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Part A: Temporal and dose-dependent transcriptional responses in the liver of fathead minnows following short term exposure to the polycyclic aromatic hydrocarbon phenanthrene

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ABSTRACT

Phenanthrene is a low molecular weight polycyclic aromatic hydrocarbon (PAH) that is composed of three fused benzene rings. PAHs are formed naturally through incomplete combustion of organic materials, and are environmental contaminants due to anthropogenic activities (e.g. oil extraction and refining, industrial and municipal effluents, fossil fuel burning). Fish exposed to PAHs such as phenanthrene have been reported to exhibit altered reproductive axis

AbbreviationsSREBF1sterol regulatory element binding transcription factor 1FASNfatty acid synthaseHSD17B12hydroxysteroid (17-beta) dehydrogenase 12ACACAacetyl-CoA carboxylase alphaCYB5Acytochrome b5 type A (microsomal)SLC27A4solute carrier family 27 (fatty acid transporter), member 4ELOVL6ELOVL fatty acid elongase 6ELOVL5ELOVL fatty acid elongase 5ELOVL4ELOVL fatty acid elongase 4TECRtrans-2,3-enoyl-CoA reductaseELOVL2elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2ELOVL1ELOVL fatty acid elongase 7, ELOVL7 elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1LRP8low density lipoprotein receptor-related protein 8, apolipoprotein e receptorNR1H3nuclear receptor subfamily 1, group H, member 3TP53tumor protein p53NR1H4nuclear receptor subfamily 1, group H, member 4AKT1v-akt murine thymoma viral oncogene homolog 1ESR1estrogen receptor 1CATcatalaseAPPamyloid beta (A4) precursor proteinMDM2Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse)PPARGC1Aperoxisome proliferator-activated receptor gamma, coactivator 1 alphaSREBF1sterol regulatory element binding transcription factor 1ADIPOQadiponectin, C1Q and collagen domain containingGCGperoxisome proliferator-activated receptor alpha, PPARAglucagonAPOA1apolipoprotein A-ILPLipoprotein lipaseMTTPmicrosomal triglyceride transfer proteinPOMCproopiomelanocortinPNPLA2patatin-like phospholipase domain containing 2SIRT1sirtuin 1SOD2superoxide dismutase 2, mitochondrialFASNfatty acid synthaseRORARAR-related orphan receptor ALMNAlamin A/CAPOBapolipoprotein B (including Ag(x) antigen)CREB1cAMP responsive element binding protein 1FOXL2CCAAT/enhancer binding protein (C/EBP), beta, CEBPB forkhead box L2NR4A1nuclear receptor subfamily 4, group A, member 1CYP1B1cytochrome P450, family 1, subfamily B, polypeptide 1NPYneuropeptide YPPARDperoxisome proliferator-activated receptor deltaCAV1caveolin 1, caveolae protein, 22 kDaLIPClipase, hepaticAGTR1angiotensin II receptor, type 1HNF4Ahepatocyte nuclear factor 4, alphaLRP5low density lipoprotein receptor-related protein 5NR3C1nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)CASP3caspase 3, apoptosis-related cysteine peptidaseCLUclusterinRARAretinoic acid receptor, alphaPTPN1protein tyrosine phosphatase, non-receptor type 1F2coagulation factor II (thrombin)CREMcAMP responsive element modulatorERN1endoplasmic reticulum to nucleus signalling 1SCARB1scavenger receptor class B, member 1GGPS1geranylgeranyl diphosphate synthase 1NR1D1nuclear receptor subfamily 1, group D, member 1PLIN2perilipin 2ACHEacetylcholinesteraseSYPsynaptophysinTHRBthyroid hormone receptor, betaCYP7A1cytochrome P450, family 7, subfamily A, polypeptide 1PLTPphospholipid transfer proteinNPC1L1NPC1 (Niemann-Pick disease, type C1, gene)-like 1SCDstearoyl-CoA desaturase (delta-9-desaturase)PDE3Bphosphodiesterase 3B, cGMP-inhibitedCYP11A1cytochrome P450, family 11, subfamily A, polypeptide 1RXRAretinoid X receptor, alphaHSD3B1hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1HMGCR3-hydroxy-3-methylglutaryl-CoA reductaseLDLRlow density lipoprotein receptorNR1I2nuclear receptor subfamily 1, group I, member 2PLAURplasminogen activator, urokinase receptorNR2C2nuclear receptor subfamily 2, group C, member 2PRKCBprotein kinase C, betaAPOA4apolipoprotein A-IVDHCR2424-dehydrocholesterol reductaseABCB1ATP-binding cassette, sub-family B (MDR/TAP), member 1APOEapolipoprotein ESLC27A1solute carrier family 27 (fatty acid transporter), member 1NCOA6nuclear receptor coactivator 6SOD1superoxide dismutase 1, solubleLIPElipase, hormone-sensitiveANGPTL3angiopoietin-like 3ZFP36L1zinc finger protein 36, C3H type-like 1SORT1sortilin 1SULT2B1sulfotransferase family, cytosolic, 2B, member 1DGAT2diacylglycerol O-acyltransferase 2HDAC3histone deacetylase 3LCATlecithin-cholesterol acyltransferaseLRP1low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)VLDLRvery low density lipoprotein receptorPSEN1presenilin 1CELcarboxyl ester lipase (bile salt-stimulated lipase)ARRB2arrestin, beta 2NPC1Niemann-Pick disease, type C1INSIG1insulin induced gene 1ANXA2annexin A2ACACBacetyl-CoA carboxylase betaDHCR77-dehydrocholesterol reductaseCYP51A1cytochrome P450, family 51, subfamily A, polypeptide 1STARD3StAR-related lipid transfer (START) domain containing 3ACAT2acetyl-Coenzyme A acetyltransferase 2CNBPCCHC-type zinc finger, nucleic acid binding proteinSMPD2sphingomyelin phosphodiesterase 2, neutral membrane (neutral sphingomyelinase)CYP27A1cytochrome P450, family 27, subfamily A, polypeptide 1ABCG5ATP-binding cassette, sub-family G (WHITE), member 5ABCB11ATP-binding cassette, sub-family B (MDR/TAP), member 11FABP6fatty acid binding protein 6, ilealACAT1acetyl-CoA acetyltransferase 1SCP2sterol carrier protein 2SREBF2sterol regulatory element binding transcription factor 2AMFRautocrine motility factor receptor, E3 ubiquitin protein ligaseINSIG2insulin induced gene 2PZPpregnancy-zone proteinACBD3acyl-CoA binding domain containing 3HSD3B7hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7TOMM20translocase of outer mitochondrial membrane 20 homolog (yeast)APODapolipoprotein DFABP1fatty acid binding protein 1, liverFDFT1farnesyl-diphosphate farnesyltransferase 1HMGCS13-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) LIPAlipase A, lysosomal acid, cholesterol esteraseSLC10A2solute carrier family 10 (sodium/bile acid cotransporter family), member 2OSBPoxysterol binding proteinEHMT2euchromatic histone-lysine N-methyltransferase 2CH25Hcholesterol 25-hydroxylaseNPC27Niemann-Pick disease, type C2LBRlamin B receptorMBTPS1membrane-bound transcription factor peptidase, site 1RNF139ring finger protein 139TMEM97transmembrane protein 97CYP46A1cytochrome P450, family 46, subfamily A, polypeptide 1LSSlanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)UGT2B4UDP glucuronosyltransferase 2 family, polypeptide B4EBPemopamil binding protein (sterol isomerase)UFD1Lubiquitin fusion degradation 1 like (yeast)ABCA2ATP-binding cassette, sub-family A (ABC1), member 2LAMA5laminin, alpha 5GNMTglycine N-methyltransferaseMANFmesencephalic astrocyte-derived neurotrophic factorSQLEsqualene epoxidaseSOAT2sterol O-acyltransferase 2SEC14L2SEC14-like 2 (S. cerevisiae)LRP4low density lipoprotein receptor-related protein 4

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endpoints, however the mechanisms that underlie these responses are not fully characterized. To better understand effects at the mechanistic level, we applied transcriptomics to identify molecular pathways altered after acute exposure to phenanthrene on both a dose and temporal scale. Female fathead minnow (*Pimephales promelas*) were exposed to an average measured concentration of either 0, 29.8, 389 or 943 μg phenanthrene/L for 24, 48, and 72 h in a static-renewal bioassay. Ovaries were assessed for oocyte distribution as well as *in vitro* 17β-estradiol production and gene expression for transcripts related to steroidogenesis and estrogen signalling. In addition, the liver transcriptome was measured as this tissue is the primary source of the egg yolk precursor protein vitellogenin. Exposure to 29.8 μg phenanthrene/L increased proportions of the cortical alveolar stage in the ovaries after 48 h while the proportion of cortical alveolar oocyte were decreased in fish exposed to 943 μg phenanthrene/L for 48 h. Phenanthrene did not affect 17β-estradiol production at any time or dose, and did not affect transcripts associated with hormone synthesis nor signalling pathways. In the liver, the transcriptome showed fewer genes in common across time when compared to those transcripts affected by concentration at a single time point. Cholesterol metabolism was the only pathway perturbed in the liver following all comparisons in both the dose and time course experiments. Our data suggest that transcriptome networks associated with hepatic lipid metabolism are rapidly affected by phenanthrene, and this may indirectly reduce resources available for reproductive efforts.

1. Introduction

Phenanthrene is a polycyclic aromatic hydrocarbon (PAHs) that is composed of three benzene rings and is also one of the most abundant PAHs found in the aquatic environment (Anyakora et al., 2005; Zhang et al., 2011). Phenanthrene can be a significant component of crude oil (Kreitsberg et al., 2010) and is a bi-product of incomplete combustion, including vehicle exhaust (Marr et al., 1999), cigarette smoke (Forehand et al., 2000) and waste incineration (Johansson and Bavel 2003; Maliszewska-Kordybach 1999). The concentration of phenanthrene in aquatic environments can increase substantially due to runoff and atmospheric deposition around urban environments and oil spills (Lima et al., 2003). For example, phenanthrene has been detected at 0.52–0.73 mg/L in ground water around wood treatment and storage locations in Canada (Irwin, 1997). Groundwater samples taken around a coal and oil gasification plant in Seattle, USA had phenanthrene concentrations up to 0.13 mg phenanthrene/L (130 μg phenanthrene/L) (Irwin, 1997), and concentrations as high as 1460 μg phenanthrene/L have been detected in surface water near a crude oil exploration area in Nigeria (Anyakora et al., 2005). Phenanthrene in sediments from Boston harbour were determined to be 23 μg phenanthrene/g dry weight (Boston, USA, Wang et al., 2001). In addition, atmospheric transport has distributed PAHs, including phenanthrene, all over the world, and concentrations in relatively pristine areas are reported to be ~0.1–8 ng/L (both in sediment and surface water) (Menzie et al., 1992; Giannarelli et al., 2017). Phenanthrene continues to be a contaminant of global concern and has been listed as one of the 16 priority PAHs by the Environmental Protection Agency (EPA; Bojes and Pope 2007).

PAHs affect multiple physiological systems, such as those involved in detoxification, development, and circulation. PAHs induce CYP1A activity through the aryl hydrocarbon receptor (AHR) signalling pathway, leading to oxidative damage, immune dysfunction, and impaired reproduction in teleost fishes (Loughery et al., 2014; Möller et al., 2014; Bender et al., 2016; Wiseman et al., 2013). The AHR ligand pyrene causes anemia, peripheral vascular defects, and neuronal cell death in fish (commonly referred to as 'blue sac disease'; Incardona et al., 2004). However, not all PAHs induce CYP1A activity or bind to AHR and could have effects on fish through different mechanisms. Phenanthrene binds to AHR only weakly or not at all (Barron et al., 2004; Billiard et al., 2002) but it affects heart development and heart conductance in larval zebrafish, leading to craniofacial deformities, pericardial and yolk sac edema, and spinal curvature in developing larvae (Incardona et al., 2004). Additionally, unlike other PAHs, phenanthrene induces a phase II detoxification response, demonstrated by an increase in glutathione-related enzyme activity and the presence of glucuronide conjugated phenanthrene-1,2-diol as the main metabolite in fish bile (Varanasi et al., 1989; Jee and Kang, 2005). These differences in responses to phenanthrene likely means that it has a different mode of action when compared to other PAHs.

Phenanthrene also affects reproductive processes in fish but its effects have been variable. This PAH reduces the production of androstenedione and 17β-estradiol in ovarian explants from flounder (*Platichthys flesus* L.), likely due to the inhibition of cytochrome p450 17, 17β-hydroxysteroid dehydrogenase, and aromatase (Monteiro et al., 2000b). Decreased circulating 17β-estradiol concentrations were found in male and female common carp (*Cyprinus carpio*) after 72 h, but only in fish exposed to concentrations ≥1.5 mg phenanthrene/L (Han et al., 2010). A dose response experiment conducted for 80 days with 0.06–60 μg phenanthrene/L decreased vitellogenic oocyte proportions and downregulated *aromatase* and *estrogen receptor α* mRNA levels in the ovary of marine medaka (*Oryzias melastigma*) (Sun et al., 2015). In contrast, zebrafish exposed to phenanthrene over three generations did not have altered reproductive hormones or gonad size (Horng et al., 2010). The impact of phenanthrene on the reproductive axis is therefore not entirely clear and warrants further investigation.

Research has recently focused on predictive indicators of adverse health effects in aquatic species. Adverse health effects are induced by molecular initiating events; for example, Bisphenol A, a 17β-estradiol agonist, binds to estrogen receptors and decreases plasma E_2 and vitellogenin in female fish (Perkins et al., 2013). A strength of transcriptomics is that one can identify clusters of genes and pathways that are perturbed by a chemical exposure and that are associated to an adverse health effect over time. To better characterize the mechanisms of action of phenanthrene, transcriptomic analyses were conducted herein to determine if early exposure to this PAH induced changes within the steroidogenic pathway or negatively impacts steroid receptor signalling.

This study used the fathead minnow (FHM; *Pimephales promelas*) as a model to investigate the effects of phenanthrene on reproductive endpoints because its reproductive biology is well characterized at both the physiological and molecular levels (Jensen et al., 2001). We hypothesized that phenanthrene would negatively affect reproductive axis endpoints that spanned molecular targets to the whole animal. In the ovary, we took a focused approach as this tissue is the primary site of steroidogenesis. As such, transcripts involved in steroid hormone receptor signalling and the steroidogenic biosynthesis pathway were measured for steady state levels following phenanthrene exposure. In addition, *in vitro* E_2 production was measured as well as a detailed examination of oocyte proportions using histology. Liver, a tissue that is actively involved in reproduction, metabolism, and detoxification, was chosen to investigate whole transcriptome responses to phenanthrene and these responses were characterized for different doses and on a temporal scale. The objectives were to (1) identify genes or pathways that may be useful in predictive ecotoxicology for phenanthrene exposure, as well as other PAHs; and (2) examine the temporal and concentration-related variability in expression patterns in the liver after exposure to a model PAH.