AN ABSTRACT OF THE THESIS OF

Annika J. Swanson for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on May 19, 2014. Title: Identification of potential new biomarkers of oxygenated polycyclic aromatic hydrocarbon (OPAH) exposures in zebrafish.

Abstract approved: _____
Robert Tanguay

Oxygenated polycyclic aromatic hydrocarbons (OPAHs) are a class of environmentally prevalent compounds with little known information on their effect *in vivo.* In a recent study conducted using zebrafish as a model, 38 OPAHs were screened for toxicity. This investigation focuses on two of these OPAHs, 7,12-B[a]AQ and BEZO. Both 7,12-B[a]AQ and BEZO show AHR2-dependent toxicity, however only 7,12-B[a]AQ consistently and significantly induces expression of cyp1a protein. Cyp1a is a diverse xenobiotic metabolizing protein that is used as a biomarker for many AHR-dependent toxicity studies in vertebrates. As 7,12-B[a]AQ and BEZO differentially express cyp1a, this study focused on four genes, ctsl.1, wfikkn1, ponzr3, and ponzr4, identified by RNA-sequencing as potential alternative biomarkers for AHR-dependent OPAH toxicity. A time point analysis of gene expression of ctsl.1 and wfikkn1 concluded that the stage of development is important in selecting alternative biomarkers. Preliminary localization of all four gene mRNA transcripts using *in situ* hybridization suggests that expression is sensitive to both stage of development and specific OPAH exposure. Investigating the candidacy of potential alternative biomarkers for AHR-dependent OPAH toxicity would help determine the pathway of toxicity of OPAHs in any environmental sample.

Key words: OPAH, toxicity, biomarker, AHR, environment
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Identification of Potential New Biomarkers of Oxygenated Polycyclic Aromatic Hydrocarbon (OPAH) Exposures in Zebrafish

by

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Annika J. Swanson, Author

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CONTRIBUTION OF AUTHORS

Several people at Oregon State University contributed to the four-year study described in this thesis. Britton Goodale and Andrea Knecht contributed to the organization of the toxicity screen of 38 OPAHs, the selection of environmentally relevant compounds, morpholino injections, oxidative stress analysis, and RNA-sequencing. Jane LaDu trained and assisted me with RNA extraction, qRT-PCR, and *in situ* hybridization. Robert Tanguay provided the necessary guidance and resources for this project.

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DEDICATION

This thesis is dedicated to my parents for their love, support, and encouragement throughout my life and to my sister, Abigail, for joining me along the Camino and for always sticking by my side through the bad and the good times.

Identification of Potential New Biomarkers of Oxygenated Polycyclic Aromatic Hydrocarbon (OPAH) Exposures in Zebrafish

INTRODUCTION

Exposures to polycyclic aromatic hydrocarbons (PAHs) are associated with toxic effects in a variety of organisms. PAHs are molecules consisting of a least two hydrocarbon benzene rings and are present throughout the environment. They are formed through incomplete combustion, for example, in automobile exhaust, industrial waste, wood burning, and tobacco smoke. Toxicity of PAHs varies widely and depends on their structure, but some have been shown to cause adverse effects including cancer, genetic mutations, and mortality in certain organisms (Lundstedt *et al*., 2007). One pathway of PAH toxicity is initiated by binding and activating the aryl hydrocarbon receptor (AHR), the initiating event of a well conserved vertebrate signaling pathway. Ligand binding to this receptor can induce the expression of specific genes and subsequent proteins such as cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1). This protein is typically expressed in tissues such as the skin, gastrointestinal tract, and in lung epithelial cells (Walsh *et al.*, 2013). One of the main functions of CYP1A1 is to metabolize a diverse set of compounds including xenobiotics, steroids, and fatty acids (Uniprot, 2014). Most notably, CYP1A1 is known for oxidizing 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene, and various PAHs in the environment. Often, metabolizing these compounds creates reactive metabolites that are also toxic. Because expression of this protein is a common biomarker for AHR-dependent toxicity, many studies have characterized the CYP1A1 response with respect to chemical carcinogenicity, pharmaceutical safety, and environmental health (Walsh *et al.*, 2013).

Until recently, there had been little research centered on the hazard potential of oxygenated polycyclic aromatic hydrocarbons (OPAHs). These compounds are formed during incomplete combustion similar to PAHs; however, OPAHs can also be created from the transformation of PAHs. Some earlier studies suggested that OPAHs might represent less toxic "dead-end products" resulting from degradation of the parent compounds. However, the opposite is now known to be closer to the truth; the degradation products may be as toxic as the parent material and easily produced during remediation efforts that promote PAH degradation. Added to this, OPAHs demonstrate relatively high environmental mobility and persistence (Lundstedt *et al*., 2007, p.475) and they can be formed directly during incomplete combustion processes. Together, these characteristics would suggest that OPAHs may pose a substantial human and environmental health hazard.

We evaluated the developmental toxicity of 38 OPAHs in zebrafish. The zebrafish model was chosen because the embryos are small in size, nearly transparent, and develop quickly. The human and zebrafish genome are approximately 75% similar; therefore, the information learned using zebrafish is often highly translatable to humans including understanding processes of development and unraveling causes of human disease (Howe *et al.,* 2013).

Two of the original 38 OPAHs were pursued further because of their high environmental relevance and their predicted high toxicity (Knecht, Swanson *et al.,* 2013, Layshock *et al.*, 2012, p.2453; Sienra, 2006; Kurihara, 2005). Identifying biomarkers that are associated with exposure to these OPAHs would be of significant practical value and became the focus for these studies.

Benz(a)anthracene-7,12,dione (7,12-B[a]AQ) and 1,9-Benz-10-anthrone (BEZO) are both 4-ring OPAHs that are widely present in the environment. BEZO is present as particulates in the atmosphere as a result of coal and wood burning and has been detected in exhaust from automobiles. It is predicted that long term exposure could lead to the formation of skin tumors (Dwivedi *et al.*, 2013). 7,12-B[a]AQ is also present in combustion products and at industrial waste sites. According to a study in Beijing, 7,12-B[a]AQ was one of the highest concentrated OPAHs measured in the urban air in that region (Wnag Jariyasopit, 2011). The parent PAH, benz(a)nthracene (BAA) is listed as one of the 16 priority PAH pollutants by the EPA (Incardona Day *et al.*, 2006).

The goal of this study was to identify a set of common biomarkers for AHR-dependent OPAH toxicity. Ideally, genes unlike cyp1a that are consistent and reliable in gene expression for a range of OPAHs could be used as gene expression indicator tools and to help determine the pathway of toxicity of OPAHs in any environmental sample.

MATERIALS AND METHODS

Fish Husbandry

All spawning and management of adult zebrafish were conducted in the Sinnhuber Aquatic Research Laboratory on a recirculating water system with a temperature of 28 ± 1ºC. Fish were housed in a controlled environment with a 14 hour light and 10 hour dark period daily. Embryos spawned from numerous adult zebrafish were collected in the morning and screened for quality and similar age. Zebrafish and experiments were carried out with respect to the Oregon State University Institutional Animal Care and Use Committee regulations.

Chemical Preparation

Benz[a]athracene-7,12-dione (7,12-B[a]AQ) was purchased at >95% purity from Sigma-Aldrich and 1,9-benz-10-anthrone (BEZO) from Fluka also at >95% purity. Chemicals were dissolved to 10mM concentration in 100% dimethyl sulfoxide (DMSO) and stored at room temperature in the dark as stock solutions. Before use, the stock solutions were sonicated in a water bath for 15min.

Embryo Exposure

Embryos were rinsed with fish water multiple times and separated into glass vials at a temperature of 28ºC with 40 embryos per container. Each vial held 4ml of exposure solution with the appropriate amount of DMSO-solubilized chemical stock in fish water (1% DMSO in all samples). Vials containing fish were covered in foil to protect the chemical from photooxidation then placed in a 28ºC incubator and placed on a rocker

until each time point of embryo collection. At each collection time, certain vials of embryos exposed to 1% DMSO, 10µM 7,12-B[a]AQ, or 10µM BEZO were removed from the incubator and embryos were rinsed in standard embryo media four times and placed in individual petri dishes. If 48hpf or younger, embryos were carefully dechorionated using forceps and then placed on ice and euthanized with MS-222 (tricane methanesulfonate) for RNA isolation and *in situ* hybridization, respectively. In relation to the older embryos, at age 48hpf, all embryos were removed from the chemical and washed with embryo media and placed back in the incubator until the correct time point.

RNA Isolation

Embryos were placed in separate 1.5ml microtubes with 20 fish in each and placed on ice for 15min. Embryo media was removed from euthanized embryos and 0.5mm zirconium oxide beads and 500µL RNAzol was added to each tube. Fish were homogenized using a bullet blender and samples were stored at -80ºC. When ready for total RNA extraction, samples were thawed and RNA was isolated using water, isopropanol, and ethanol. Sample pellets were solubilized in 25µL of RNAase-free water and concentration and quality was determined using a SynergyMx microplate reader and Gen5 Take3 module to determine OD 260/280 ratios for RNA. All RNA samples were diluted to 200ng/µL for cDNA synthesis.

Quantitative RT-PCR

cDNA was formed from 2µg of total RNA using a MultiScribe Reverse Transcriptase ABI master mix reagents and protocol. The cDNA was then diluted to 100ng/µL and stored at -20ºC for later use. qPCR was conducted using a 2X SYBR®

Green master mix in a 12µL reaction using gene-specific primers. Amplification of specific gene products was performed at the following cycling conditions: 95ºC for 10min and 15sec min during the holding stage, 35 cycles at alternating 60ºC for 1min and 95ºC for 15sec, and finally a holding stage at 95ºC once the amplification is complete. Melt curves were generated at three degree increments for analysis of product quality. Results were analyzed using the Pflaffl Method (Pfaffl, 2001) by normalizing to B-actin Ct values. Three biological replicates were used as well as technical replicates depending on standard error results. Log2 fold-change expression data for OPAH-exposed animals compared to DMSO control fish were assessed for significance using the software GraphPad Prism 5 to generate a one-way ANOVA using a Dunnett's test with significance defined as p-values of *p>0.05, **p>0.01, and ***p>0.001.

Paired-end mRNA Sequencing and Analysis

Total RNA was extracted from whole embryos exposed to 1% DMSO, 10µM 7,12-B[a]AQ, or 10µM BEZO. mRNA was isolated from these samples which were then fractionated and sequenced using a 50bp paired-end method with an Illumina HiSeq 2000 Sequencer. Samples were filtered depending on quality scores, analyzed to determine quality with FastQC analytical software, and trimmed if the reads were of low quality. Sample reads were aligned with the *Danio rerio* genome (Zv9.70) using TopHat 2.0.7. Analysis was carried out using 50bp minimum intron length, 10000bp maximum intron length, 200 mate pair inner distance, 150bp mate pair inner distance standard deviation and without the mixed alignment option (Goodale, 2013).

In situ Hybridization Probe Synthesis

A Novegen KOD-Hotstart reaction mixture was made for the creation of the templates for antisense RNA probe synthesis. cDNA was amplified using T3-tagged gene-specific primers in a 100µL KOD-PCR reaction with the following parameters: 95ºC for 2min, 33 cycles of 95ºC for 20sec, 61ºC 10sec, 70ºC 15sec, and a final step of 72ºC for 5min. Resulting template DNA samples were purified using a QIAquick PCR Purification kit and then 100ng was added to a reaction mixture for antisense-probe synthesis. The reaction mixture was incubated for 2hrs at 37ºC and then the reaction was halted by the addition of 2µL of RQ1 Dnase (Promega, Cat No. M610A). After an additional 15min incubation at 37ºC, 10% sterile 0.5M EDTA in water was added to the sample. Next, the probe product was purified using a Sigmaspin post-reaction purification column (Sigma, Ct No. S-5059). Concentration and purity was evaluated using SynergyMx microplate reader and Gen5 Take3 module. Probe samples were diluted to 50ng/µL with hybridization solution and stored at -20ºC.

Whole-mount In Situ Hybridization

This *in situ* hybridization protocol was adapted from the Thisse lab (2010). Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4ºC and then dehydrated in 100% methanol and stored at -20ºC for later use. Multiple 24-well plates and sterile homemade embryo-carrying baskets were prepared for a series of washes and incubation over a 3-day period of experimentation. 15-30 embryos were placed in each well basket. Embryos were first rehydrated at room temperature in a series of four decreasing dilutions of methanol in 1X PBS and were eventually washed in 100% 1X

PBST. Embryos were transferred to 10µg/ml Proteinase K (ProK) for an age-dependent digestion. 24hpf embryos received 15min, 48hpf 25min, and 72-96hpf 35min in the ProK solution. To stop the digestion, embryos were placed in 4% PFA for 20min and then washed in PBST. Embryos were pre-hybridized in hybridization buffer for 2hrs at 70ºC in order to decrease non-specific binding. Embryos in baskets were placed in 0.5ng/µL hybridization solution of antisense DIG-labeled RNA probe overnight under a tight seal with gentle rocking at 70ºC. A positive control probe for the gene-specific RNA probe, sonic hedgehog (shh) was used to ensure the experiment and staining was executed well.

On day two, sample washing solutions were prepared and heated to 70ºC before the addition of embryos. At this point in the protocol, extra pre-hybridized fish were stored at -20ºC for later use. With the remaining fish, each basket group was washed quickly in hybridization solution, without the addition of tRNA or heparin, and then placed in a series of four decreasing dilutions of cheap hybridization solution in 2X SSC for 10min each. Washes in 0.2X SCC for two 30min increments were then conducted at 70ºC as well. At room temperature, the embryos then underwent a series of washes in four decreasing concentrations of 0.2X SSC in PBST for 10min each. At the end of day two, fish were added to blocking buffer (2mg/ml BSA and 2% sheep serum in PBST) for 3-4hrs at room temperature. During this time, the extra pre-hybridized embryos are placed in 1:1000 anti-DIG antibody solution to absorb any undesirable particulates before diluting and adding to the *in situ* fish in baskets. The fish in blocking buffer were put in pre-absorbed 1:5000 anti-dig antibody solution overnight at 4ºC on a gentle rocker.

Day three consists of two fast washes in PBST and then placement of the embryos in Fast Red Buffer (Tris-HCl with NaCl, pH 8.2) for two 30min increments, rocking at

room temperature. After that, embryos were removed from baskets and placed in new empty wells with Fast Red stain (dissolve 1 Fast Red SIGMA*FAST*™ F4648-50SET tablet for every 2mls Fast Red Buffer). Plates were covered in foil to protect from light and left stationary at room temperature for 2hrs. Fast Red stain was then removed and embryos were washed several times in PBST and stored in the dark at 4ºC for imaging with a Zeiss fluorescent microscope. Approximately five embryos from each experiment group/well were segregated into 1.5ml microtubes for IHC.

Immunohistochemistry

Since embryos from the *in situ* hybridization protocol were already permeabilized, the five fish in each microtube were washed with 1ml PBST for 30min at room temperature on a rocker. Then, the PBST was replaced with a blocking solution (10% normal goat serum in PBST) for one hour rocking at room temperature. Fish were then transferred into 1:500 CYP1A primary antibody solution (mouse anti-fish CYP1A antibody, Biosense Laboratories, Bergen Norway, C02401101-500) in 10% NGS/block and stored overnight rocking at 4ºC. On day two, the primary antibody was removed, fish were washed for 1hr and 30min in PBST and then placed in 1:1000 secondary antibody (goat anti-mouse Alexa 488, GFP) for 2hrs at room temperature. Fish were rinsed and stored in PBST at 4ºC for imaging using a Zeiss microscope. For fish that were tagged with a sonic hedgehog probe during *in situ*, 1:4000 acetylated tubulin antibody was used as a positive control for immunohistochemistry stain success.

RESULTS

Effect of OPAHs on CYP1A1 protein expression

Immunohistochemistry (IHC) was conducted on the two selected OPAHs, 7,12-B[a]AQ and BEZO, to determine CYP1A protein expression. Studies comparing the two selected 4-ring OPAHs, found that 7,12-B[a]AQ induced CYP1A1 expression significantly throughout the vascular system and this differed greatly from BEZO which had no visible expression of CYP1A using IHC. Significant malformation levels due to 7,12-B[a]AQ exposure consistently occurred at approximately 5µM with mortality above 20µM. Although malformations were different, BEZO exposure led to similar malformation levels at 5µM with mortality above 20µM (Swanson *et al.,* HHMI, 2012, Goodale *et al.,* 2013). Since minimal malformations occurred at 10µM, this concentration was chosen for use in future studies involving 7,12-B[a]AQ and BEZO and their pathways of toxicity.

Effect of OPAHs on CYP1A1 protein expression in AHR2-null zebrafish

A novel AHR2-null fish line for use in studies examining the toxicity of environmental pollutants such as PAHs was characterized at OSU's Sinnhuber Aquatic Research Laboratory. These zebrafish have a mutation in the gene *ahr2*^{hc3335} which is a gene that codes for the aryl hydrocarbon receptor 2 (AHR2) protein. The mutation causes the AHR2 receptor to become non-functional.

Immunohistochemistry was performed using AHR2-null fish (Goodale *et al.,* 2012) that were exposed to a range of concentrations of 7,12-B[a]AQ and BEZO for comparison to toxicity with AHR2 wild type fish. The results demonstrated that CYP1A

expression in fish exposed to 7,12-B[a]AQ is dependent on AHR2 (Swanson HHMI, 2012). Although only 7,12-B[a]AQ substantially induced CYP1A, both compounds illustrated similar degrees of malformation and markers of oxidative stress. A comparison between wild type and AHR2-null fish determined that absence of AHR2 rescued the developmental effects caused by exposures to both 7,12-B[a]AQ and BEZO (Goodale *et al.,* 2013).

OPAH influence on gene expression

An RNA sequence analysis of the zebrafish genome concluded that 7,12-B[a]AQ induces the expression of xenobiotic metabolizing genes, such as cyp1a, with more prominence than BEZO; however, BEZO exposure led to modest increase in cyp1a expression. Unlike the differential expression of CYP1A in B[a]AQ and BEZO, other genes, particularly in the class of redox-homeostasis, were consistently expressed in both OPAH exposure groups.

Based on RNA-Seq and a CuffDiff analysis regarding fish exposed to 7,12-B[a]AQ and BEZO, four genes were highlighted that were significantly affected by exposure to both compounds compared to control. Two of the four genes, *Whey Acidic Protein, follistatin/kazal, immunoglobulin, kunitz, and netrin domain containing 1* (*wfikkn1*) and *cathepsin L1c* (*ctsl.1*), were upregulated due to both OPAH exposures. The other two genes, *plac8 onzin related protein 3* (*ponzr3*) and *plac8 onzin related protein 4* (*ponzr4*), were downregulated in each OPAH exposure (Table 1).

BEZO	Log2 FC	7,12 B[a]AQ	Log2 FC
cyp1a	1.83141	cyp1a	7.80887
ctsl.1	1.62755	ctsl.1	2.44051
ponzr4	-4.41946	ponzr4	-2.27156
ponzr3	-3.408	ponzr3	-3.342
wfikkn1	2.242	wfikkn1	5.076

Table 1. Using RNA-sequencing, four genes were selected as potential new biomarkers of OPAH AHR-dependent toxicity
Paired-end RNA sequencing was conducted using an Illumina HiSeq 2000 sequencer. Differentially expressed transcripts in 7,12-B[a]AQ and BEZO-exposed animals were identified using Cuffdiff. Log2 fold changes for the genes selected in this study are depicted in the table above and correspond to changes in mRNA expression compared to 1% DMSO control fish.

qRT-PCR analysis of gene expression

To confirm the results of RNA-seq at 48hpf as well as determine gene expression at other time points, qRT-PCR was used with specific primers for each gene of interest. As depicted in Figure 1a, ctsl.1 had an increase in expression ($p<0.001$) in 7,12-B[a]AQ-exposed fish at 48hpf, in agreement with RNA-seq, however, ctsl.1 was not significantly upregulated in BEZO treated fish. At 30hpf, there was a similar trend with even higher ctsl.1 induction in 7,12-B[a]AQ-exposed fish ($p<0.001$). Surprisingly, at 24hpf ctsl.1 expression was decreased compared to controls in both BEZO ($p<0.01$) and 7,12-B[a]AQ ($p<0.05$) treated fish. Preliminary studies at 72 and 96hpf (Figure 1b) suggest that expression lowers after the spike at 30 and 48hpf, however concrete conclusions about the time course of expression cannot be formed based only on technical replicates of samples with a sample size of one.

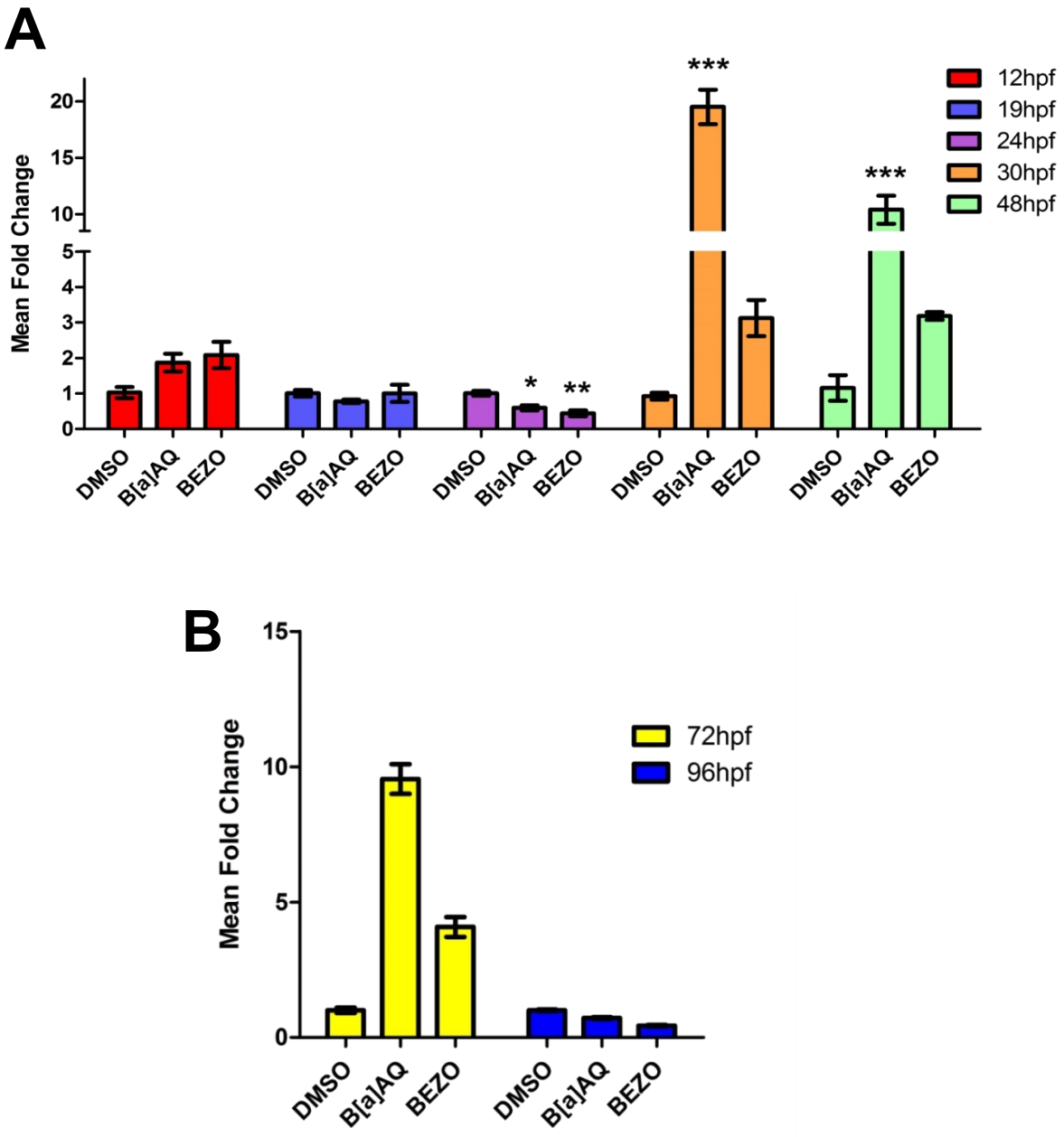

Figure 1. Differing ctsl.1 expression during early stages of development
RNA was extracted from all fish exposure groups using a total-RNA RNAzol procedure and then converted into cDNA for a SYBR® Green qPCR reaction. mRNA expression in all exposure groups were analyzed for ctsl.1. **A)** n=3 **B)** n=1. (One-way ANOVA with a post hoc Dunnett's test (N=3). *p<0.05, **p<0.01, ***p<0.001)

Wifikkn1 gene expression varies between time points and exposure groups. At 24hpf, 7,12-B[a]AQ caused wfikkn1 induction ($p<0.05$, Figure 2). Unexpectedly, at 30hpf BEZO-exposure resulted in a significant ($p<0.05$) downregulation of wfikkn1 expression compared to control. Significant upregulation of wfikkn1 gene expression at 48hpf in 7,12-B[a]AQ and BEZO-exposed fish from the RNA-seq results could not be confirmed because a larger sample size was needed.

Due to error in pipetting, qRT-PCR will need to be repeated to determine gene expression changes at different stages of development for ponzr3 and ponzr4 (data not shown).

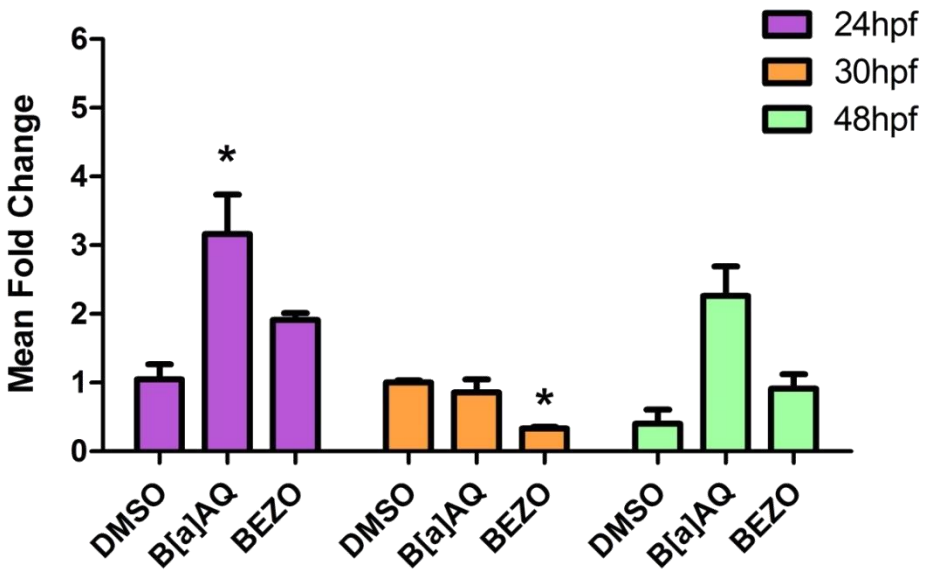

Figure 2. Differing wfikkn1 expression in each exposure compound during early stages of development
24 and 30hpf were analyzed with n=3. The experiment will need to be repeated to confirm if there is significance in gene expression compared to control at 48hpf n=2.

Localization of mRNA transcripts specific to each gene of interest

Whole-mount *in situ* hybridization was used to localize ctsl.1, wfikkn1, ponzr3, and ponzr4 mRNA in fish exposed to 1% DMSO, 10µM 7,12-B[a]AQ, and 10µM BEZO. The focus of this study centered on ctsl.1. Localization using Fast Red Stain and BM Purple was conducted on a series of ages of zebrafish including 24, 48, 72, and 96hpf. The location of ctsl.1 mRNA transcript origin changed over development and there appeared to be some slight qualitative differences in location between exposure groups.

At 48hpf, fish from each exposure group had a slightly different mRNA pattern. In DMSO-exposed fish, ctsl.1 transcripts were located throughout the heart and brain region, and at specific pinpoints along the trunk of the fish and at different points in the tail. Upon closer examination, the florescence was primarily present in the blood cells (pinpoints) along the trunk to the tip of the tail. A similar pattern of blood cell localization of ctsl.1 mRNA in the tail was found in 7,12-B[a]AQ-exposed fish. In addition, 7,12-B[a]AQ appeared to cause some transcript localization of ctsl.1in the snout and brain area, however, the fluorescence was spotted instead of a general "glow" in that area as with DMSO-exposed fish (Figure 3). BEZO exposure caused a localization of mRNA expression in the heart and had very few blood cells fluoresce with the transcript present compared to control and 7,12-B[a]AQ.

In order to localize mRNA expression, a more sensitive colorimetric stain, BM Purple, was used for wfikkn1. The age of choice based on initial expression data for *in situ* hybridization analysis was 24hpf. Control DMSO-exposed fish exhibited very little to no purple stain throughout the body. In contrast, fish exposed to both 7,12-B[a]AQ and

BEZO had strong localization of wfikkn1transcript in the eyes, snout, spinal cord, and throughout the brain (Figure 4).

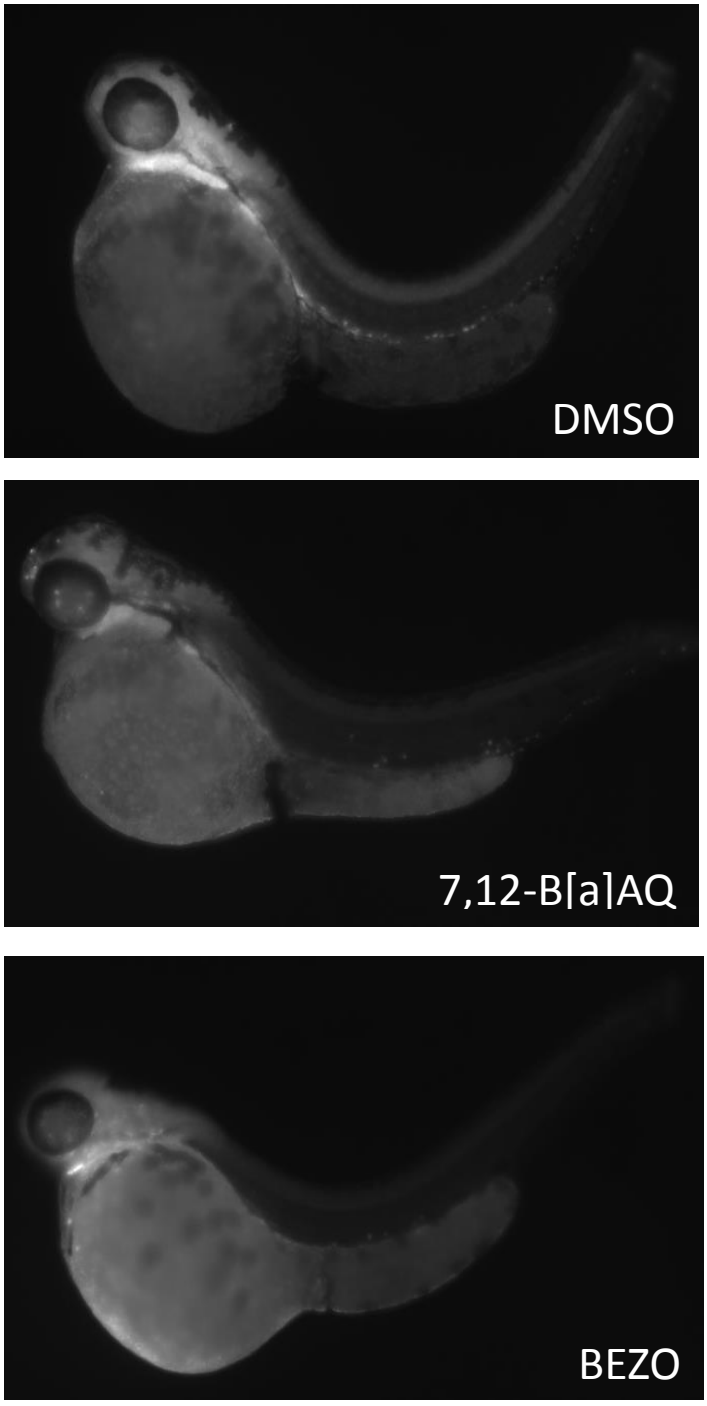

Figure 3. Localization of ctsl.1 mRNA using *in situ* hybridization
48hpf fish were exposed to 1%DMSO, 10µM 7,12-B[a]AQ, or 10µM BEZO. *In situ* fish were stained with Fast Red stain (rhodamine) for ctsl.1.

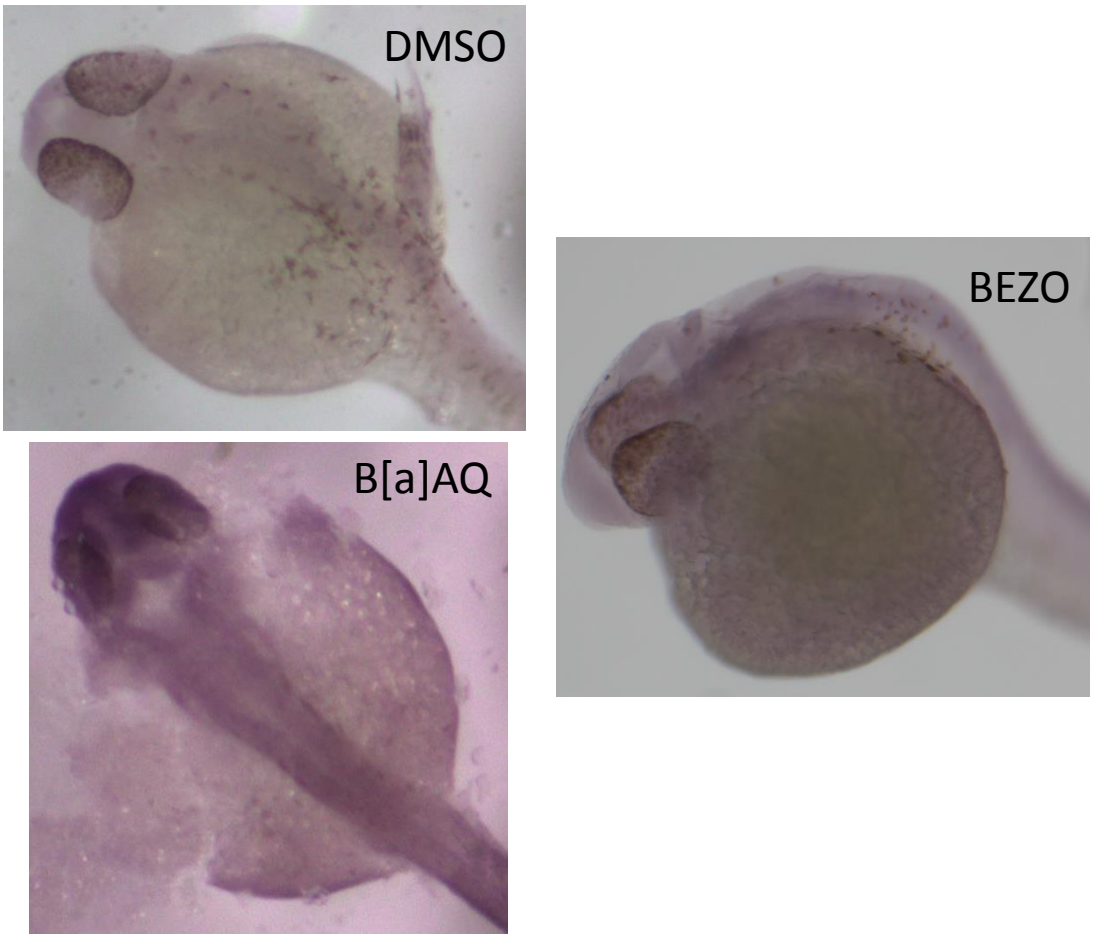

Figure 4. Localization of wfikkn1 mRNA expression
24hpf embryos were exposed to 10µM 1%DMSO, 7,12-B[a]AQ, or 10µM BEZO and used for i*n situ* hybridization and stained with BM Purple.

Initial studies on the localization of ponzr3 and ponzr4 of mRNA expression were conducted in control fish. Ponzr3 and ponzr 4 had comparable mRNA transcript locations in the tail particularly on the outer edge in DMSO-exposed fish (Figures 5 and 6, respectively). The pinpoint fluorescence pattern appeared to be individual cells, however, a more in depth analysis is needed to determine the exact location and cell type of the transcripts. The effect of OPAHs on mRNA expression of ponzr3 and ponzr4 will be investigated in the future.

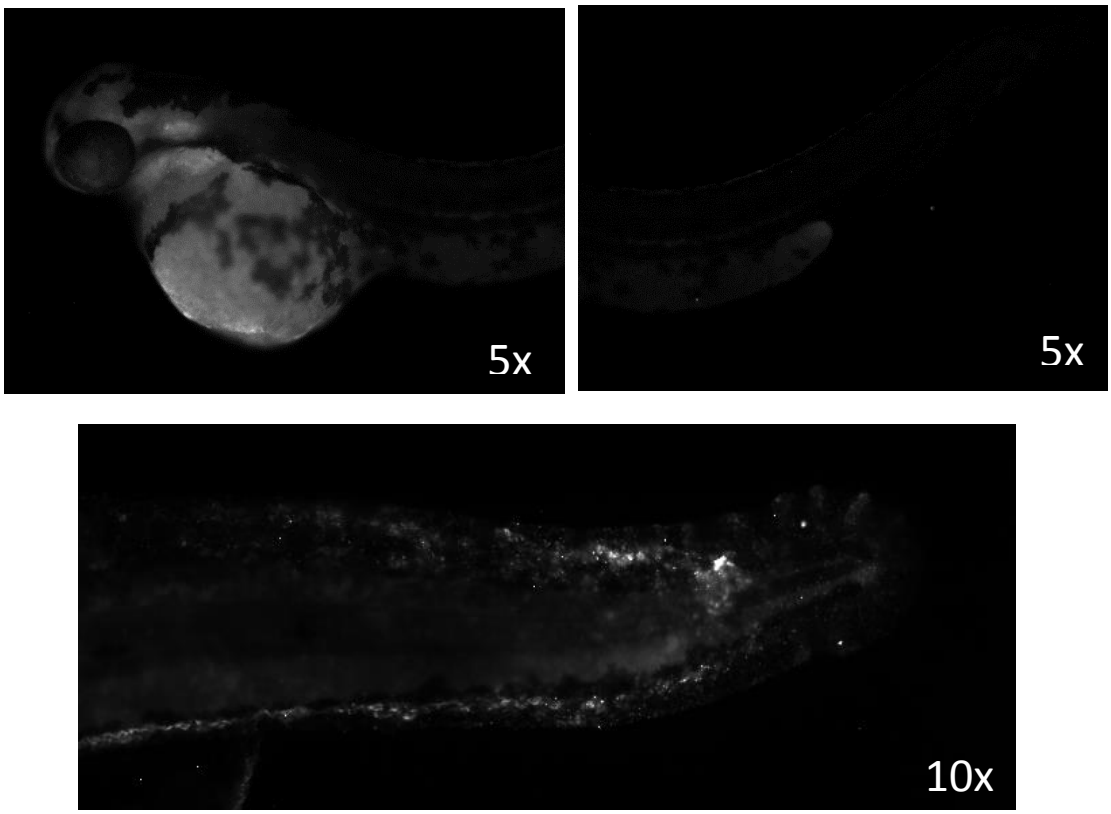

Figure 5. Localization of ponzr3 mRNA
48hpf fish were exposed to 1%DMSO and stained with Fast Red stain (rhodamine) for ponzr3.

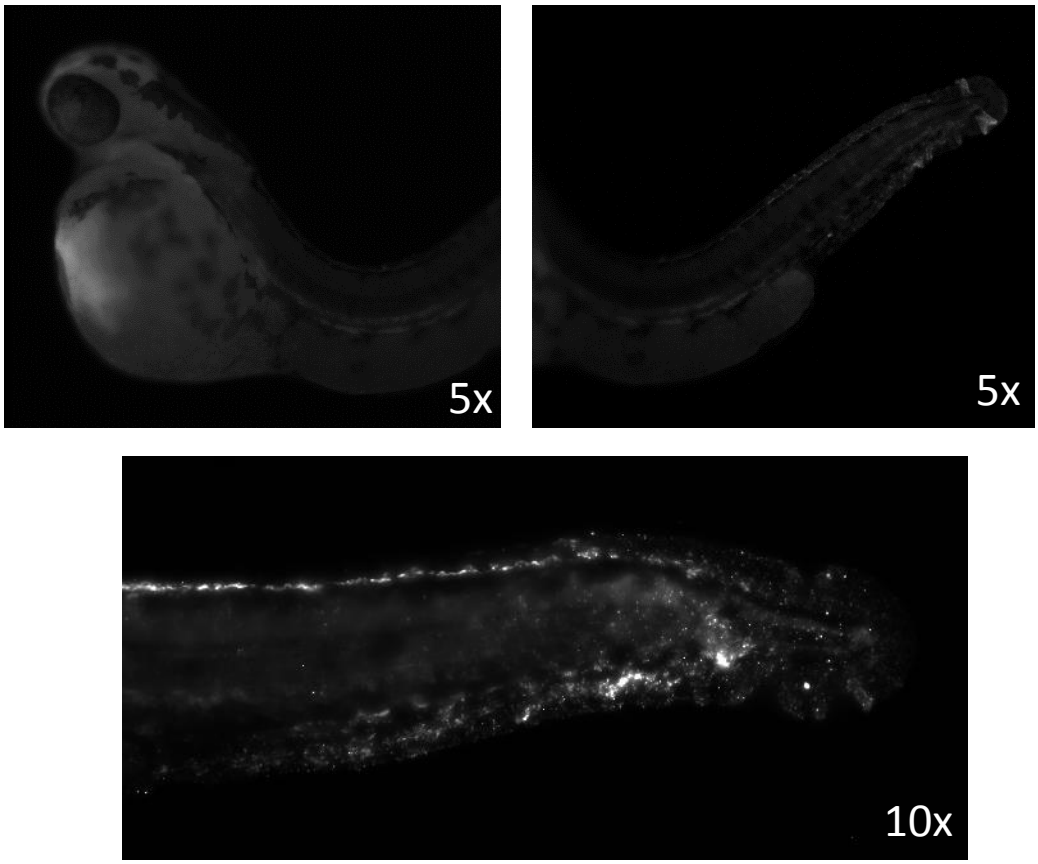

Figure 6. Localization of ponzr4 mRNA
48hpf fish were exposed to 1%DMSO and stained with Fast Red stain (rhodamine) for ponzr4.

DISCUSSION

CYP1A protein has been found to be differentially expressed in zebrafish exposed to two different AHR2-depedent OPAHs. This study centered on identifying potential alternative biomarkers that are more reliable and consistent indicators of AHR-dependent OPAH toxicity than CYP1A expression.

The OPAHs, 7,12-B[a]AQ and BEZO were selected from a screen of 38 OPAHs for toxicity of environmental relevance. The toxicity of these two OPAHs have been found to be AHR2-dependent but have differential cyp1a protein expression, yet they cause similar levels of significant malformations. A study conducted on rats and guinea-pigs ingesting BEZO showed that there was a dose-dependent decrease in glutathione (GSH) and the water-soluble vitamin ascorbic acid in the liver and several other tissues (Dwivedi *et al.,* 2001). It was determined that BEZO may cause oxidative stress possibly through the formation of reactive intermediates and the decrease of GSH and ascorbic acid in the body (Dwivedi *et al.,* 2001). Further studies confirm that BEZO is a potentially harmful compound present in the environment and presents a direct risk to workers at dye manufacturing plants as well as common exposure from air particulates (Dwivedi *et al.,* 2013). A recent study that used guinea-pigs as a model for humans concluded that the reactive oxygen species created from the exposure of BEZO to light may be contributing to photo-contact dermatitis in dye industrial workers (Dwivedi *et al.,* 2013).

These studies identified wfikkn1 as a possible biomarker for AHR2-dependent OPAH toxicity. RT-PCR and *in situ* hybridization techniques revealed that wfikkn1 may

be a good candidate biomarker although significant expression changes appear to be time dependent and yield different affects for each OPAH. A majority of the research concerning this gene, wfikkn1, describe the human version of this molecule. In an experiment done using surface plasmon resonance, human wfikkn1 was shown to bind to growth differentiation factor 11 (GDF11) as well as myosatin with high affinity (Kondás *et al.,* 2011). Myostatin is a member of the TGFβ family and functions as a negative regulator of muscle growth. By binding with high affinity, wfikkn1 acts as an effective antagonist of GDF11 and myostatin. In an investigation using mice, lacking functional myostatin can lead to increased muscle mass (Szláma *et al.,* 2013). Because of this antagonistic property of wfikkn1, this protein is currently being evaluated as a potential agent of antimyostatic therapy and in preventing the deterioration and muscle loss in cancer patients (Szláma *et al.,* 2013). Although some 7,12-B[a]AQ-exposed fish exhibited fast red staining of wfikkn1 in the muscles of the tail at 24hpf, this was not representative in all fish so further experiments are needed with a larger sample size to confirm transcript location in the muscle. Primarily, wfikkn1 mRNA was localized in the brain , eyes, and spinal cord which suggests wfikkn1 may have other functions besides being a myostatin and GDF11 antagonist.

Ctsl.1 was also identified as a potential new biomarker. Out of the four genes of interest, this study confirmed that ctsl.1 is the best candidate biomarker for AHR-dependent OPAH toxicity. Identified as another potential biomarker for OPAH toxicity, ctsl.1, codes for a tissue-specific protein that functions as a lysosomal cysteine protease (PubMed Gene). This protein acts with other members of the cathepsin family to degrade endogenous and exogenous proteins marked for turnover elimination and housed in

lysosomes. Ctsl.1 is particularly important for the degradation of muscle in fish during development, starvation, and migration (Kim *et al.,* 2011). According to another study conducted using zebrafish (Tingaud-Sequeira *et al.,* 2006), RT-PCR results in the form of gel electrophoresis showed bands present, illustrating expression occurring in 120hpf larvae. Expression of ctls.1 was also found in the intestine, kidney, gills, and testis of adult fish. No expression was visible as gel bands at 72hpf or in individual embryonic organ systems. These results directed our investigation toward testing later time points for ctsl.1 instead of stopping at 48hpf as was used in RNA-seq. Younger embryos exhibited mRNA expression in the brain and tail in DMSO and 7,12-B[a]AQ-exposed fish. At later time points, specifically 96hpf, mRNA in fish from all exposure groups was localized in a region of the intestine and kidneys. Although closer examination is needed, this finding supports previous research that ctsl.1 is localized in these areas using RT-PCR in adult fish (Tingaud-Sequeira *et al.,* 2006).

Two other genes, *ponzr3* and *ponzr4*, were significantly down-regulated due to OPAH exposure. To the best of our knowledge, this is the first investigation of the ponzr3 and ponzr4 genes. Preliminary results using *in situ* hybridization suggest that localization of transcript in both ponzr3 and ponzr4 is present in the tail. In a paper published investigating ponzr1, a distantly related gene, in zebrafish, the role of this protein was found to aid in the development of the glomerulus (Bedell *et al.,* 2012). This is a structural part of the kidney that helps maintain homeostasis by filtering blood. Using *in situ* hybridization, Bedell *et al.* localized ponzr1 transcript in the developing kidney and pharyngeal arches. *In situ* also revealed faint ponzr4 mRNA expression in the

pharyngeal arches in DMSO-exposed fish which could be realistic expression, as ponzr1 and ponzr4 are in the same family of genes and may have conserved functions.

This study investigated a series of genes related to redox-homeostasis that are potential candidates as alternative biomarkers for OPAH ARH-dependent toxicity. Although ponzr3 and ponzr4 need more thorough investigation, wfikkn1 and ctsl.1 both exhibited significant gene expression changes resulting from exposure to 7,12-B[a]AQ and BEZO at different stages of development as well as some unique transcript localization between exposure groups. There was differential expression of wfikkn1 and ctsl.1 at multiple time points in fish exposed to 7,12-B[a]AQ and BEZO, as shown using RT-PCR, and this suggests that age and development stage is to be considered with great care when identifying new possible biomarkers for toxicity. Future studies will involve testing a broader range of AHR-dependent OPAHs to determine if ctsl.1, wfikkn1, ponzr3, and ponzr4 have similar expression as seen with 7,12-B[a]AQ and BEZO and will hopefully confirm whether this suite of genes could be useful as biomarkers of toxicity.

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