferase reporter assay showed minimal induction of human AHR, which is more similar to the zfAHR1 isoform. Due to the possibility

of false negatives in AhR testing, we surveyed a AhR docking interactions and AhR-independent endpoints. Our observed interactions at AhR ligands appear similar to that of some 2-3-ring PAHs[398] where molecular size governs the binding affinities.[34] Additional research may be needed to compare effects of greener candidate fuels with activation of the constitutive androstane receptor (CAR),[399] involved in behavior, oxidative stress, and adverse neurological responses in larval fish. zebrafish.[354]

Materials and Methods

Chemical compounds used in this study were: furan (CAS 110-00-9, Sigma-Aldrich #185922), 2,3-dimethylfuran(CAS 14920-89-9,Sigma #428469) 2,-5 dimethylfuran (CAS 625-86-5, Sigma-Aldrich 177717), 2-methylfuran(CAS 534-22-5, Sigma #M46845), 2-pentylfuran (CAS 3777-69-3,Sigma-Aldrich #W331708), 2,3-dihydrofuran(CAS 1191-997-7,Sigma #200018). We tested two combustion products: indene (CAS 95-13-6, Sigma #193828), furfural (CAS 98-01-1,Sigma W248924). β-napthoflavone (CAS#6051-87-2;Sigma-Aldrich #N3633), and benzo(a)pyrene (CAS 50-32-8,Sigma-Aldrich B1760) Because of volatile properties, biofuel candidates and furan- analogs (≥97%) were stored in paraffin sealed containers at - 20C in dark bottles.

Low dose Toxicant exposures

We followed existing protocols for high-throughput multiple endpoint zebrafish developmental toxicity [133,400]. Briefly, 5D wild type zebrafish were obtained from Sinnhuber Aquatic Research Laboratory, Oregon State University. Tanks were housed at 28˚C with a 14 hour light/10 hour dark cycle. At 2hpf, embryos were examined for quality and dechorionated at 4hpf using pronase enzyme and a series of washes.[133] At 6hpf, 8 embryos per treatment were examined and plated in embryo medium, on replicate plates for a total of 32 embryos per concentration. The exposure concentrations (50uM, 13.9uM, 3.87uM, 1.08uM, 0.3uM) were in a half log-dilution range which was twenty-fold lower than published 48hr LC_{50} for furan congeners. A final DMSO concentration was maintained throughout the experiment of 0.64 % (v/v).

To prevent evaporation and transfer of chemicals, all exposure plates were sealed using parafilm and wrapped in aluminum foil to prevent light exposure. During this development period, control zebrafish embryos adapt to the dark and develop normally, and treatment samples develop in the presence of toxicant. Both control and treatment samples plates were stored in 28 °C incubator until 24hpf, and then replaced into the incubator for the LPR readings at 120hpf (5dpf).

Developmental Toxicity Screening

The zebrafish developmental toxicity screening was performed using established protocols to detect key morphological endpoints following exposures [133,400]. Developmental

endpoints were assessed by absence/presence of deformities in the following morphological forms: yolk sac edema, bent body axis, eye deformity, snout, jaw, otic, pericardial edema, somite, pectoral fin, caudal fin, pigmentation, circulation, trunk length, swim bladder, and notochord); data was collected and entered into Zebrafish Acquisition and Analysis Program (ZAAP) (Truong et al. 2014).

AhR reporter assay and virtual docking zfAhR-LBD homology models

We verified AhR-dependent regulation of XRE-driven luciferase reporters using established protocols [38] in human HepG2 cells. Cells were cultivated in DMEM and 10%fetal bovine serum prior transfection of the XRE element. XRE-MMTV-Luc contained a synthetic XRE oligonucleotide upstream of the MMTV viral promoter. Chemical concentrations of 1uM, 10uM, and 100uM of each compound were added to cells plated from the density of 0.75 x 105 cells/well grown overnight. Cells were exposed to chemical, DMSO, or TCDD control for 24h, followed by determining absorbance of $\boldsymbol{\beta}$-galactosidase activity at A_{405}.

Virtual ligand screening models previously published for zebrafish AhR-LBD isoforms were used. [401] To assess the most stable conformations of ligand binding to the three zebrafish AhR isoforms (zfAhR1, zfAhR1A, zfAhR2)- TCDD and Leflunomide were used as the most potent agonist to compare stabilization of each compound in the binding pocket. Binding energies were calculated using previously published virtual docking parameters.

Embryonic PMR Analysis

We followed previously established protocols for detection of photomotor response (PMR), which is a nonvisual motor behavior that can be blocked by chemicals that disrupt excitation of hindbrain neurons.[154] Photomotor response (PMR) is defined by spontaneous behavior (before eye formation, and swimming phenotypes) following delivery of pulses of light in 24hpf fish. There are three recorded intervals: "excitatory" (E), "background" (B), and refractory patterns (R) obtained following delivery of a pulse of light. [124,154,274] A hypoactive response is measured by reduced movement in response to a first pulse of light that is received in the brain at optic neurons. The pattern of activity within each B, E. or R interval is compared to the negative control activity using a percent change (-50% change from control for hypoactivity, 75 % change from control for hyperactivity) and a Kolmogorov–Smirnov test (Bonferroni-corrected p value threshold = 0.05/5 concentrations= 0.01). The percent change thresholds for hypo- and hyperactivity were parameterized so that the distributions of negative control responses were equivalent across activity-associated chemicals (i.e., "hits").

Secondary Behavioral test (Larval photomotor Response)

The larval phase of development produces another locomotor phenotype that includes both spontaneous swimming and phototaxis. [275] The movement in the dark is higher for zebrafish than in the light,[126,402] a characteristic of conserved anxiety and avoidance responses that are observed in mammals.[262] A Viewpoint Zebrabox monitoring system (Viewpoint Life

Sciences) was used to detect swim behavior in light and dark intervals, as used previous protocols on exposed zebrafish as described above.[133] Automated motion tracking software recorded distance moved in each well of a 96 well plate during a 25 minute assay. The average movement per minute for each fish was recorded for over 25 minutes. Increased motor activity in light would be significant for neuro-active compounds and may indicate neurotoxicity.[132] In the analysis of each behavior chamber, at each concentration, customized R scripts were used to determine area under the curve (AUC), and a multiple comparison test to compare median locomotor activity per minute (distance moved, in mm) in treatment versus control, followed by a Dunn's multiple comparison post test. Significance was determined as $p < 0.05$ and change in the AUC of $> 40\%$.

Potency ranking

We ranked the relative potency by comparing responses of furan candidates to the most toxic congener within the class (i.e., dioxin, furan). [403,404] Because of the potential for false negatives in AhR screening, we utilized multiple endpoints (*in vitro, in silico*, developmental and behavioral) to validate these rankings.

CHAPTER TWO
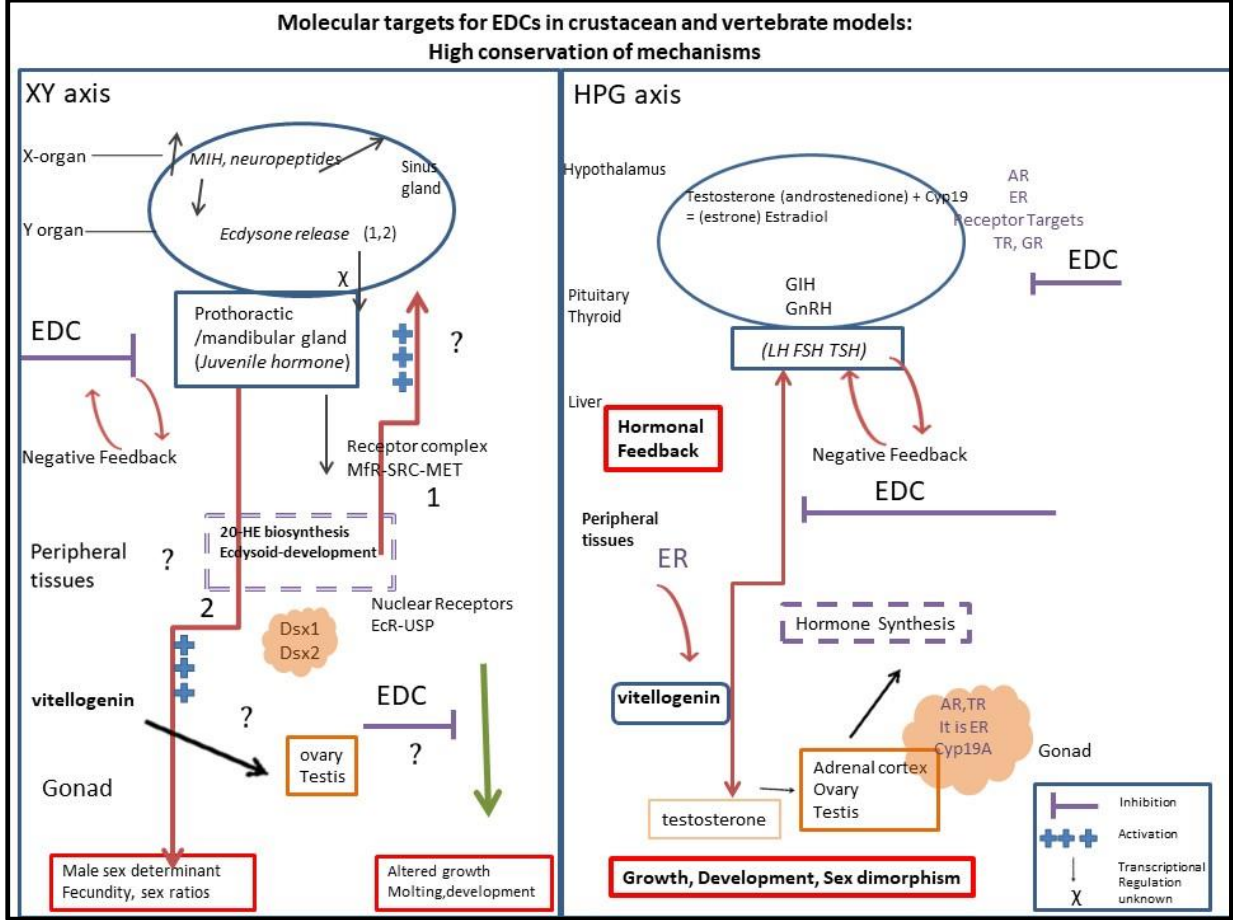
Figure 1.1 Crustacean X-Y and Vertebrate HPG Reproductive Axis Sketch
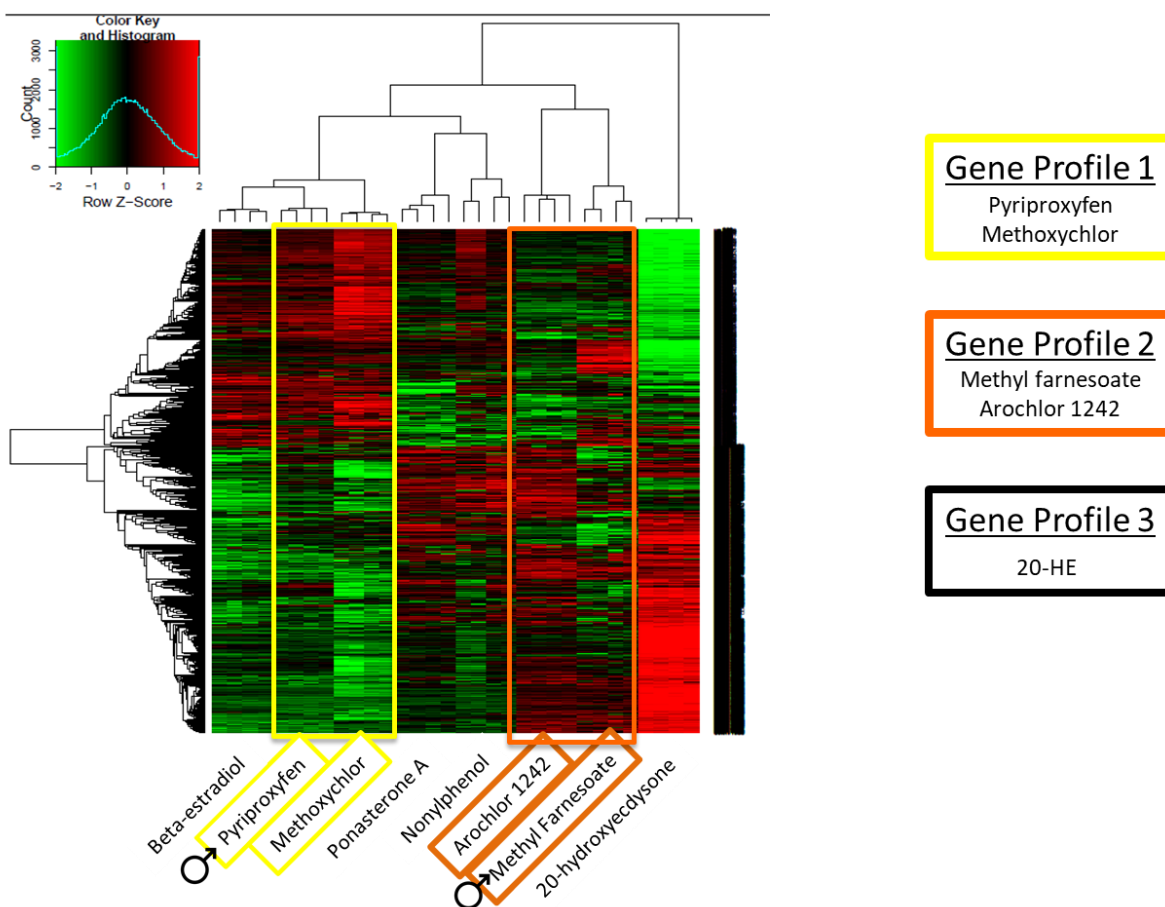
Figure 2. Inverse patterns among male-producing compounds in hierarchical clustering
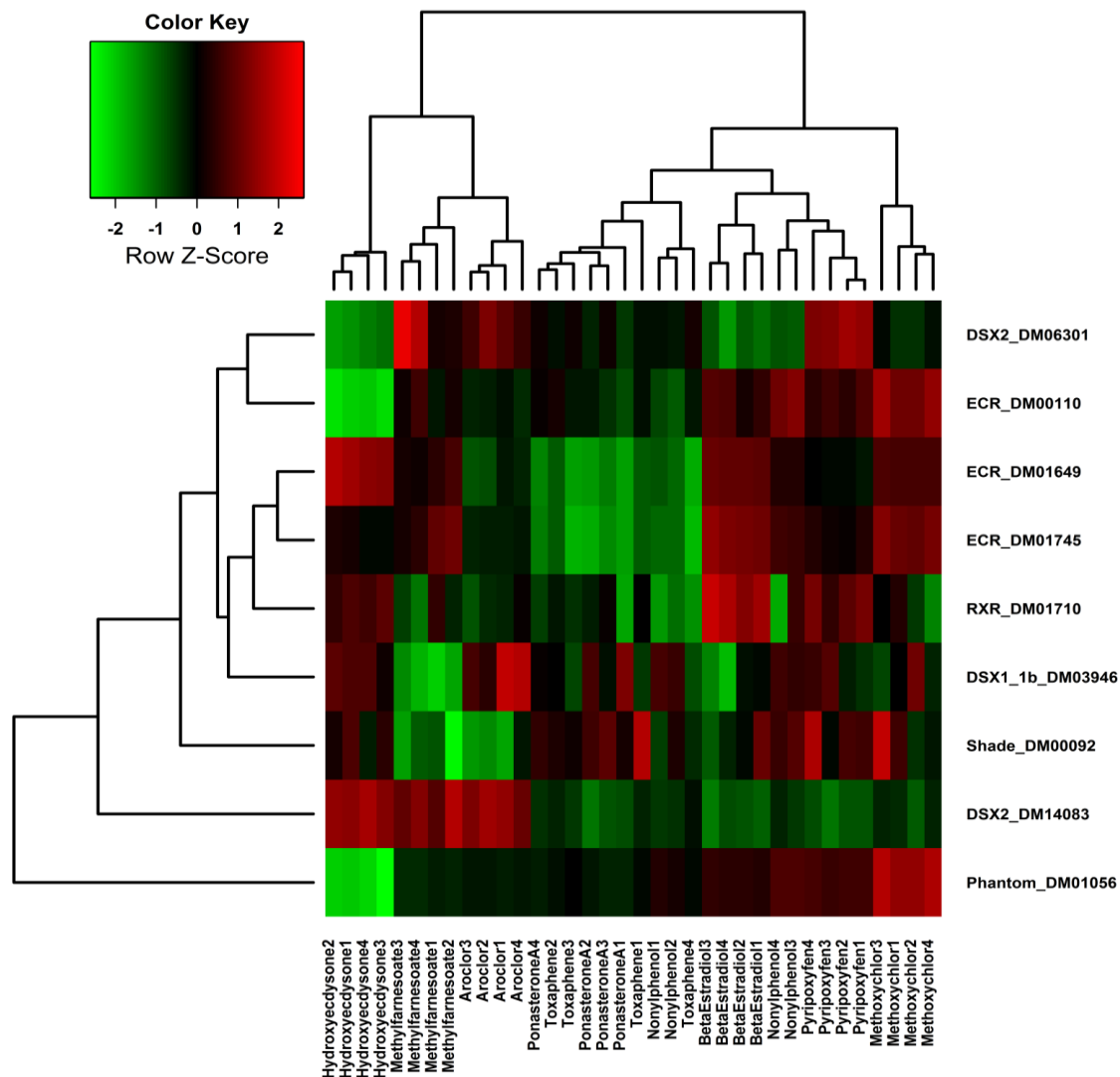
Figure 3. Z-score transformation of gene expression for Daphnia endocrine target genes.
Daphnia ecdysteroid receptor isoforms (EcR_DM00110(EcRA1), EcR_DM01649(EcR-B),
EcR_DM01745(EcRA1), Daphnia RXR orthologs, doublesex gene (dsx1, dsx2), ecdysteroid
biosynthesis genes (shade, phantom). Two-color heat map key: green=reduced gene expression,
red=increased expression, black = no change)
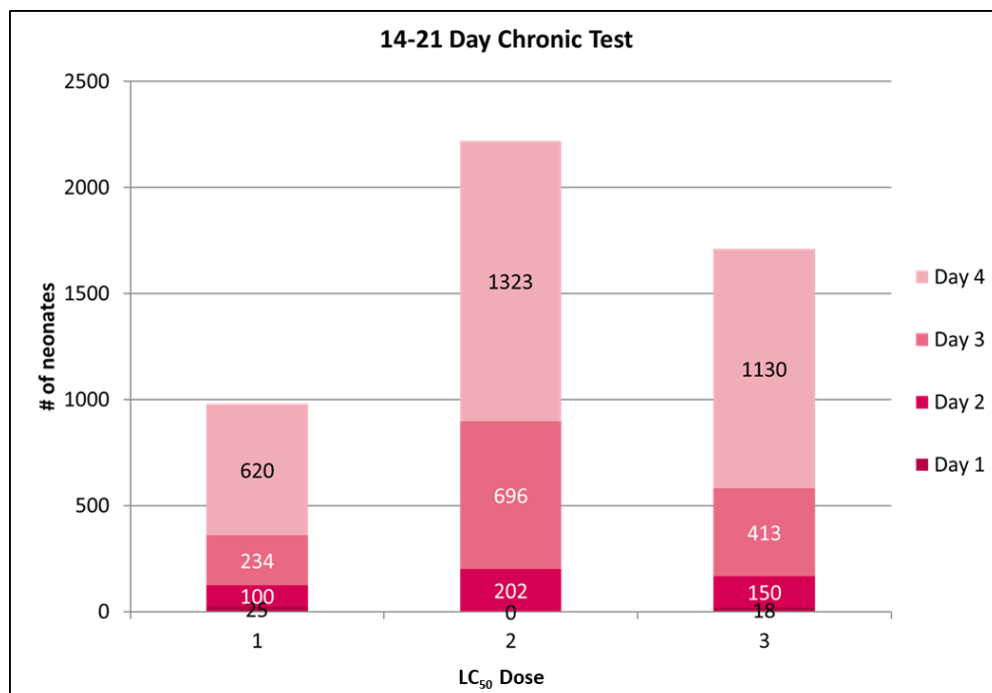
Figure 4. Daphnia Chronic Reproductive Test. Number of neonates counted after exposure to acetone (control) and Benzo(a)pyrene. Chemical Dose: 1) Control 2) 2.5% LC_{50} and 3) 5% of the LC_{50}
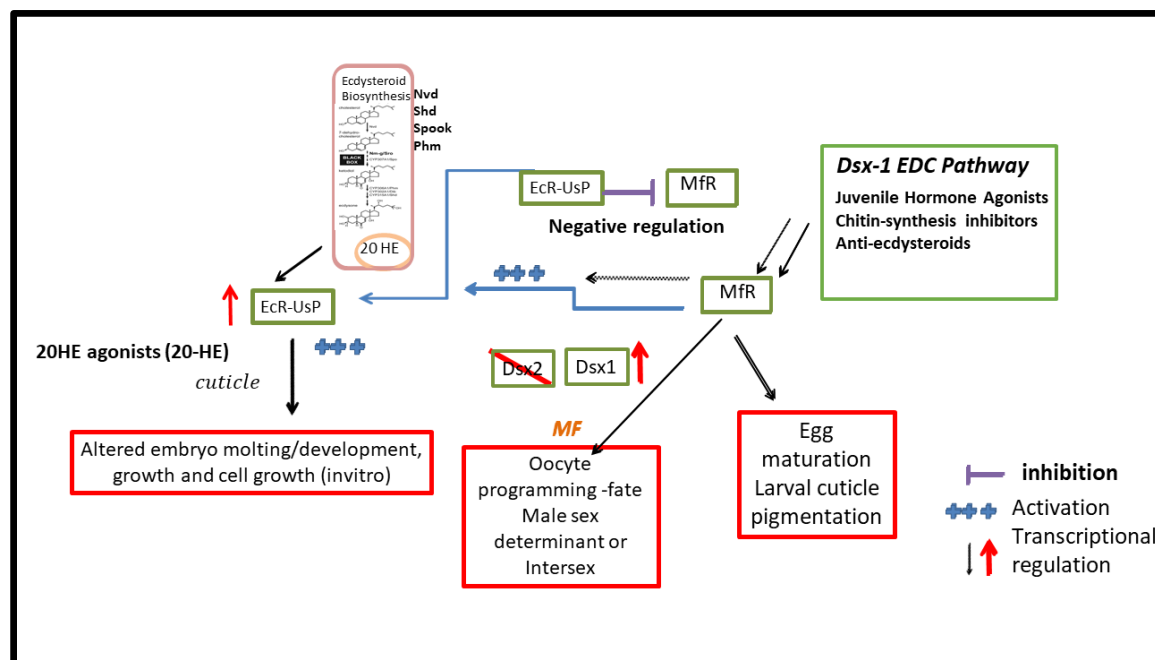
Figure 5. Current scientific consensus for Daphnia EDC Mechanisms
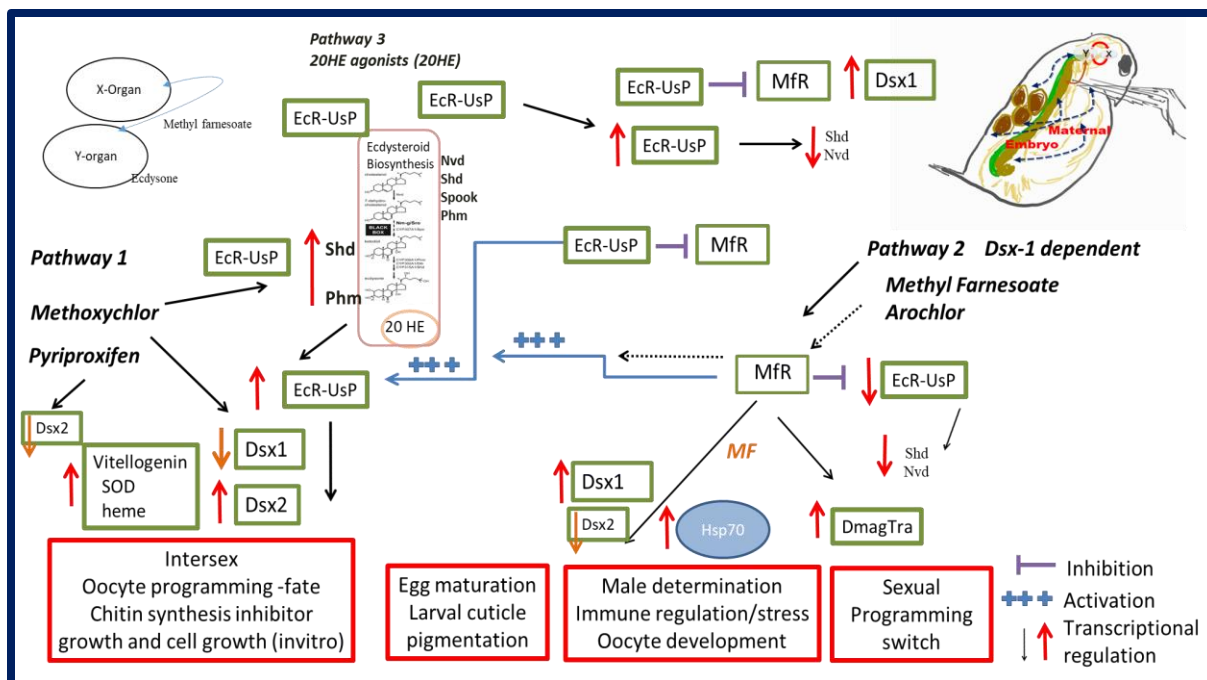
Figure 6. Alternate Daphnia EDC model incorporating transcriptomic profiling of JHAs
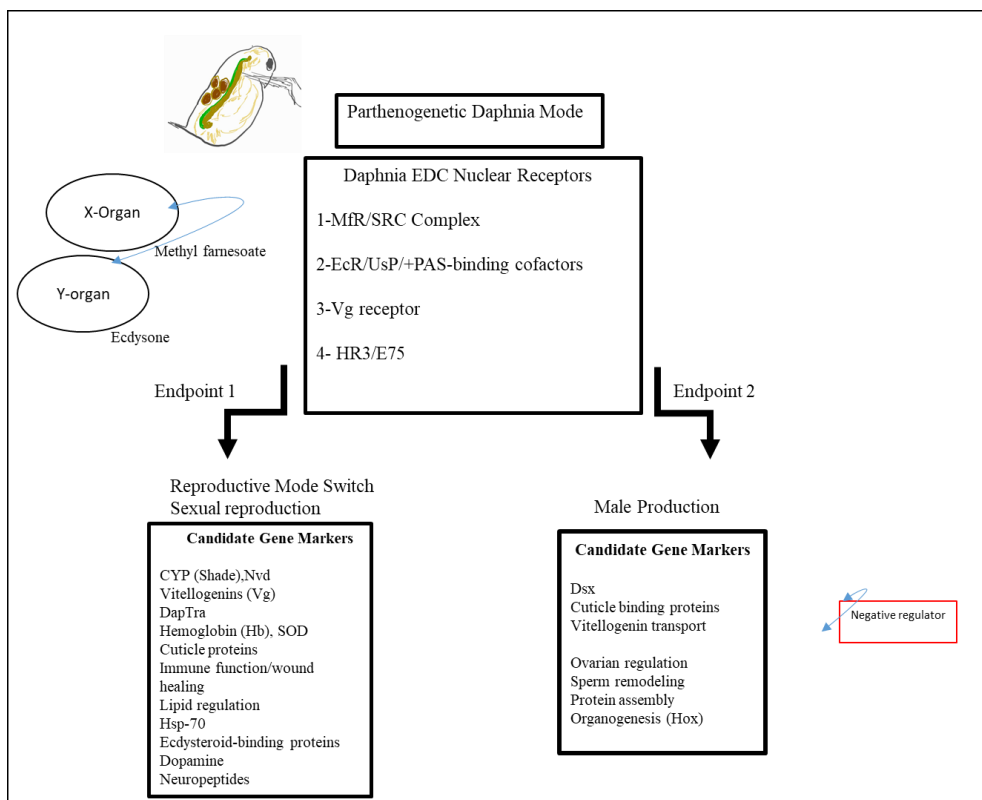
Figure 7. Reproductive Mode Switch and Male production: Placement of Key EDC gene regulators in Daphnia X-Y Reproductive Axis

Table 1. Modes of action for Endocrine Disrupting Chemicals (EDCs)

Insecticides	Mode of Action	Citation
Pyriproxyfen	Juvenile hormone growth regulator	76
	Sex determination/ male production	99
Methyl Farnesoate	Anti-Ecdysteroid	98,405
Methoxychlor	Vitellogenin repression	237
	ovary development	72
20-hydroxyecdysone	Molting, development, ecdysteroid	86
	Antennae development	99
Ponasterone A		
4-Nonylphenol		

Table 2. Representative up-regulated genes in response to methoxychlor/pyriproxyfen (Cluster 1)

Probe name	EDC Endpoint	Gene Function	Fold change
DM13610	Development/morphogenesis	Cuticle development	28.26
DM08004	Precursor of yolk	Vitellogenin, lipid transport	13.25
DM02255	Lipid transport yolk proteins	Lipid transport, yolk proteins	10.85
DM11670	Vitellogenin, lipid transport		7.96
DM11390		SOD domain	6.51
DM10524	Behavior	Neurotransmitter: dopamine	26.31
DM01546	Chitin metabolism and vitellogenesis synthesis antioxidant	SOD domain Vitellogenin Hemolectin Coagulation	5.94

DM05269	Heme binding	Hemoglobin subunit	22.31
DM06388	Protein metabolism	Protein metabolism	22.01
DM03029	Energy metabolism	Acyl CoA metabolism	15.31
DM03167	Respiration	Mitochondrial Respiration NADH:ubiquinone oxidoreductase	4.91
DM05876	Testis specific regulation	Sperm chromosome remodeling	10.46
DM11085	Would healing, Hemolymph coagulation	Hemolectin vitellogenin precursors	9.30
DM07136	Lipid transport	Hemolymph coagulation/wound healing	7.51
DM00608	Pathogen recognition sequence/Immunity	Pathogen recognition sequence/Immunity	8.83

Table 2A. Representative down-regulated genes in response to methoxychlor and pyriproxyfen (Cluster 1).

Probe name	EDC Endpoint	Gene Function/ InterproScan	Gene expression
DM00092 DM01056	Ecdysone Production	Shd /Cyp314A1 Phm/Cyp306a	upregulated
DM13610 DM08004	Egg development Vitellogenin	Dyl IPR001507 SOD domain	downregulated
DM01839	Secretory proteins	IPR001283	downregulated
DM03946	Sex determination	Dsx1	downregulated
DM11495	Hemoglobin	Hb2	downregulated

Table 3. Representative downregulated genes in response to similar juvenile hormone-agonists (Cluster 1)

Gene Target	EDC Endpoint	Gene function	Fold change
DM13610	Metamorphosis/Development/ cuticle regulation Growth, chitin binding Embryonic trachea formation	cuticle protein	-6.21
DM12038		ribosomal protein	-2.97
DM02979		cuticle protein	-4.36
DM11934		transcription repressor	-4.05
DM02749		cuticle protein	-5.61
DM06347		collagen	-4.96
DM06747	Development, tissue formation	collagen helix	-4.78
DM11892	Movement	troponin	-4.49
DM00445		myosin complex,Mhc1	-3.39
DM04779	Nerve growth	I Q motif, glycoprotein binding	-4.39
DM05102	Stress, Apoptosis, cell fate	IRE-1 protein sensor	-3.86
DM03051	Metabolism Mitochondrial respiration	NAD(P)binding superfamily oxidoreductase	-3.10
DM12265		NADH:ubiquinone oxidoreductase,subunit 1	-6.53

Table 4. Commonly upregulated genes between juvenile agonists (Methyl farnesoate and Pyriproxifen)

Gene Target	EDC Endpoint	Gene function	Fold change
DM01839	Immune	Insect allergen ortholog	20.23
DM14169	Cell regulation	Nucleotide binding	10.92
DM12954	Metabolism	Mitochondrial origin	
DM12099	Protein co-factor	PWWP domain	2.76
DM02992	Development	Heme-dependent peroxidase, also DmeI (Distalless minimal element), homeobox	18.69
DM08009	Chitin development	Chitinase chitin binding	12.74

Table 5. Representative upregulated genes in response to methyl farnesoate and arochlor (Cluster 2)

Gene Target	EDC Endpoint	Gene function	Fold change
	Cellular Metabolism		
DM02485	Heme binding protein	DOMON domain	149.76
DM07499	Fatty acid binding	conserved FABP	111.07
DM01033	sulfate transporter	Pendrin ortholog	18.66
DM02721	Fatty acid binding	Calycin superfamily	37.42
	Development		
DM10932	Development	Collagen alpha	51.97
DM01911	Organogenesis	Ppn ortholog	6.62
DM06652	embryonic development	cuticle development	33.30
DM01995	embryonic development	bagpipe ortholog	5.61
	Ovarian-embryonic regulation		
DM06992	Vitellogenin	vitellogenin precursor,von willebrand factor	13.56
DM11938	lysosomal transport	DmeI ortholog	13.10
DM00998	Female-altered reproductive mode	Daphnia Transformer gene RNA recognition	7.97
DM05030	oocyte regulation	ovarian	5.37
	Immune-Wound response		
DM00419	Immune, stress response	autophagy Atg9 ortholog	7.87
DM11143	oxidative stress	steroid hydroxylase	6.11
DM01960	Stress	heat shock protein-70	6.39
DM00631	stress response	DmeI	5.52

CHAPTER 3

Figure 1. Chemical structures of test compounds. 24-hr acute toxicity tests were conducted with 14-day old daphnia to obtain LC_{50} value for each compound.
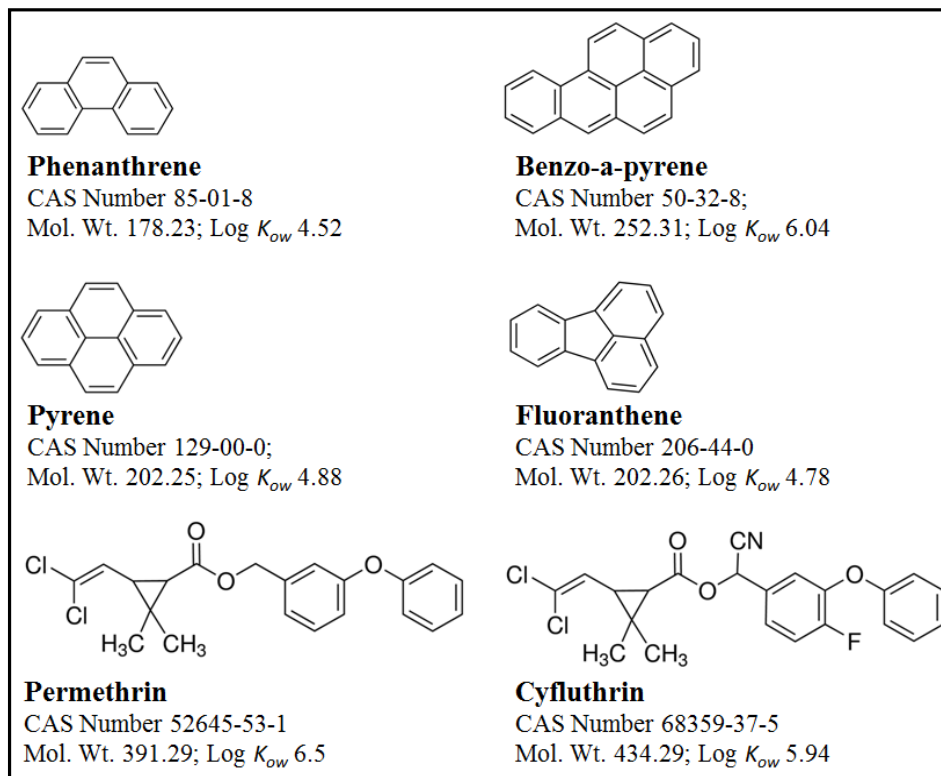
Phenanthrene
CAS Number 85-01-8
Mol. Wt. 178.23; Log K_{ow} 4.52

Benzo-a-pyrene
CAS Number 50-32-8;
Mol. Wt. 252.31; Log K_{ow} 6.04

Pyrene
CAS Number 129-00-0;
Mol. Wt. 202.25; Log K_{ow} 4.88

Fluoranthene
CAS Number 206-44-0
Mol. Wt. 202.26; Log K_{ow} 4.78

Permethrin
CAS Number 52645-53-1
Mol. Wt. 391.29; Log K_{ow} 6.5

Cyfluthrin
CAS Number 68359-37-5
Mol. Wt. 434.29; Log K_{ow} 5.94

Figure 2. Swim activity in DMSO, acetone, and media at C1 swim interval.

Significant differences (SD) across all intervals, between (BG) and within (WG) groups in different speed classes (C1 – C10) is shown. All controls tested at 1 and 24-hr. The color codes

for BG indicate: no SD (green), SD either time point (orange), or SD at both the time points (red). Within group comparisons (WG) color codes represent coefficient of variations corresponding to ≤ 10% (green), between 10 - 20% (orange) and > 20% (red).
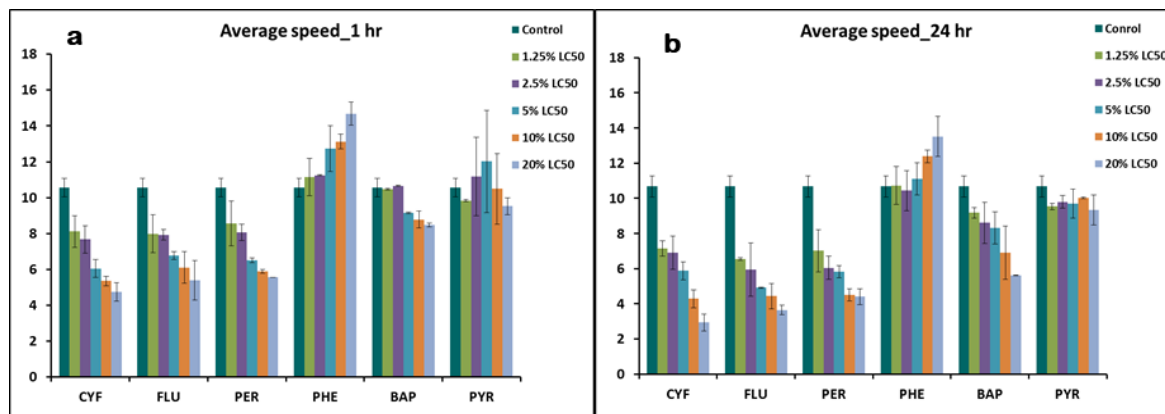
Figure 3. Changes in swim speeds over 1-hour (a) and 24-hr (b) from PAHs and pyrethroids. Reduced swim speed from cyfluthrin (CYF), fluoranthene (FLU), benzo(a) pyrene, and permethrin (PER) after one hour; phenanthrene increased swim speed.
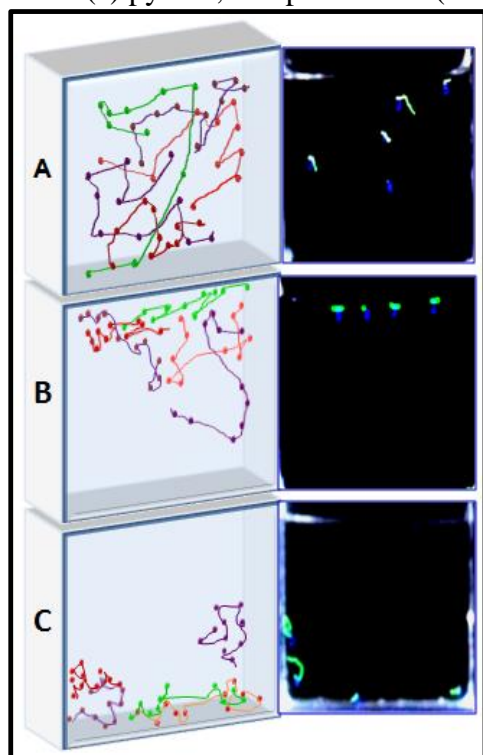
Figure 4. Daphnia swim behavior responses detected with video-tracking. Locomotor patterns: (A) Basal level in control media (B) Surface swimming observed in benzo(a)pyrene, pyrene, phenanthrene exposure (C) Bottom-tank swimming patterns observed in cyfluthrin, permethrin and fluoranthene.

Figure 5. GO-term processes regulated by phenanthrene exposure.
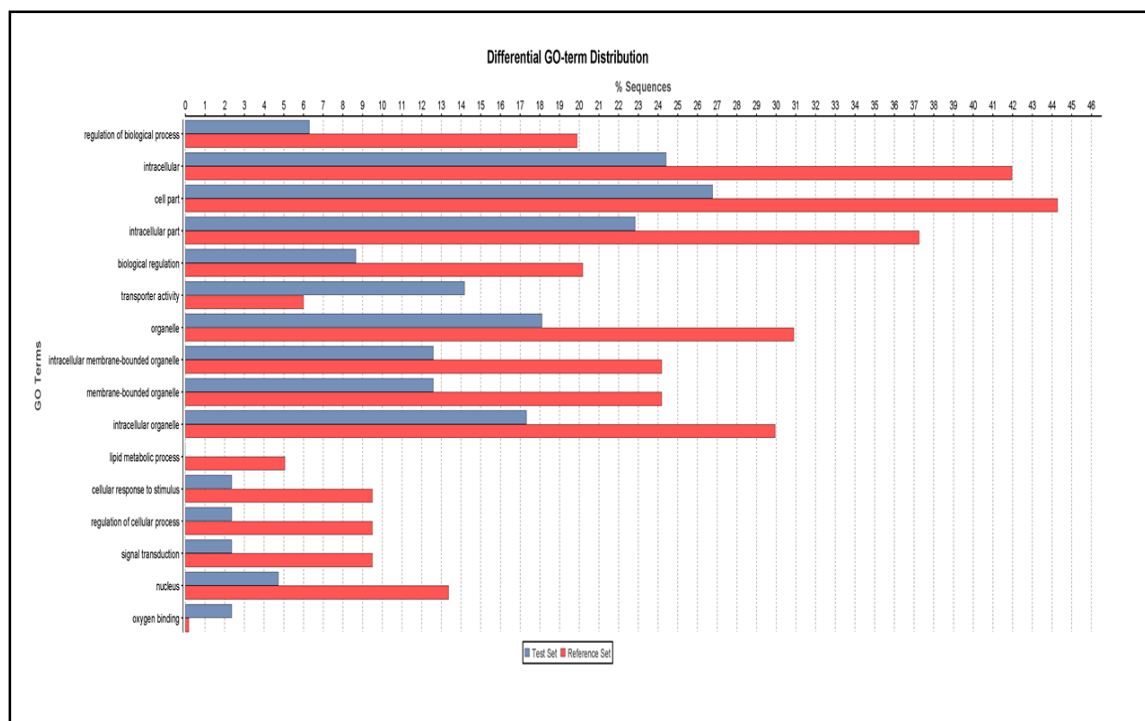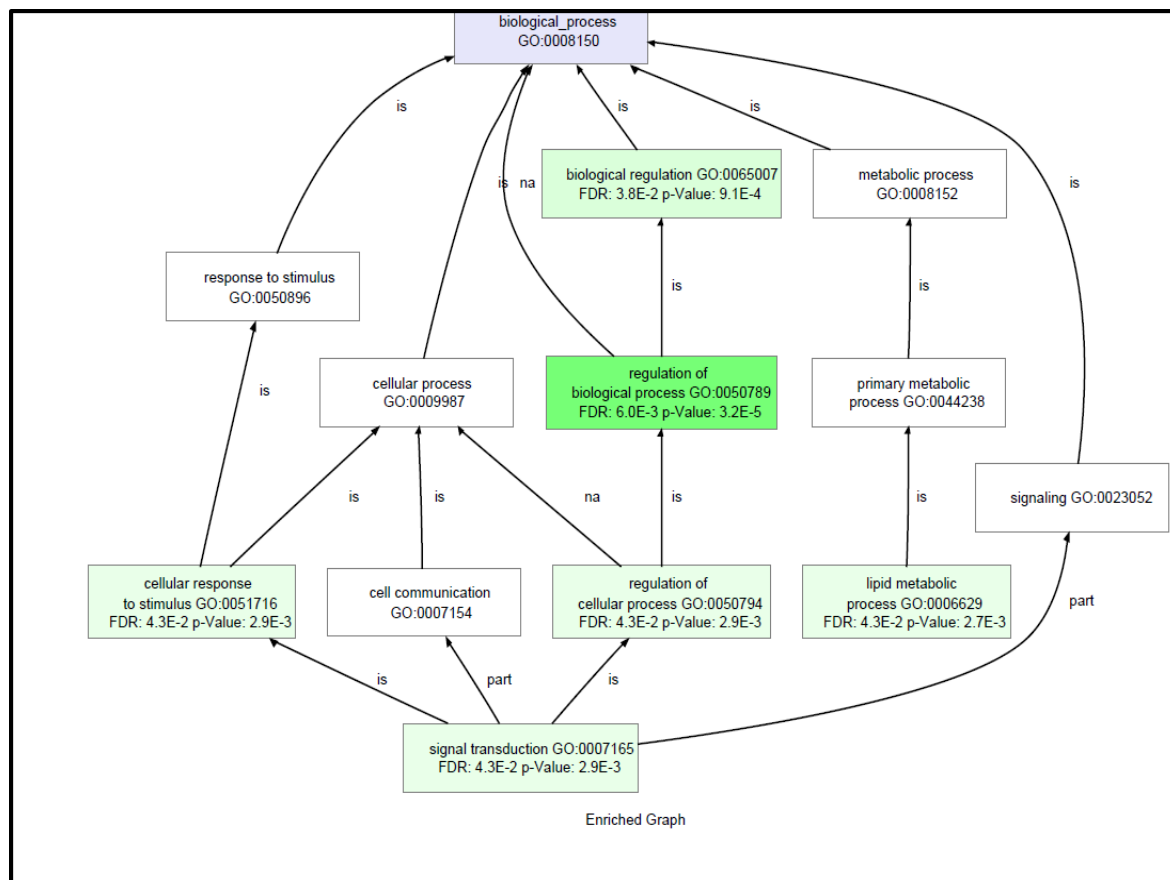
Figure 6. Enriched graph of pyrene-induced genes, and FDR for GO biological process.
Genes in biological regulation involve circadian-genes involve 9 genes (FDR: 3.8E-2 p-Value: 9.1E-4)
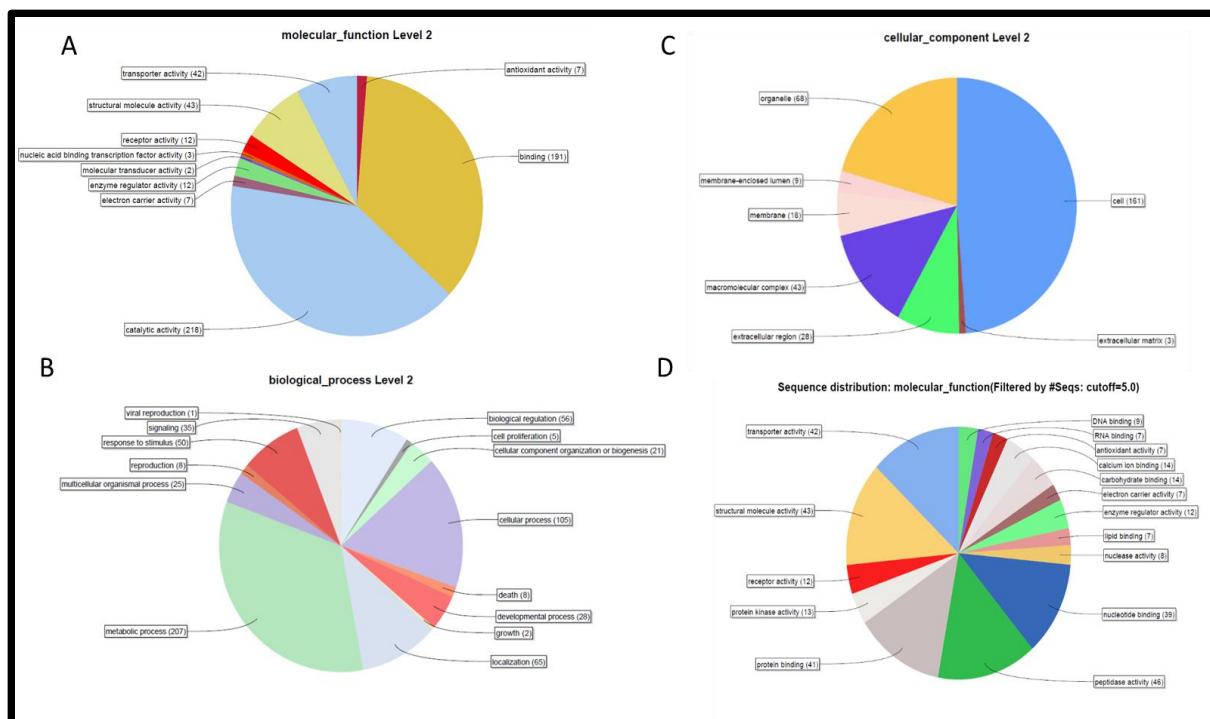
Figure 7. KEGG pathway analysis of transcription data for daphnia PHE exposures. A). Molecular function. B) Biological process C) Cellular component. D) Sequence distribution of 1654 genes significantly regulated in an estimated 58 pathways.
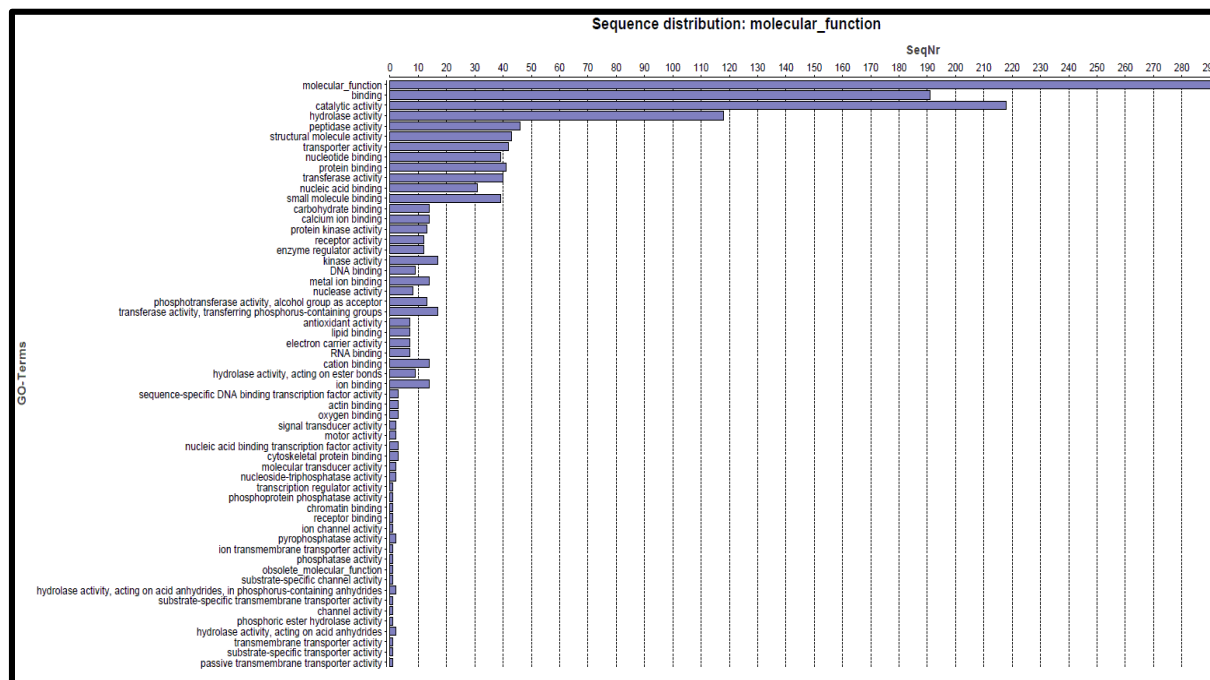
Figure 8. Distribution of PHE-induced sequences for functional pathway. 54 gene pathways affected involving ion transport, electron carrier, calcium ion binding, actin binding, oxygen binding, and transcription factor activity are represented.
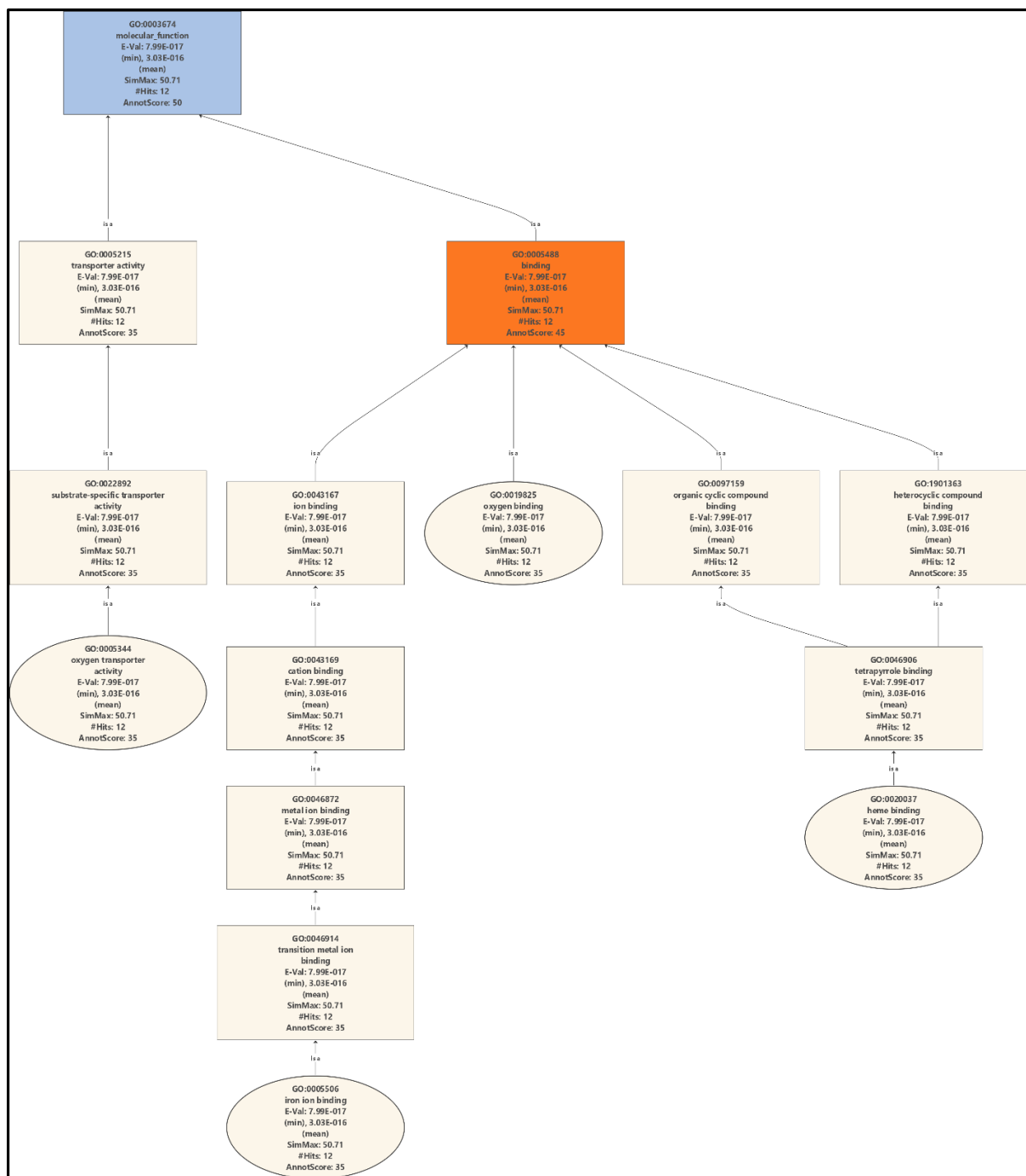
Figure 9. GO-terms (Molecular function) for DEGs of fluoranthene exposed Daphnia. Reduced expression of genes from GO:0016491 (oxidoreductase activity), GO:0016209 (antioxidant activity), GO: 1901363 (heterocyclic binding), and GO:0022892,GO:0005215 (transporter activity).
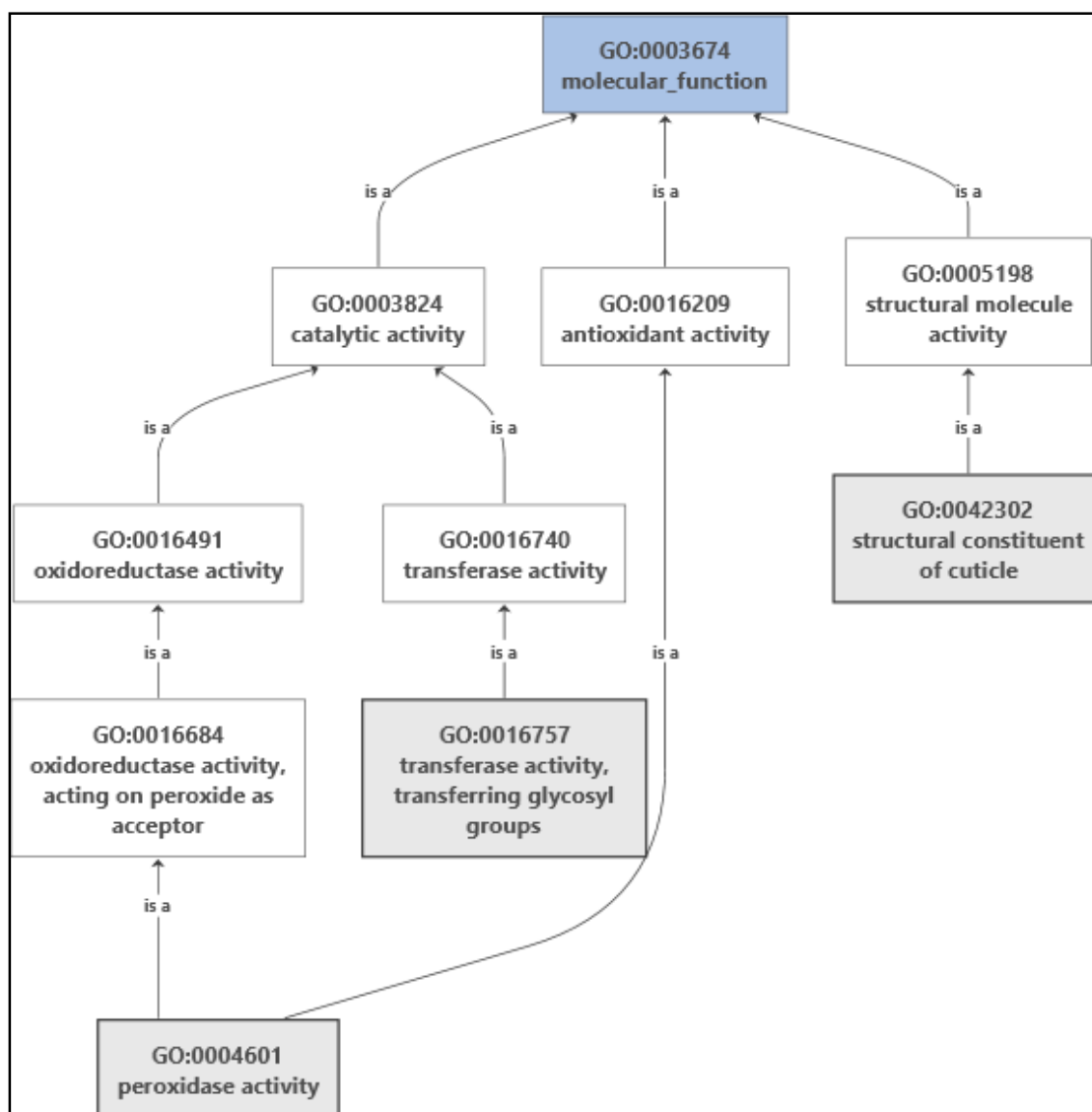
Figure 10. GO-graph enrichment analysis of benzo(a)pyrene gene cluster. Branches of the GO hierarchical tree without significant enriched GO terms are not shown. Black solid edges stand for relationship between enriched and unenriched terms, black dashed edges stand for relationship between two unenriched GO terms
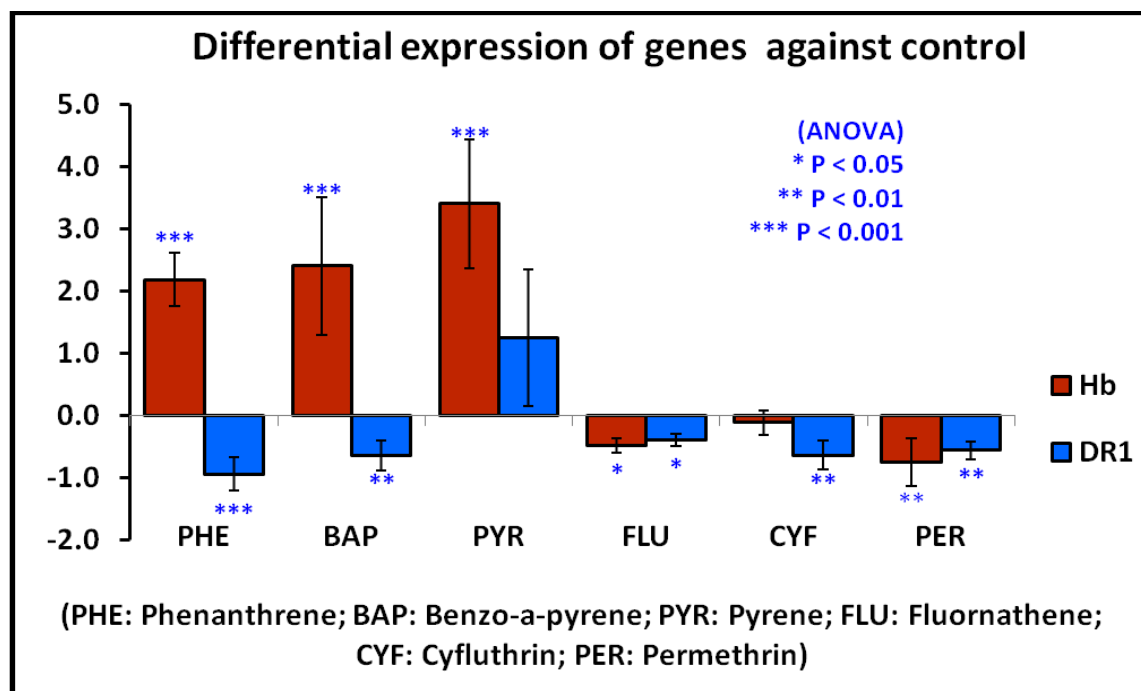
Figure 11. Changes in expression levels of *Dhb* and *D1R* genes in Daphnids following 24-hr exposure to the test agents. Values are significantly different from control (Student's *t*-test) at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0^{001}$ (***)
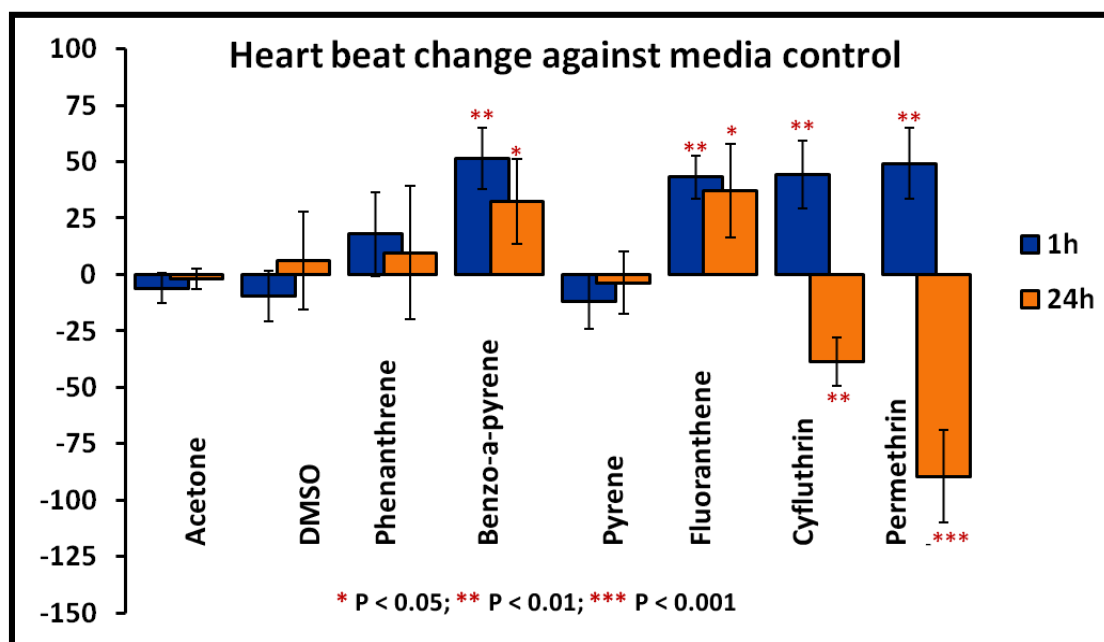
Figure 12. Changes in heart rate following 1and 24hr exposure to the different test agents. Values are significantly different from control (Student's *t*-test) at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.^{001}$ (***).

Table 1. LC$_{50}$ test values for 14-day old *Daphnia magna*.

Chemical	Solvent	LC$_{50}$	Molarity	Type
Phenanthrene	DMSO	2.92 mg/L	16.38 µM	PAH
Benzo-a- pyrene	Acetone	43.65 µg/L	173.00 nM	PAH
Fluoranthene	DMSO	12.08 µg/L	59.73 nM	PAH
Pyrene	Acetone	3.00 µg/L	14.83 nM	PAH
Permethrin	DMSO	4.84 µg/L	12.37 nM	Pyrethroid
Cyfluthrin	Acetone	3.24 µg/L	7.47 nM	Pyrethroid

Table 2. Speed class and locomotor distance for Daphnia behavior (mm)

Speed class	1	2	3	4	5	6	7	8	9	10
Thresholds (mm)	>0 <0.5	≥0.5 <1.0	≥1 <2	≥2 <4	≥4 <18	≥8 <16	≥16 <32	≥32 <64	≥64 <100	≥100

Table 3. Primers sequences for Daphnia hemoglobin (*Dhb*) and dopamine receptor (*D1R*) genes

Dhb	Forward sequence	5' – CACCACTGTGACTACCACTG – 3'
	Reverse sequence	5' – CAGCCTTCTTGAGGTTTTTG – 3'
D1R	Forward sequence	5' – TTACCTGCGACGACAAGGAT – 3'
	Reverse sequence	5' – GCCGAATAGGCGTACATCAT – 3'

Table 4A. Gene enrichment analysis for phenanthrene. GO elements are: Biological process (P), molecular function (F), and cellular component (C) (FDR-adjusted p<.005 for multiple tests in phenanthrene exposures. Number of probes in that Go category (#Test). Over/under indicates whether the term was overrepresented or underrepresented based on Fisher's exact test.

Probe ID	GO-ID	Category	#Test	Term	Gene functions	P-Value	Over/ Under
DM02485	GO:0050789	P	8	regulation of biological process	LDL receptor ligand-binding repeat bearing protein/ARP2_G108	3.24E-05	under
DM02485	GO:0065007	C	3	Biological regulation	Mbn12 circadian rhythm essential for photoreceptor	9.09E-04	under
DM00859 DM03585, DM14425, DM02499, DM02493	GO:0044464	C	34	cell part	Cryptochrome-biological rhythm	8.05E-05	under
DM04678, DM04676, DM05760, DM03586	GO:0044424	C	29	intracellular part	intracellular part	7.23E-04	under
DM05760, DM03586, DM02493, DM02490,	GO:0065007	P	11	biological regulation	Ras-related protein rab-14/ARP2_G1788	9.09E-04	under
DM03589	GO:0005215	F	18	transporter activity	OATP4A1,Solute carrier organic anion transporter family member 4A1	0.0010	Over
DM05875	GO:0005575	C	5	Cell part	Pfdn3/ Mgr protein	0.0028	Over
DM08856	GO:0003824 GO:0005488 GO:0005515	F		Catalytic activity	Dopamine beta-hydroxylase Neurexin-4	0.0025	Over
DM06542 DM04369 DM01567 DM01561, DM02553	GO:0019825	F	3	oxygen binding	oxygen binding	0.0034	Over
DM04667, DM10062, DM14418	GO:0043229	C	22	intracellular organelle	intracellular organelle	0.0020	under

DM03522, DM01347, DM10004, DM00245,	GO:0006629	P	0	lipid metabolic process	lipid metabolic process	0.0027	under
DM00299, DM01383, DM12220	GO:0051716	P	3	cellular response to stimulus	cellular response to stimulus	0.0029	under
DM03561	GO:0050794	P	3	regulation of cellular process	guanine nucleotide-binding protein gamma-1 subunit precursor/ARP2_G3211	0.0029	under
DM02493, DM02490, DM03566	GO:0007165	P	3	signal transduction	signal transduction	0.0029	under
DM04670, DM02493	GO:0005634	C	6	nucleus	nucleus	0.0029	under
DM06542, DM04369, DM01567, DM01561, DM02553	GO:0019825	F	3	oxygen binding	oxygen binding	0.0034	Over

Table 4B. Daphnia phenanthrene exposure (Phen v DMSO) significant GO pathways (enriched in test group compared to reference group using fishers exact test).

GO-ID	Term	Category	FDR	P-Value	#Test	#Ref	#not Annot Test
GO:0050789	**regulation of biological process**	**P**	**0.00598**	**3.24E-05**	**8**	**756**	**119**
GO:0005622	**intracellular**	**C**	**0.00598**	**7.03E-05**	**31**	**1595**	**96**
GO:0044464	**cell part**	**C**	**0.00598**	**8.05E-05**	**34**	**1683**	**93**
GO:0044424	**intracellular part**	**C**	**0.03833**	**7.23E-04**	**29**	**1416**	**98**
GO:0065007	**biological regulation**	**P**	**0.03832**	**9.09E-04**	**11**	**767**	**116**
GO:0005215	**transporter activity**	**F**	**0.03833**	**0.001031**	**18**	**228**	**109**
GO:0043226	**organelle**	**C**	**0.04318**	**0.001639**	**23**	**1174**	**104**
GO:0043231	**intracellular membrane-bounded organelle**	**C**	**0.04318**	**0.001985**	**16**	**919**	**111**
GO:0043227	**membrane-bounded organelle**	**C**	**0.04318**	**0.001985**	**16**	**919**	**111**
GO:0043229	**intracellular organelle**	**C**	**0.04318**	**0.002034**	**22**	**1138**	**105**
GO:0006629	**lipid metabolic process**	**P**	**0.04318**	**0.002699**	**0**	**192**	**127**
GO:0051716	**cellular response to stimulus**	**P**	**0.04318**	**0.002879**	**3**	**361**	**124**
GO:0050794	**regulation of cellular process**	**P**	**0.04318**	**0.002879**	**3**	**361**	**124**

GO:0007165	signal transduction	P	0.04318	0.002879	3	361	124
GO:0005634	nucleus	C	0.04318	0.002905	6	508	121
GO:0019825	oxygen binding	F	0.04677	0.003356	3	7	124

Table 5. PHE-related genes mapped to Kegg Pathways in GO enrichment of DEGs. 97 pathways (55 non-redundant) were estimated for this assessment.

Pathway	Seqs in Pathway	Enzyme	Enzyme ID	Seqs of Enzyme	Seqs
Aminobenzoate degradation	1	phosphatase	ec:3.1.3.1	1	DM03465
Naphthalene degradation	1	dehydrogenase	ec:1.1.1.1	1	DM12118
Chloroalkane and chloroalkene degradation	2	epoxide hydrolase	ec:3.3.2.10	1	DM01452
Flavone and flavonol biosynthesis	1	beta-glucuronide glucuronohydrolase glucuronidase	ec:3.2.1.31	1	DM03118
Nitrogen metabolism	8	dehydratase	ec:4.2.1.1	3	DM01414, DM13238, DM00457
Nitrogen metabolism	8	synthase (ammonia)	ec:6.3.4.16	1	DM01155
Nitrogen metabolism	8	oxidase	ec:1.9.3.1	2	DM01577, DM01629
Nitrogen metabolism	8	glutaminase I	ec:3.5.1.2	1	DM01178
Nitrogen metabolism	8	reductase (H+translocating)	ec:1.6.5.3	1	DM14949
Pyruvate metabolism	2	carboxylase	ec:6.4.1.2	1	DM00113
Phenylpropanoid biosynthesis	6	lactoperoxidase	ec:1.11.1.7	6	DM04090, DM01860, DM01544, DM05947, DM03159, DM05293
Aminoacyl-tRNA biosynthesis	1	ligase	ec:6.1.1.9	1	DM03709
Glycolysis / Gluconeogenesis	4	dehydrogenase	ec:1.1.1.1	1	DM12118
Glycolysis / Gluconeogenesis	4	aldolase	ec:4.1.2.13	1	DM01691
Glycolysis / Gluconeogenesis	4	glucose 6-phosphate phosphatase	ec:3.1.3.9	2	DM09565, DM14949
Arginine and proline metabolism	3	decarboxylase	ec:4.1.1.17	1	DM01270
Arginine and proline metabolism	3	synthase (ammonia)	ec:6.3.4.16	1	DM01155
Arginine and proline metabolism	3	glutaminase I	ec:3.5.1.2	1	DM01178
Pentose and glucuronate interconversions	1	beta-glucuronide glucuronohydrolase glucuronidase	ec:3.2.1.31	1	DM03118
Methane metabolism	7	aldolase	ec:4.1.2.13	1	DM01691

Methane metabolism	7	lactoperoxidase	ec:1.11.1.7	6	DM04090, DM01860, DM01544, DM05947, DM03159, DM05293
Phenylalanine metabolism	6	lactoperoxidase	ec:1.11.1.7	6	DM04090, DM01860, DM01544, DM05947, DM03159, DM05293
Fatty acid metabolism	1	dehydrogenase	ec:1.1.1.1	1	DM12118
Amino sugar and nucleotide sugar metabolism	9	chitodextrinase	ec:3.2.1.14	8	DM03350, DM00952, DM01236, DM03662, DM01372, DM00116, DM01427, DM00651
Amino sugar and nucleotide sugar metabolism	9	deacetylase	ec:3.5.1.41	1	DM00834
Purine metabolism	4	kinase	ec:2.7.1.20	1	DM01078
Purine metabolism	4	phosphatase	ec:3.6.1.15	1	DM10948
Purine metabolism	4	adenylyltransferase	ec:2.7.7.4	1	DM00609
Purine metabolism	4	synthase	ec:6.3.4.4	1	DM05054
Purine metabolism	4	kinase	ec:2.7.1.25	1	DM00609
Glycine, serine and threonine metabolism	1	dehydrogenase	ec:1.1.1.1	1	DM12118
Carbon fixation in photosynthetic organisms	1	aldolase	ec:4.1.2.13	1	DM01691
Selenocompound metabolism	1	adenylyltransferase	ec:2.7.7.4	1	DM00609
Glutathione metabolism	3	decarboxylase	ec:4.1.1.17	1	DM01270
Glutathione metabolism	3	dehydrogenase (NADP+dependent, decarboxylating)	ec:1.1.1.44	1	DM01085
Glutathione metabolism	3	ligase	ec:6.3.2.2	1	DM05418
Oxidative phosphorylation	5	oxidase	ec:1.9.3.1	2	DM01577, DM01629
Oxidative phosphorylation	5	ATPase	ec:3.6.3.6	2	DM00969, DM02049
Oxidative phosphorylation	5	reductase (H+translocating)	ec:1.6.5.3	1	DM14949
Terpenoid backbone biosynthesis	1	reductase (NADPH)	ec:1.1.1.34	1	DM00758
T cell receptor signaling pathway	1	protein-tyrosine kinase	ec:2.7.10.2	1	DM03608
Propanoate metabolism	1	carboxylase	ec:6.4.1.2	1	DM00113
Tyrosine metabolism	**1**	**dehydrogenase**	**ec:1.1.1.1**	**1**	**DM12118**

Pentose phosphate pathway	**3**	**aldolase**	**ec:4.1.2.13**	**1**	**DM01691**
Pentose phosphate pathway	**3**	**dehydrogenase (NADP+dependent, decarboxylating)**	**ec:1.1.1.44**	**1**	**DM01085**
Pentose phosphate pathway	**3**	**dihydroxyacetonetransferase**	**ec:2.2.1.2**	**1**	**DM00585**
Fatty acid biosynthesis	2	carboxylase	ec:6.3.4.14	1	DM00896
Tryptophan metabolism	2	2,3-dioxygenase	ec:1.13.11.11	2	DM00255, DM05645
Other glycan degradation	1	alpha-D-mannosidase	ec:3.2.1.24	1	DM00090
Retinol metabolism	1	dehydrogenase	ec:1.1.1.1	1	DM12118
Tetracycline biosynthesis	1	carboxylase	ec:6.4.1.2	1	DM00113
Porphyrin and chlorophyll metabolism	2	beta-glucuronide glucuronohydrolase glucuronidase	ec:3.2.1.31	1	DM03118
Porphyrin and chlorophyll metabolism	2	ligase	ec:6.1.1.17	1	DM03709
Alanine, aspartate and glutamate metabolism	3	synthase	ec:6.3.4.4	1	DM05054
Alanine, aspartate and glutamate metabolism	3	carbamoyltransferase	ec:2.1.3.2	1	DM01155
Alanine, aspartate and glutamate metabolism	3	glutaminase I	ec:3.5.1.2	1	DM01178
Valine, leucine and isoleucine degradation	1	carboxylase	ec:6.4.1.3	1	DM00113
Thiamine metabolism	1	phosphatase	ec:3.6.1.15	1	DM10948
D-Glutamine and D-glutamate metabolism	1	glutaminase I	ec:3.5.1.2	1	DM01178
Folate biosynthesis	1	phosphatase	ec:3.1.3.1	1	DM03465
Sulfur metabolism	2	adenylyltransferase	ec:2.7.7.4	1	DM00609
Sulfur metabolism	2	oxidase	ec:1.8.3.1	1	DM01339
Sulfur metabolism	2	kinase	ec:2.7.1.25	1	DM00609
Sphingolipid metabolism	2	acylsphingosine deacylase	ec:3.5.1.23	1	DM00880
Sphingolipid metabolism	2	sulfotransferase	ec:2.8.2.11	1	DM11369
Drug metabolism - other enzymes	1	beta-glucuronide glucuronohydrolase glucuronidase	ec:3.2.1.31	1	DM03118
Glyoxylate and dicarboxylate metabolism	3	hydratase	ec:4.2.1.3	1	DM00819
Glyoxylate and dicarboxylate metabolism	3	carboxylase	ec:6.4.1.3	1	DM00113
Glyoxylate and dicarboxylate metabolism	3	phosphatase	ec:3.1.3.18	1	DM01327

Lysine degradation	2	5-dioxygenase	ec:1.14.11.4	2	DM00604, DM00582
Drug metabolism - cytochrome P450	**1**	**dehydrogenase**	**ec:1.1.1.1**	**1**	**DM12118**
Galactose metabolism	2	glucose 6-phosphate phosphatase	ec:3.1.3.9	2	DM09565, DM14949
Metabolism of xenobiotics by cytochrome P450	**2**	**dehydrogenase**	**ec:1.1.1.1**	**1**	**DM12118**
Metabolism of xenobiotics by cytochrome P450	**2**	**epoxide hydrolase**	**ec:3.3.2.9**	**1**	**DM01452**
Citrate cycle (TCA cycle)	2	hydratase	ec:4.2.1.3	1	DM00819
Citrate cycle (TCA cycle)	2	carboxylase	ec:6.4.1.1	1	DM00896
Phosphatidylinositol signaling system	1	kinase C	ec:2.7.11.13	1	DM00304
Fructose and mannose metabolism	2	aldolase	ec:4.1.2.13	1	DM01691
Fructose and mannose metabolism	2	endo-1,4-beta-mannosidase	ec:3.2.1.78	1	DM03147
Glycerophospholipid metabolism	2	O-acyltransferase	ec:2.3.1.51	1	DM03384
Glycerophospholipid metabolism	2	dehydrogenase	ec:1.1.5.3	1	DM05671
Starch and sucrose metabolism	3	beta-glucuronide glucuronohydrolase glucuronidase	ec:3.2.1.31	1	DM03118
Starch and sucrose metabolism	3	glucose 6-phosphate phosphatase	ec:3.1.3.9	2	DM09565, DM14949
Glycosaminoglycan degradation	1	beta-glucuronide glucuronohydrolase glucuronidase	ec:3.2.1.31	1	DM03118
Glycerolipid metabolism	3	lipase	ec:3.1.1.3	2	DM07667, DM01737
Glycerolipid metabolism	3	O-acyltransferase	ec:2.3.1.51	1	DM03384
Pyrimidine metabolism	1	carbamoyltransferase	ec:2.1.3.2	1	DM01155
Pyrimidine metabolism	1	carbamoylaspartic dehydrase	ec:3.5.2.3	1	DM01155
Arachidonic acid metabolism	1	epoxide hydrolase	ec:3.3.2.10	1	DM01452
Cysteine and methionine metabolism	2	dioxygenase	ec:1.13.11.20	1	DM00085
Cysteine and methionine metabolism	2	(cytosine-5-)-methyltransferase	ec:2.1.1.37	1	DM12099
Carbon fixation pathways in prokaryotes	3	hydratase	ec:4.2.1.3	1	DM00819
Carbon fixation pathways in prokaryotes	3	carboxylase	ec:6.4.1.3	1	DM00113
Carbon fixation pathways in prokaryotes	3	carboxylase	ec:6.4.1.2	1	DM00113

Carbon fixation pathways in prokaryotes	3	carboxylase	ec:6.4.1.1	1	DM00896
Taurine and hypotaurine metabolism	1	dioxygenase	ec:1.13.11.20	1	DM00085

Table 6. Pyrene-regulated Daphnia transcripts and fold change

Probe ID	Gene Function	fold change
DM01769P2	cysteine-rich protein/ARP2_G11407: Acetylation,LIM domain,Metal-binding,Zinc	-2.76
DM10919P2	scaffold_75:165913-169539:+ : spinster type iv/ARP2_G17112	-2.51
DM03530P1	Metalloprotease,Protease,Repeat,Secreted, Signal,Transmembrane,Zinc,Zymogen	-2.32
DM09968P3	Acetylation,Apoptosis,Nucleus,Phosphoprotein Beta-catenin-like protein 1	-2.30
DM03897P3	Nucleosome core,Nucleus,Phosphoprotein,Ubl conjugation H2a/j,Histone H2A,Histone H2A.J	-2.17
DM01857P2	vitellogenin-1 precursor/ARP2_G1053	-2.60
DM01036P1	alpha methylacyl-coa racemase/ARP2_G3423; CaiB/baiF CoA-transferase family protein C7orf10,CaiB/baiF CoA-transferase family protein C7orf10 homolog,EC=2.	2.22
DM03506	ldl receptor ligand-binding repeat bearing protein/ARP2_G108	2.5
DM08040P1	conserved protein/ARP2_G113	2.10
DM00260P2	vitellogenin-1 precursor/ARP2_G1053	2.70
DM08794P1	expressed protein/ARP2_G24729	2.51
DM12948P1	laminin beta-1 chain precursor/ARP2_G3834 : tissue formation	105.88
DM02202P1	26s protease regulatory subunit/ARP2_G5773	4.86
DM07102P4	neurexin IV/ARP2_G19	15.69
DM11931P1	class b scavenger receptor cd36 domain./ARP2_G1276	2.04
DM01998	Cytoglobin,Hemoglobin subunit beta-H0	13.5

Table 7. Fluoranthene regulated *D.magna* genes. FDR-adjusted p<.005 for multiple tests. GO categories: Function (F) P(process)C(cell component). Estimated gene regulation that is higher than control is "over'; lower than control gene expression is "down."

Probe ID	GO Category	Gene function	Regulation
DM00347	GO:0005215/F:transporter activity,GO:0005488/F:binding GO:0005575	ATP sensitive potassium channel	over
DM03815	GO:0015378/F: transporter GO:0006814	sodium chloride importer: chloride symporter	down
DM15146	GO:0005575 GO:0006810/P:transport	Adenylate cyclase ARP2_G2249	down
DM02928	GO:0004601:P: GO:006979 F: peroxidase activity P: response to oxidative stress F: heme binding	Antioxidant stress	down
DM12885	GO:0005198/F:structural molecule activity GO:0019538	cuticle protein metamorphosis	down
DM08082	Not determined	Unknown expressed protein/ARP2_G13423	down
DM05929	GO:0003723 F:RNA binding GO:0015031/P:protein transport SRP72_HUMAN homolog signal recognition particle kda protein/ARP2_G1988	Secretory protein of rough ER. signal recog. particle RNA binding	down

Table 8. Representative up-regulated genes in response to benzo(a)pyrene treatment (p<0.05).

Gene Target	Gene Function	Fold change (unlogged)
DM11495	Oxygen transport	19.31
DM06909 DM06310	C-type lectin domain family 4-member K Immune response, response to stimulus	3.98 2.26
DM05875	Dopamine activity Histidine catabolic process	4.18
DM12999	Transmembrane signaling receptor Ligand-gated ion channel activity	2.47
DM09561 DM00060	Protein de-ubiquitation: ubiquitinyl hydrolase activity catabolic process UDP-glucosyltransferase	3.66 2.22
DM12302 DM00747 DM05983	Cuticle protein 5a Cuticle assembly Cuticle component	2.71 2.41 2.55
DM09204	Brain specific serine protease; trypsin-like serine protease	2.67
DM15205 DM08696	Sulfotransferase Oxidoreductase	2.98
DM03541	Organic cation transporter	2.36
DM13742	Biotin protein ligase	2.23

Table 9A. FDR Tests and enrichment analysis for cyfluthrin. GO terms: biological process (P), molecular function (F), and cellular component (C) (FDR-adjusted p<.005 for multiple tests in cyfluthrin exposures. Number of probes in that Go category (#Test). Over/under indicates whether the term was overrepresented or underrepresented based on Fisher's exact test.
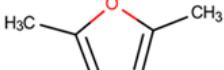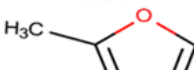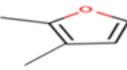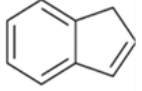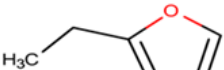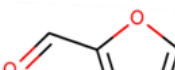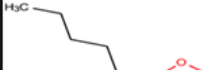
GO Term	Name	Type	FDR	single test p-Value	# in test group	# in Ref group	# non Annot test	# non Annot Ref group	Over/ Under
GO:0050789	regulation of biological process	P	1.4E-4	6.8E-6	3	761	90	3073	under
GO:0005198	structural molecule activity	F	5.1E-4	3.0E-5	15	175	78	3659	over
GO:0008233	peptidase activity	F	1.5E-3	1.2E-4	17	248	76	3586	over
GO:0005515	protein binding	F	2.3E-3	2.0E-4	10	1040	83	2794	under
GO:0043234	protein complex	C	5.6E-3	5.5E-4	4	630	89	3204	under
GO:0044267	molecular protein metabolic process	P	1.6E-2	1.7E-3	3	511	90	3323	under
GO:0031981	nuclear lumen	C	2.5E-2	4.0E-3	0	249	93	3585	under
GO:0006996	organelle organization	P	2.5E-2	4.0E-3	0	251	93	3583	under
GO:0003676	nucleic acid binding	F	3.0E-2	4.9E-3	2	399	91	3435	under
GO:0005739	mitochondrion	C	3.8E-2	6.4E-3	0	239	93	3595	under

9B. **Functional enrichment analysis for cyfluthrin.** GO terms: biological process (P), molecular function (F), and cellular component (C) (FDR-adjusted p<.005 for multiple tests in cyfluthrin exposures. Number of probes in that Go category (#Test). Over/under indicates whether the term was overrepresented or underrepresented based on Fisher's exact test.

Probe ID	GO-ID	Category	#Test	Gene type	Gene Function	FDR pvalue	Over/ under
DM00116	GO:0044464	C	16	Ephrin type-B receptor 2 precursor, putative,	RTK signaling	6.06E-08	Under
DM00859 DM00585, DM02819, DM03321, DM01611	GO:0009987 GO:0044237 GO:0044260	P	19	Cryptochrome 2	biological rhythm	1.10E-06	under

DM05113 DM01260	GO:0065007	P	3	Conserved protein/ARP2_G789	Heat shock protein	4.21E-06	Under
DM00915	GO:0044260	P	3	Tyrosyl-trna synthetase/ARP2_G3 601	ATP activated production of tRNA. translation/synthesis	5.15E-04	under
DM03586	GO:0005634	C	1	transcription initiation factor iib general transcription factor tfiib/ARP2_G2847	RNA polymerase II co-factor for transcription GTF2B	6.00E-05	under
DM04621	GO:0043234	C	4	Calcium/calmodulin-dependent protein kinase type ii alpha chain/ARP2_G964	CaMKII potassium channel	5.48E-04	under
DM01675	GO:0008233	F	17	Cath D	regulation of α-Synuclein; misfolded protein interaction	4.54E-04	over
DM02423	GO:0005739	C	23	mitochondrial	succinyl-coa synthetase beta chain	0.00642	under

Chapter Four

furan		2,3-dihydrofuran	
2,5-dimethylfuran		2-methylfuran	
2,3-dimethylfuran		indene	
2-ethylfuran		furfural	
2-pentylfuran			

Figure 1. Chemical structures for furan, candidate biofuels, and combustion products
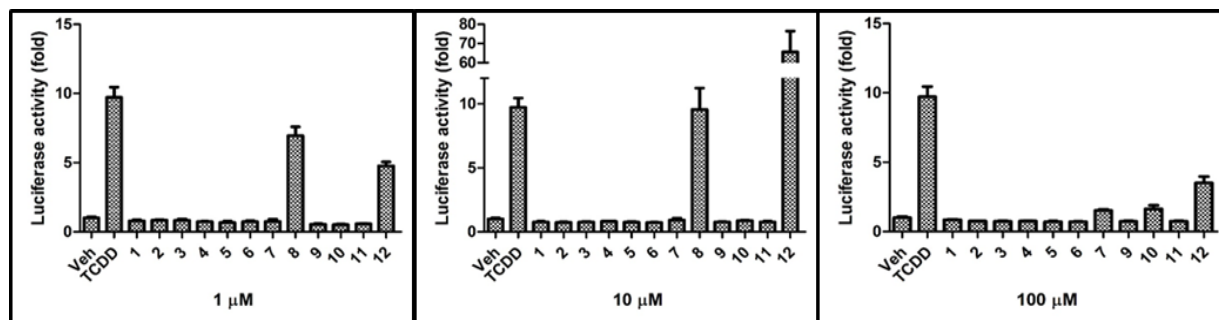
Figure 2. Reporter assay in human cells for AHR-XRE activation. In vitro activation of human AHR with aromatic compounds at 1uM,10uM, and 100uM. The compounds screened were: compound 1: 2,5-dimethylfuran; compound 2: 2,3-dimethylfuran; compound 3: 2-methylfuran; compound 4: 2-ethylfuran; compound 5: 2,3-dihydrofuran; compound 6: furan; compound 7: furfural; compound 8: benzo(a)pyrene; compound 9: furan; compound 10: indene; compound 12: **β**-napthoflavone. Strong activation was observed with furfural, benzo(a)pyrene, and **β**-naphthoflavone.
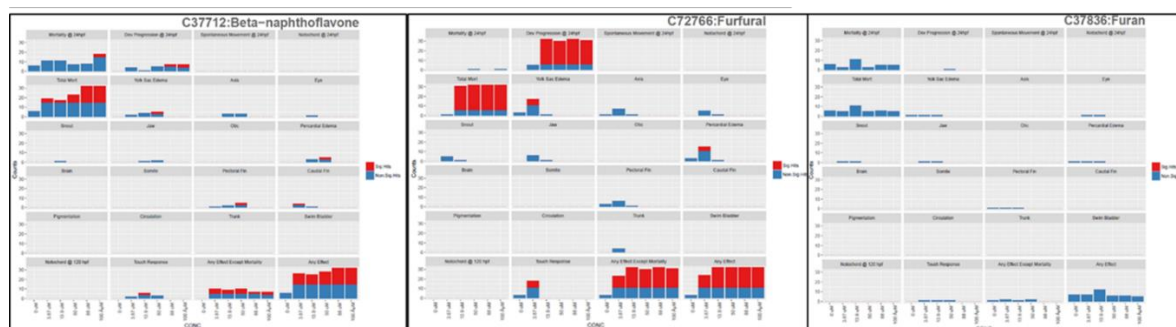
Figure 3. Developmental toxicity of furan and combustion intermediate. In addition to high mortality, furfural significantly affected zebrafish developmental progression, caudal fin, touch response, yolk sac edema, and pericardial edema. Beta-napthoflavone disrupted development at markers for embryonic yolk sac, and pericardial edema. Higher mortality was observed in furfural and beta-napthoflavone than furan.
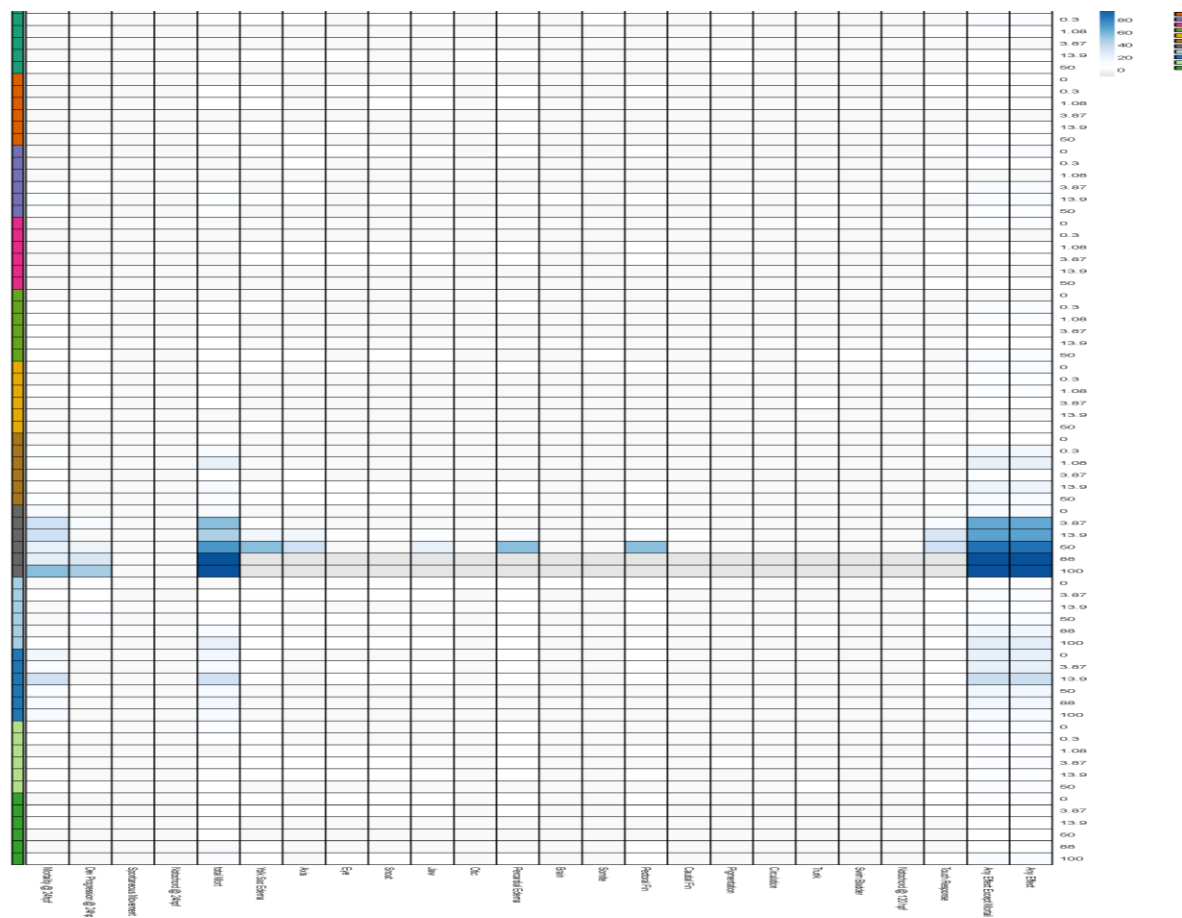
Figure 4. Developmental toxicity endpoints for zebrafish embryos. zfAHR2 agonists beta-napthoflavone and benzo(a) pyrene did not produce equivalent effects; Toxicity to pectoral fin, pericardial edema, yolk sac edema was present for beta-napthoflavone but not benzo(a) pyrene. No significant toxic effects to zebrafish from biofuel candidates.

Furan analogs
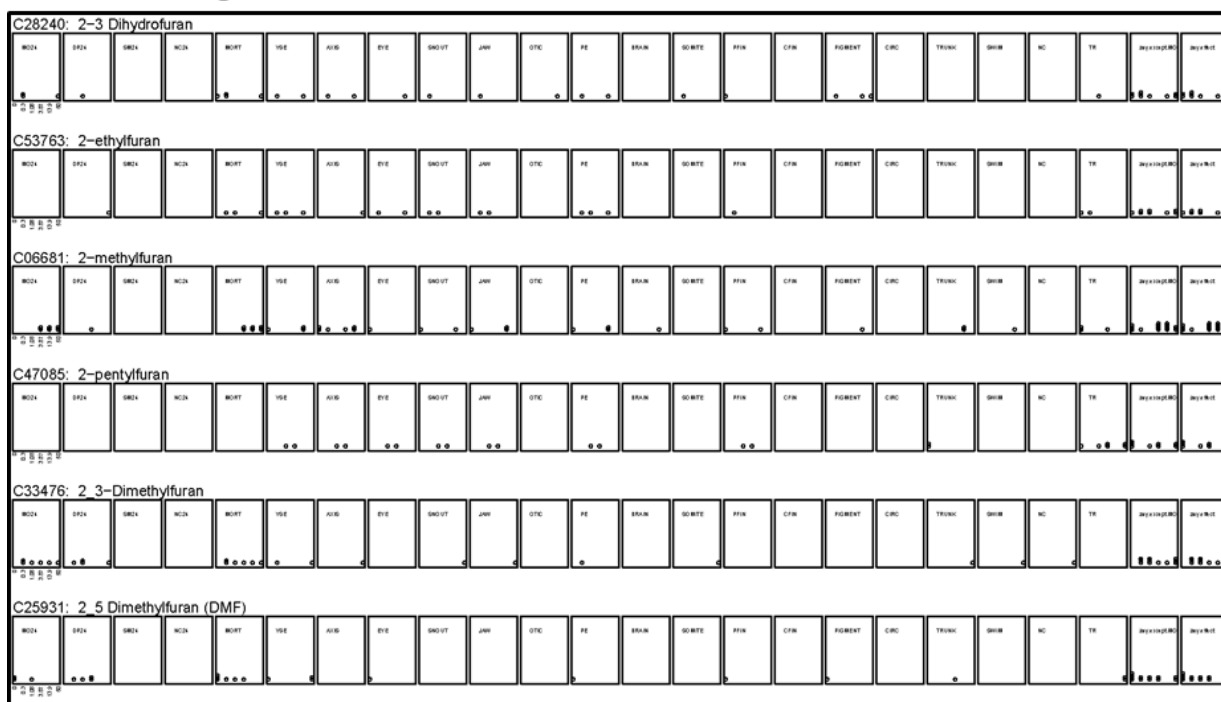
AHR agonists

Figure 5. Assessment of developmental toxicity endpoints in zebrafish. Adverse morphological effects not significant for furan biofuel compounds. AHR-dependent developmental toxicity is significant for BNF but not BAP in our study.
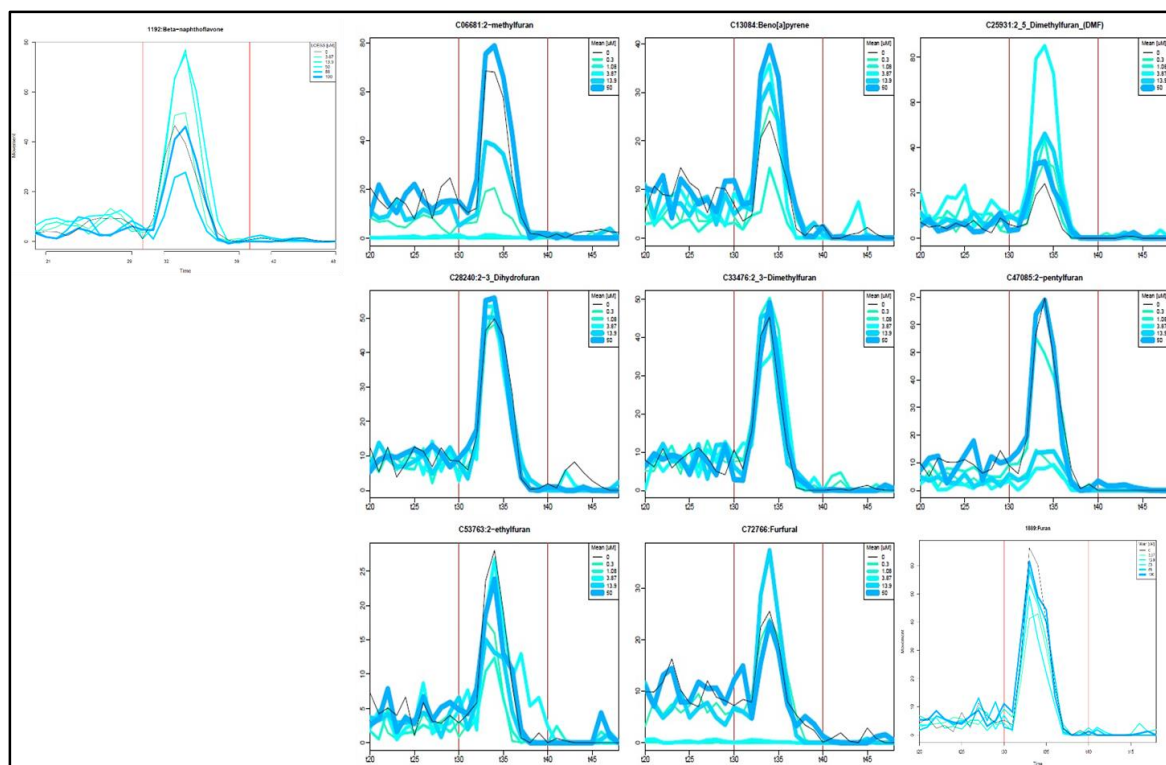
Figure 6. Embryonic photomotor responses of biofuel candidate and AHR agonists. Significant hypoactivity is observed from 2-pentylfuran and 2-methylfuran at .3uM. Benzo(a)pyrene and betanapthoflavone also ellicits hypoactive embryonic behavior; while higher doses of 2,-5 dimethylfuran, furfural, and bethanapthoflavone produced hyperactivity in the E interval.
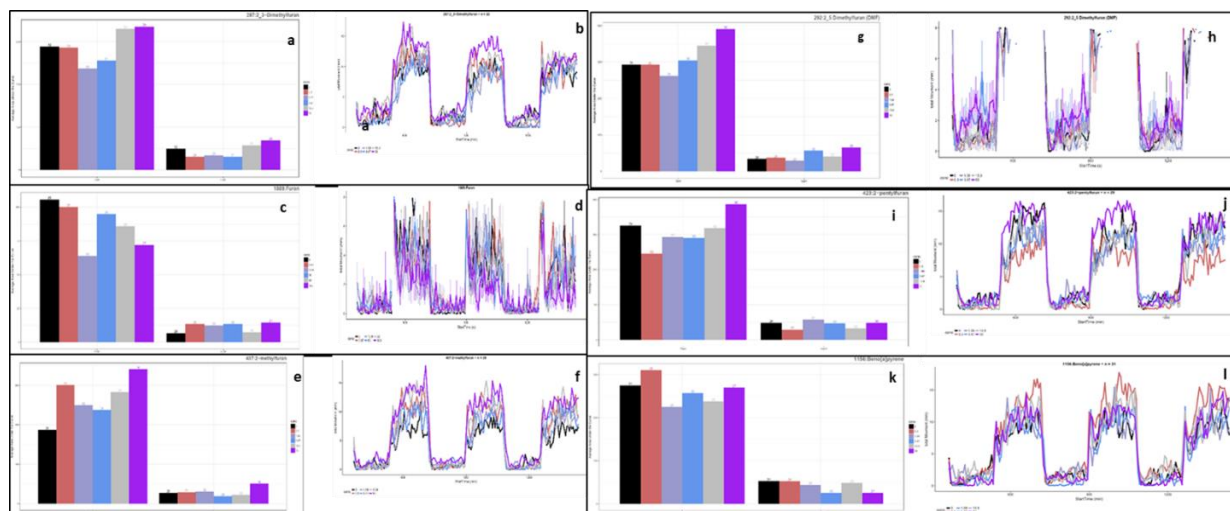
Figure 7. Larval photo-locomotor behavior resusulting from candidate biofuels. Hyperactivity for a) 2,3-dimethyfuran , e) 2-methylfuran, and g)2,5 dimethylfuran and Increased total movement (mm) in dark. Light phase hyperactivity for i)2-pentylfuran, l)benzo(a)pyrene, and b)2,5-dimethylfuran

		Embryonic Photo Response			Larval Photo Response		Morphology		
		EPR–Background	EPR–Excitatory	EPR–Refractory	LPR–Dark Cycle	LPR–Light Cycle	Development	P. edema	24h mortality
Biofuel derivatives	2-ethylfuran	---	---	---		1.08			
	2,3-dihydrofuran	---	---	---	1.08	1.08			
	2,3-dimethyfuran	---	---	---	1.08	13.9			
	2-pentylfuran	---	---	.3		13.9			
	2-methylfuran	.3		.3-13.9	.3	.3			
	2,5-dimethylfuran	1.08	1.08			3.87			
	furan		3.87		3.87	13.9			
Combustion products	furfural		13.9						
	indene		3.87			3.87			
AHR agonists	benzo(a)pyrene	1.08	13.9			3.87			
	β-napthoflavone		13-50	88	3.87	3.87			

Assays
EPR
LPR
Morphology

Figure 8. Profile for AHR vs non-AHR targeted chemicals in zebrafish. Biofuel candidates do not produce expected AHR developmental toxicity. Furfural is a developmental toxicant produced from biofuel combustion, comparable to potent AHR agonist beta-napthoflavone.

Table 1. Activation of AhR-LBD by compounds used in virtual docking into zfAHR1 and zfAHR2

Compound		ZF Model	Binding energy	Induction of AhR transcriptional activity
AHR agonist	TCDD	Zb AHR2	-21.86	High
	BNF		-17.96	High
	BAP		-4.88	Low
AHR agonists	Leflunomide TCDD BNF BAP	Zb AHR1A	-19.4 -8.24 -8.75 -11.68	Moderate
Furan candidates	2,5 DMF	Zb AHR1A	-11.84	Moderate
	2-MF		-9.63	
	2-PF		-13.19	
AHR agonist	TCDD	Zb AHR1B	-17.48	High
	BNF		-8.67	High
	BAP *** Not Docked properly		*****	*****

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