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Role of Biotransformation in the Developmental Toxicity of Hydroxychrysenes in Early Life Stages of Fish

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by

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Dedication

To my parents, Vera and Toshiya Tanabe and my brothers, Nicholas Tanabe and Ivan Semenov. Thank you for always believing in me and for your endless support.

ABSTRACT OF THE DISSERTATION

Role of Biotransformation in the Developmental Toxicity of Hydroxychrysenes in Early Life Stages of Fish

by

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Dr. Daniel Schlenk, Chairperson

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants that can enter aquatic environments through runoff, atmospheric deposition, accidental discharge, and oil spills. These compounds can be oxidized photochemically or biologically into oxygenated PAHs (oxy-PAHs) which have been shown to be more toxic compared to parent PAHs. The polar properties of oxy-PAHs increase their mobility within the environment which increases the risk of exposure to fauna and flora compared to parent PAHs. Regioselective toxicity has been observed in several oxy-PAHs and the oxidation state of the oxygen on a specific PAH can have dramatic impacts on the toxicity. Previous studies have found that exposure to hydroxychrysenes at critical developmental time-points in fish models impairs red blood cell concentrations in a regioselective manner, with 2-hydroxychrysene (2-OHCHR) being more potent than 6-hydroxychrysene (6-OHCHR). The mechanisms of toxicity of oxy-PAHs are largely unknown and we aimed to characterize the pathways of toxicity of 2- and 6-OHCHR in fish embryos. Our first aim was to characterize the toxic effects in Japanese medaka embryos and to find a

sensitive window of development to hydroxychrysenes. We found that 2-OHCHR caused anemia and mortality in medaka embryos in contrast to in zebrafish embryos, where 2-OHCHR caused only anemia and 6-OHCHR only caused mortality. A sensitive window to 2-OHCHR toxicity was found between 52-100 hpf which closely coincided with liver development. This led us to our second aim, exploring the metabolism and toxicokinetics of the hydroxychrysenes. We found that although 6-OHCHR was taken up 97.2% ± 0.18 more rapidly than 2-OHCHR, it was also eliminated 57.7% ± 0.36 faster as a glucuronide conjugate. Pretreatment with the general cytochrome P450 inhibitor ketoconazole reduced anemia by 96.8% ± 3.19 and mortality by 95.2% ± 4.76 of 2-OHCHR treatments. In addition, formation of the 1,2-catechol was also reduced by 64.4% ± 2.14. However, while pretreatment with the UGT inhibitor nilotinib reduced glucuronidation of 2-OHCHR by 52.4% ± 2.55 and of 6-OHCHR by 63.7% ± 3.19, it did not alter toxicity for either compound. These results indicated that CYP mediated activation, potentially to the oxidatively active metabolite 1,2-catechol, may be driving the isomeric differences in toxicity. Previous studies have found 2-OHCHR to be a four-fold more potent aryl hydrocarbon receptor (AhR) agonist compared to 6-OHCHR. Therefore, in aim 3, we explored the role of the AhR and oxidative stress in 2-OHCHR toxicity. While treatments with the AhR agonists PCB126 and 2-methoxychrysene (2-MeOCHR) did not cause significant anemia or mortality, pretreatments with AhR antagonist CH-223191 reduced anemia by 97.2% ± 0.84 and mortality by 96.6% ± 0.69. AhR inhibition was confirmed by a significant reduction (91.0% ± 9.94) in EROD activity. Thiobarbituric acid reactive substances (TBARS) concentrations were 32.9% ± 3.56 higher ($p<0.05$) in 2-OHCHR

treatments at 100 hpf compared to controls, indicating oxidative stress. Staining with 2',7'-Dichlorofluorescin diacetate (DCFDA) revealed 42.6% ± 2.69 of embryos exhibiting high concentrations of ROS in caudal tissues, which is a site for embryonic hematopoiesis. Both muscle and skeletal tissues were affected, as well as some caudal vasculature. Overall, our findings indicate that AhR may mediate 2-OHCHR toxicity, upregulating CYP and potentially forming the 1,2-catechol that generates ROS in the embryos within caudal tissues, potentially disrupting hematopoiesis leading to anemia and subsequent mortality. Further studies should investigate additional key events and construct adverse outcome pathways for oxy-PAHs.

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List of Acronyms

1,2-CAT	1,2-catechol
1,2-CHQ	1,2-chrysenequinone
2-MeOCHR	2-methoxychrysene
2-OHCHR	2-hydroxychrysene
5,6-CAT	5,6-catechol
5,6-CHQ	5,6-chrysenequinone
6-OHCHR	6-hydroxychrysene
ACN	Acetonitrile
AhR	Aryl hydrocarbon receptor
AKR	Aldo-keto reductase
AUP	Animal use protocol
BaP	Benzo(a)pyrene
BHT	Butylated hydroxytoluene
BLD	Below the limit of detection
BPQ	Benzo(a)pyrene-3,6-quinone
CAT	Catechol
CH	CH-223191
CHQ	Chrysenequinone
CYP	Cytochrome P450
DCFDA	2',7'-dichlorofluorescein diacetate
DI	De-ionized
DMBA	7,12-Dimethylbenz(a)anthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon

EC	Excitation-contraction
EH	Epoxide hydrolase
EPA	Environmental protection agency
ER	Estrogen receptor
EROD	Ethoxyresorufin-O-deethylase
Gluc	Glucuronidase
hpf	hours post-fertilization
HPLC	High performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee
IARC	International Agency for Research on Cancer
Keto	Ketoconazole
MeOCHR	Methoxychrysene
mono-ITP	Mono-substituted isopropylated triaryl phosphate
NAC	N-acetylcysteine
ND	Not detected
NIEHS	National Institute of Environmental Health Sciences
Nil	Nilotinib
NOAA	National Oceanic and Atmospheric Administration
OHCHR	Hydroxychrysene
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SEM	Standard error of mean
SOD	Superoxide dismutase
SPE	Solid phase extraction

Sulf	Sulfatase
SULT	Sulfotransferase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCDD	2,3,7,8 -Tetrachlorodibenzo-p-dioxin
UDP	Uridine diphosphate
UGT	UDP glucuronosyltransferase
US	United states
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
Vit E	Vitamin E
XRE	Xenobiotic response element

Chapter 1: Introduction

1.1 Oil and PAHs in the Environment

1.1.1 Oil Spills

Over the past 60 years, there have been over 44 major oil spills affecting U.S. waters (NOAA, 2017). The Torrey Canyon and Santa Barbara oil spills of 1967 and 1969 were some of the earliest in U.S. history and have consequently pioneered the efforts to study the effects of oil spills on the environment (Teal and Howarth, 1984). The 1969 crash of the barge Florida in Falmouth, Massachusetts resulted in a spill of roughly 630 tons of No. 2 fuel oil (Teal and Howarth, 1984). The spill caused immediate kill of local fauna which were extensively documented. The 1976 Argo Merchant spill in Nantucket resulted in 7.7 million gallons of No. 6 fuel oil entering the waters (Grose and Mattson, 1977). The damage control and cleanup efforts were both failures as most, if not all of the cargo entered the waters, and the spread of the oil could not be contained. The Exxon Valdez oil spill of 1989 in the Prince William Sound was at the time the largest oil spill in U.S. history. This event was a major turning point in increased scientific efforts to evaluate the risks of crude oil to embryos and juvenile fish. Before this event, it was thought that crude oil largely caused toxicity to fish through acute mortality following direct contact with the organism. It is now known that crude oil can cause developmental abnormalities in fish at concentrations much lower than those that were previously considered to be safe (Short, 2003). This spill instigated the development of toxicity

testing using both juvenile and adult fish in assessing risks of crude oil in the aquatic environment. In 2010, an explosion on the Deepwater Horizon drilling rig in the northern Gulf of Mexico caused a large spill of crude oil at a depth of 1522 meters (Beyer et al., 2016). The spill lasted for 87 days and released over 3 million barrels of oil, overtaking the Exxon Valdez spill as the largest oil spill in U.S. history (Beyer et al., 2016). The Gulf of Mexico is a prolific fishery in the U.S. that is abundant in ecologically and economically important fish species, such as mahi-mahi (*Coryphaena hippurus*), bluefin tuna (*Thunnus thynnus*), and yellowfin tuna (*Thunnus albacares*) (Gibbs and Collette, 1959; Palko et al., 1982; Muhling et al., 2012; Rooker et al., 2012). Furthermore, the spill occurred in April which coincides with many of these important pelagic species' habitat utilization and spawning patterns (Arocha et al., 2001; Block et al., 2005; Rooker et al., 2012). These factors made the location and time of the spill to be disastrous, with the economic and ecological effects still being evaluated to this day. Lastly, developing fish embryos have been shown to be particularly sensitive to crude oil exposure, with the heart being the most commonly affected organ (Incardona et al., 2012). Consequently, there is great interest in better characterizing the effects of crude oil on fish development.

1.1.2 PAHS in Crude Oil

Polycyclic aromatic hydrocarbons (PAHs) are the most studied components of crude oil. They can cause toxicity through several pathways that seem to be distinct based on the number of rings they contain (Incardona et al., 2004). While crude oil can greatly vary in chemical composition depending on its geological source (Kerr et al., 1999;

Wang et al., 2006), PAHs typically make up roughly 0.5-1.5% of crude oil composition by weight (Aas et al., 2000; Deepthike et al., 2009) and one or two ring species are more abundant compared to PAHs with larger number of rings (Saeed and Al-Mutairi, 2000). However, lower molecular weight PAHs and single ring compounds tend to be more volatile and transition out of the water column compared to larger molecular weight PAHs. This causes the PAH profile in the water column to shift overtime. Loh et al (2017) have observed that immediately following the Wu Yi San oil spill, two ring PAHs were the most abundant species but after 4 days, three ring PAHs were the major component. The oil from the Deepwater Horizon spill was composed of 74% saturated hydrocarbons, 16% aromatic hydrocarbons, and 10% polar hydrocarbons (Reddy et al., 2012). PAH profiles in surface waters at the Deepwater Horizon spill site collected from May 9-16, 2010, were 9.65% two ringed PAHs, 79.18% three ringed PAHs, 10.7% four ringed PAHs, and 0.54% PAHs with five rings or more (Diercks et al., 2010). It should be noted that the water samples for this study were analyzed several weeks after the spill began which could yield a much different profile compared to that of day 0 of the spill.

Crude oil may consist of more than 20,000 compounds and parent PAHs are a small fraction of the mixture (Marshall and Rodgers, 2004). In addition to parent PAHs, there are alkylated, nitrated, and oxygenated variants, as well as those containing nitrogen, sulfur, and oxygen heteroatoms in the ring system, each having unique biological effects (McElroy et al., 1989). The United States Environmental Protection Agency (US EPA) currently lists 16 priority pollutant PAHs which pose the most significant threat to human health. The original list was created in 1976 and research

groups focus on the fate and effects of these PAHs only because they are listed by the US EPA as priority PAHs. Given the advancements in analytical technologies and a growing body of research detailing higher toxicities of PAH variants, metabolites, or degradates, there has been a push for an updated list of priority PAHs which includes substituted and heterocyclic PAHs (Andersson and Achten, 2015).

1.2 Fate of PAHs

1.2.1 Water Solubility

The solubility of PAHs in water is mainly determined by their hydrophobicity. The most commonly used measure of hydrophobicity is the octanol-water partition coefficient, or K_{ow}. Generally, higher molecular weight PAHs have lower water solubility (higher K_{ow}) and partition into organic carbon. A review of 24 most commonly observed PAHs found a range of K_{ow} values of almost four orders of magnitude (Meador et al., 1995), suggesting that certain PAHs may be more soluble and, thus, more bioavailable to organisms in the water column. Two ringed PAHs such as naphthalene are the most water soluble and make up the largest fraction of PAHs in the water column immediately after an oil spill. These PAHs are also the most volatile and, as a result, mostly removed from the water column through volatilization (Gros et al., 2014).

1.2.2 Sediments

Due to their hydrophobicity, PAHs tend to be adsorbed to organic carbon constituents and transition into sediments. As a result, a separate partition coefficient, K_{oc}, is often used to predict sediment adsorption of PAHs by normalizing for organic carbon content. Mass balance studies suggest that sediments act as a sink for PAHs and that the net movement of PAHs in the water column is into the sediment as opposed to volatilization (Gevao et al., 2000; Mackay and Hickie, 2000). Once bound to sediment, bioavailability is diminished, and the time spent in the environment (characterized as half-life) is enhanced. Tansel et al. (2011) reported a half-life three-fold higher for chrysene in sediment compared to that in water, and a thirty-fold higher half-life for pyrene in sediment relative to water. The differences in half-lives may contribute to a shift in PAH profiles overtime, potentially affecting exposure and subsequent risk to organisms. Most cases of PAH bioaccumulation in fish have been observed in benthic or bottom feeding species and the amount of bioaccumulation correlated with PAH concentration in the sediment (Baumann, 1998; Frapiccini et al., 2018).

1.2.3 Microbial Degradation

Microbes play a major role in PAH degradation in the environment. Several bacterial, fungal, and algal species have shown capacities to degrade PAHs, but bacterial and fungal degradation are the most well studied (Ghosal et al., 2016). Microbes can catalyze hydroxylation, as well as both aerobic and anaerobic ring cleavage reactions

(Mallick et al., 2011). Bacteria tend to favor aerobic environments for PAH degradation using oxygenase enzymes, most commonly dioxygenases (Mallick et al., 2011). This pathway is unique from cytochrome P450 oxygenation because dioxygenases form *cis*-dihydrodiols (Mallick et al., 2011), while P450 form *trans*-dihydrodiols (Sutherland et al., 1990; Moody et al., 2004). Bioremediation and bioaugmentation are techniques used to expedite the biological breakdown of PAHs either through the addition of PAH-transforming microbes, nutrients to promote the growth of endemic PAH-transforming microbes, or a combination of the two. These techniques have been utilized throughout several major oil spills, including Exxon Valdez and Deepwater Horizon. However, surfactants are often added to bioremediation products which disperse the oil and increase surface area for bacteria to adhere to but may be toxic themselves and cause deleterious effects to local fauna and flora.

1.2.4 Photolysis

PAHs can also be degraded through photolysis which is a complex series of reactions that can vary between different PAHs. Miller and Olejnik (2001) examined the photolytic degradation of benzo(a)pyrene (BaP), chrysene, and fluorene, and concluded that not only were there differences in the rate of decomposition between the chemicals, but that chrysene and BaP degrade through a different mechanism than fluorene. The degradation rates of BaP and chrysene were affected by pH, inhibited by the presence of tert-butyl alcohol, and decreased in the absence of oxygen, while the degradation rate of fluorene was unaffected by pH and tert-butyl alcohol and degraded faster when the

oxygen was removed. Jacobs et al. (2008) have observed that dissolved organic carbon (DOC) decreases the rate of photodegradation for several PAHs while nitrates increase the rate. This trend has been thought to occur due to light screening by DOC and through generation of hydroxyl radicals from nitrate photolysis. While several chemicals and variables have been studied, there are currently few models predicting photolytic degradation of PAHs. The most commonly produced degradation products are hydroxylated and oxygenated PAHs which some groups believe to be "dead-end" products of PAH degradation (Cerniglia, 1993; Kochany and Maguire, 1994; Cerniglia, 1997). This warrants further investigation into the fate and effects of the degradates in the environment.

1.3. Effects of Crude Oil on Wildlife

1.3.1 Cancer

Crude oil exposure has been shown to be carcinogenic to fish. Brown et al. (1973) observed a higher frequency of tumors in fish from the Fox River watershed, which was contaminated with crude oil, compared to non-polluted waters. Several studies have also found a strong correlation between aromatic hydrocarbons in sediment and liver tumors in demersal fish species in the Puget Sound (Malins et al., 1984; 1985 a&b). The major carcinogenic chemicals in crude oil were determined to be PAHs (GESAMP 1977). Later studies have observed that the covalent binding of PAHs to DNA was a molecular initiating event in carcinogenesis within fish (Farber and Sarma, 1987). Since then,

numerous studies have observed DNA adducts following PAH exposure and have begun using adducts as a biomarker of crude oil exposure (Varanasi, 1989; Sikka et al., 1991; Collier et al., 1993; Ericson et al., 1999; Pampanin et al., 2016). Several groups have later found that there is a positive correlation between PAH concentrations in sediment and hepatic DNA-adducts in wild fish (Stein et al., 1992; Collier et al., 1993).

1.3.2 Reproduction

Reproductive effects have also been observed in fish following crude oil exposure. Abnormal ovarian development was observed in plaice (*Pleuronectes platessa*) that were exposed to crude oil after the Amoco Cadiz oil spill in France (Stott et al., 1983). Johnson et al. (1988) reported similar results in English sole (*Parophrys vetulus*) from PAH contaminated waters of Puget Sound, where the fish had impaired gonadal recrudescence compared to those from a reference site. Thomas and Budiantara (1995) also observed impaired ovarian growth in female Atlantic croaker (*Micropogonias undulatus*) following chronic exposure to water soluble fractions of oil. Ovarian atresia is associated with reduced oogenesis and increased oocyte weight which can decrease fecundity. Decreases in testosterone concentration in blood were also found in crude oil exposed landlocked salmon (*Salmo salarsepago*) and winter flounder (*Pseudopleuronectes americanus*) (Truscott et al., 1983). Reduced plasma estradiol concentrations have also been observed in English sole and dolly varden trout following crude oil exposure (Johnson et al., 1988; Sol et al, 2000). Casillas et al. (1991) reported that gravid English sole (*Parophrys vetulus*) from habitats with higher sediment PAH

concentrations were less likely to spawn and had reduced oocyte viability compared to those from waters with lower sediment PAH concentrations.

1.3.3 Immune Suppression

Immunotoxicity has been observed in fish exposed to crude oil. Juvenile salmon from an urban estuary were compared to a non-urban estuary or hatcheries and were found to have a suppressed immunological memory compared to those from the non-urban estuary or hatcheries (Arkoosh et al., 1991). PAHs were also found to suppress B-cell immunity in juvenile chinook salmon (Arkoosh et al., 1994), as well as increase susceptibility to *Vibrio* bacterial pathogens (Arkoosh et al., 2001). Suppression of macrophage function has also been observed in oyster toadfish (*Opsanus tau*) from urban estuaries (Seeley and Week-Perkins, 1991) as well as after exposure to a single PAH, (Seeley et al., 1997). While several studies have observed deleterious immunological responses, the mechanism by which these effects occur are still not well understood.

1.3.4 Developmental Toxicity

Some of the earliest observations of crude oil developmental toxicity in fishes were after the Exxon Valdez oil spill. Pacific herring larvae from oiled sites had higher incidences of spinal abnormalities, optic malformations, craniofacial deformities, as well as pericardial and yolk sac edema (Kocan et al., 1996 a&b; Marty et al., 1997). Laboratory exposures to crude oil and petroleum-based tar creosote resulted in similar deformities

(Middaugh et al., 1998; Vines et al., 2000; Couillard, 2002). These morphological features have become classic phenotypes of crude oil exposure in fishes and are commonly assessed in oil and PAH research. While several developmental defects have been observed following crude oil exposure, the heart and vasculature have been suggested to be the primary targets of crude oil toxicity in fish with other morphological abnormalities occurring as a consequence of cardiac malformation (Incardona et al., 2004). Brette et al. (2014) have observed cardiac excitation-contraction impairment in juvenile tuna following exposure to crude oil collected from the Deepwater Horizon oil spill. Jung et al. (2013) reported similar results in zebrafish following exposure to crude oil from several different sources, suggesting a conserved cardiotoxic phenotype in fish regardless of the crude oil source. This has been attributed to certain components in all crude oil that have similar water solubility acting through a common mechanism (Incardona et al., 2004). Similar cardiac effects were observed after exposure to three-ringed PAHs such as phenanthrene (Incardona et al., 2004). Cardiotoxicity was proposed to occur through blockage of specific calcium and potassium ion channels in cardiac myocytes (Brette et al., 2017). However, cardiac toxicity has also been observed in herring embryos treated with phenanthrene before hatching after completion of cardiac development, suggesting that PAHs may exert cardiotoxic effects through multiple mechanisms (Incardona et al., 2009). In general, mixtures of PAHs with a higher proportion of three ringed PAHs used to simulate weathered oil were found to cause more cardiotoxicity than at proportions during the beginning of a spill (Incardona et al., 2004). PAHs with more than three rings have been shown to cause very different effects. Pyrene,

a four membered PAH commonly observed in oil spill sites, has been shown to cause loss of peripheral circulation, anemia, and cell death in developing zebrafish (Incardona et al., 2004). Pyrene is an agonist of the aryl hydrocarbon receptor (AhR) and the toxic effects observed with pyrene are very similar to those observed in zebrafish treated with tetrachlorodibenzo-*p*-dioxin (TCDD), a potent AhR ligand (Henry et al., 1997; Belair et al., 2001; Dong et al., 2001; Dong et al., 2002; Teraoka et al., 2002). Furthermore, both chemicals caused toxicity after 3 days post-fertilization, suggesting a similar mechanism of stage-specific developmental toxicity. In contrast, chrysene and other PAHs were not shown to have developmental toxicity in zebrafish or medaka (Diamante et al., 2017; Tanabe et al., 2021).

1.4. Toxicokinetic Properties of PAHs

1.4.1 Absorption

In order for toxicity to occur within biota, the chemical must first be absorbed into the organism. There are several routes of PAH absorption in adult fish. The primary route of uptake from aqueous exposure is through the gills (Thomas and Rice, 1981). Randall et al. (1998) have reported that for chemicals that have a $\log K_{ow}$ between 2 and 6.5, absorption through the gills is the dominant route of uptake in fish when exposed in aqueous environments. Most of the commonly found PAHs in the water column fall within this range and are expected to follow this trend, which is supported by results from Namdari and Law (1996) showing branchial absorption of pyrene to be the dominant

route of uptake into rainbow trout (*Oncorhynchus mykiss*) adults. Another route of entry is through dietary exposure. Several studies have reported low dietary uptake efficiencies for several PAHs, ranging from 2 to 30% in rainbow trout (*Oncorhynchus mykiss*) (Niimi and Palazzo, 1986; Niimi and Dookhran, 1989). However, these studies did not account for metabolism which could have led to underestimations of uptake efficiencies. Furthermore, publications on PAH-like chemicals that are not readily metabolized, such as polychlorinated biphenyls (PCBs), reported higher transfer efficiencies ranging from 38 to 56% (Madenjian et al., 1999). This provides further evidence for underestimation due to metabolism of PAHs. Dermal absorption is another vector of PAHs into the body but varies greatly between chemicals and species and is not considered to be a major route of absorption.

Embryos are thought to absorb PAHs by passive diffusion through the chorion (Hodson, 2017). The rate of accumulation is generally lower in embryos compared to larvae due to a lower rate of transport through the chorion compared to larval gills (Petersen and Kristensen, 1998). Hydrophobic chemicals have been shown to partition into the oil globule in eggs of Atlantic croaker (*Micropogonias undulates*) (Ungerer and Thomas, 1996). Hornung et al. (2007) reported similar findings in medaka embryos exposed to benzo(a)pyrene.

1.4.2 Distribution

PAHs are lipophilic and are expected to be distributed to lipid rich tissues (Sijm and van der Linde, 1995). In adult fish, the highest concentrations of PAHs are often

found in the liver and gall bladder due to metabolites accumulating in the bile. Zhao et al. (2014) reported that the bile had the highest concentration of PAHs in several edible lake fish, while the hepatic tissues had higher concentrations compared to extrahepatic tissues. Interestingly, they found a very weak correlation between PAH concentration and lipid contents in tissues, suggesting that lipid content is not an important factor that determines PAH accumulation within adult fish tissues.

In embryos, the oil globule and yolk are a major sink for lipophilic xenobiotics which then get distributed throughout the embryo as the yolk is absorbed throughout development. Hornung et al (2007) have characterized the toxicokinetics of BaP in medaka embryos which was found to be isolated to the oil globule from 3 hours post-fertilization (hpf) to 24 hpf, after which it was detected in the yolk as well. By 2 days post-fertilization (dpf), the concentration of benzo(a)pyrene dropped in the oil globule and increased in the yolk. By 3-5 dpf, high concentrations were detected in the hepatic biliary system. By day 7, the distribution was similar to 3-5 dpf but with a stronger overall signal. Interestingly, they did not observe a decrease in hepatic biliary benzo(a)pyrene signal until post-hatch, suggesting that while the PAH was being metabolized, it was not being readily excreted.

1.4.3 Metabolism

PAHs undergo both phase I and phase II metabolism in fish, mainly in the liver. Phase I metabolism typically results in bioactivated metabolites which can be more toxic than the parent compound. There are three main pathways of PAH bioactivation: the

cytochrome P450 / epoxide hydrolase (CYP/EH) pathway, CYP peroxidase pathway, and aldo-keto reductase (AKR) pathway. These pathways are summarized in Figure 1 using BaP as a model PAH. In the CYP/EH pathway, a PAH first undergoes epoxidation, mainly by CYP1A, followed by hydrolysis by microsomal epoxide hydrolase to form a dihydrodiol. Another epoxide can be formed adjacent to the dihydrodiol by further CYP-catalyzed oxidation, resulting in a diol-epoxide. If this diol-epoxide is in a bay region of a PAH, it can be particularly mutagenic (Kapitulnik et al., 1978) and is most famously observed in BaP activation. It should be noted that epoxides formed by CYP can sometimes be protonated and collapse into monohydroxylated metabolites before epoxide hydrolases can hydrolyze them into dihydrodiols. These metabolites can still be further epoxidized or hydroxylated which can result in additional hydroxyl groups on the aromatic ring. CYP can also have peroxidase-like activity (Hrycay and Bandiera, 2012) which can catalyze single-electron oxidations of a PAH to produce radical cations that can react with DNA much like diol-epoxides (Cavalieri and Rogan, 1985; Devanesan et al., 1992). In the AKR pathway, a PAH is first metabolized into a dihydrodiol much like in the CYP/EH pathway but instead of further epoxidation, the dihydrodiol is dehydrogenated into a catechol or hydroquinone by AKR. In the presence of dioxygen and reducing equivalents, the catechol or hydroquinone can be autoxidized into a quinone or semiquinone radical, which can form DNA adducts and generate reactive oxygen species. Although not a direct enzymatic metabolism, PAHs can be co-oxidized following peroxidation of unsaturated fatty acids by prostaglandin H synthase where a lipid peroxyl radical can strip a proton and electron from a nearby PAH, resulting in

oxidation (Schirmer et al., 2000). It should be noted that oxidation by CYP and co-oxidation can yield similar products but unique differences in stereochemistry between the two have been observed (Panthananickal et al., 1983). Phase II metabolism plays a major role in detoxification and excretion of PAHs. Once a PAH is hydroxylated by CYP or CYP/EH, it becomes a good substrate for conjugation by UDP-glucuronosyltransferases (UGTs) or sulfotransferases (SULT), yielding large, non-reactive metabolites to be excreted through the bile. Electrophilic metabolites like epoxides, radical cations, and semiquinone radicals can be conjugated by glutathione-S-transferases into non-reactive metabolites which can also be excreted through the bile. Quinones can also be reverted to catechols or hydroquinones by DT diaphorase, with subsequent glucuronidation and/or sulfation.

PAHs are readily metabolized in adult fish. Metabolic enzymes can be induced by the binding of PAHs to AhR, which can then bind to specific response elements and upregulate the transcription of CYPs, as well as phase II enzymes such as SULT and UGT. The majority of PAH metabolism occurs in the liver, with CYP1 enzymes being the most abundant hepatic isoforms that are induced by PAHs and their metabolites (Stegeman 1989; Stegeman and Hahn, 1994). Gills are another important location for metabolism. Andersson and Part (1989) have observed that about 20% of BaP taken up through rainbow trout gills was metabolized locally, mostly to dihydrodiols. Several metabolites, such as dihydrodiols, quinones, phenols, glucuronides, and sulfates have been characterized in many different fish species (summarized in Tan and Melius, 1986; Ikenaka et al., 2013).

Embryos were previously thought to have limited metabolic capacities. However, several studies show that PAHs can indeed be metabolized in fish embryos. Binder and Stegeman (1984) have observed that metabolic capacity is present at every developmental stage following the onset of circulation in killifish embryos. Furthermore, several metabolites of 7,12-dimethylbenz(a)anthracene have been characterized in rainbow trout embryos (Fong et al., 1993). CYP induction has been observed as early as 24 hpf in zebrafish embryos which also coincides with the onset of circulation (Andreasen et al., 2002). Hornung et al. (2007) detected metabolites of BaP within the yolk of Japanese medaka embryos as early as 24hpf, with the most pronounced fluorescence signal in the yolk syncytial layer. This provides evidence that the syncytial layer of the yolk is capable of PAH metabolism early in development. The same study (Hornung et al., 2007) has provided evidence for dihydrodiols, as well as sulfate or glucuronide conjugates in medaka embryos at 24hpf. However, mRNA may be derived from maternal influence into oocytes which may be translated into functional enzymes, so embryos may be relying on maternal influence for early-stage metabolism (Hsu et al., 2002).

1.4.4 Excretion

In most cases, PAHs do not bioaccumulate in adult fish and are readily excreted. This can be attributed to both rapid metabolism of the compounds and poor absorption efficiencies through dietary intake (Niimi and Dookhran, 1989). The main routes of excretion in adults are through the gills through sloughing off mucus, gastrointestinal

tract via the bile, and the kidney through urine (Varanasi et al., 1978). Conjugates are mainly excreted through the bile while unconjugated metabolites are excreted primarily through the urine or gills (Varanasi et al., 1978). Several studies have shown PAH tissue concentrations in fish exposed to oil return to background levels after a few days or weeks (Logan, 2007; Jung et al., 2011).

Fish embryos have shown little ability to excrete PAHs. The rate of elimination largely depends on the developmental stage of fishes. Petersen and Kristensen (1998) observed lower elimination rates in zebrafish embryos compared to larvae exposed to naphthalene, phenanthrene, and BaP. The differences in elimination rates ranged from a factor of 3 to 46. Guiney et al. (1980) observed that the half-life of a ^{14}C-labeled polychlorinated biphenyl, a PAH-like compound, in rainbow trout was 231 days throughout the egg and early to mid-sac fry stage, while it was 15 days in the later sac fry stage. These results point to potential bioaccumulation of PAHs within fish embryos which may add risk of developmental toxicity in early life stage fish from oil spills.

1.5. Toxicodynamics of PAHs in Fish

1.5.1 Stage-Specific Toxicity

PAHs are particularly toxic to fish in key early developmental stages. One of the earliest known stages to be affected is the early cleavage stages that develop the dorsal-ventral axis (Cherr et al., 2017). The disruption of the canonical Wnt/β-catenin signaling pathway during this stage results in hyperdorsalized embryos which rarely survive to

hatching. This stage specific signal disruption has been observed in pacific herring and sea urchins, providing evidence that this conserved pathway can be targeted by PAHs across different phyla (Pillai et al., 2003; Fairbairn et al., 2012). However, the most characterized sensitive stage is the looping of the linear tube at the atrioventricular boundary in the teleost heart (Incardona et al., 2004). Several three ring PAHs can disrupt cardiac conduction which can then lead to morphological defects in the heart at later life stages, as well as craniofacial and spinal malformations. Several groups have also observed stage-specific toxicity in Japanese medaka and zebrafish embryos to TCDD (Wisk and Cooper, 1990; Belair et al., 2001). It has been speculated that liver development may contribute to the toxicity in some way (Wisk and Cooper, 1990).

1.5.2 AhR-Dependent Pathways of Toxicity

Since PAHs are considered to be dioxin-like chemicals, studies are warranted to explore if PAHs cause similar stage-specific toxicity as TCDD. While several modes of action are proposed, the major molecular initiating event for large PAHs (4 or 5 rings) has been thought to be the activation of the aryl hydrocarbon receptor (AhR), a transcription factor which regulates several metabolically important genes. Cytochrome P450 monooxygenases (CYPs) are enzymes regulated by AhR that are of particular importance in the metabolism of PAHs. As discussed above, many PAHs have been shown to be more toxic following bioactivation by CYP, the most important being CYP1A, with eventual formation of a “bay region” diol epoxide metabolite (Stegeman and Hahn, 1994). This bay region epoxide has a longer half-life than most epoxides due

to steric hindrance from further metabolism (Wood et al., 1976). This long-lasting epoxide is extremely electrophilic and readily reacts with DNA, forming bulky adducts that can cause mutations during DNA replication (Cosman et al., 1992). These mutations can lead to cancer, especially if they occur in oncogenes, such as p53, which can prevent the damaged cell from entering apoptosis.

PAHs can also be metabolized into catechols and quinones through another activation pathway involving CYP. Catechols and quinones can also form DNA adducts but can also undergo reduction-oxidation (redox) cycling (Figure 2) and generate reactive oxygen species (ROS), which can damage nearby organelles, proteins, lipids, and DNA (discussed in detail later).

PAHs can also cause toxicity through the disruption of endocrine and immune systems. A number of studies have observed PAHs that are strong AhR agonists generally diminish steroid hormone concentrations (Nicolas, 1999; Monteiro et al., 2000; Zhang et al., 2016). While the exact mechanism is unknown, it is proposed that crosstalk between AhR and estrogen receptor (ER) pathways may play a role in this endocrine disruption (Kociba et al., 1978). Some PAHs and their metabolites have also been shown to be direct ER ligands in vitro (van Lipzig et al., 2005; Hayakawa et al., 2007; Wenger et al., 2009). In vitro experiments have observed that BaP induced cell cycle progression and cell proliferation in estrogen-sensitive cells but not in estrogen-sensitive AhR knock-out cells (Hýžd alová et al., 2018). It was found that BaP metabolites, particularly 3-hydroxybenzo(a)pyrene, were responsible for estrogen receptor activation and the subsequent effects on the cell cycle. Effects have also been observed in vivo, such as

increases in vitellogenin levels and inhibition of spermatogenesis in polar cod exposed to PAHs (Tollefsen et al., 2011; Geraudie et al., 2014). Like the endocrine disruption pathways, the immunotoxicity of PAHs has also not been well characterized. Some carcinogenic PAHs have been found to suppress humoral immune response to T-cell dependent antigens (Burchiel et al., 1988; Carlson et al., 2002). Other targets of PAH-mediated immune effects include the bone marrow, thymus, spleen, and lymphoid tissues (Thurmond et al., 1987; Heidel et al., 2000; Burchiel and Luster, 2001). Much like the endocrine disruption pathways, PAH metabolites are thought to be responsible for immunotoxic effects rather than the parent PAHs (Faisal and Huggett, 1993; Reynaud et al., 2001). CYP1A1 can be induced to very high levels in leukocytes and macrophages and may be generating high levels of active metabolites which cause the immunotoxicity (Komura et al., 2001). Several studies have also demonstrably prevented immunotoxic effects of benzo(a)pyrene and dimethylbenz(a)anthracene in spleen cells and T-cells through co-treatment with alpha-naphthoflavone, a CYP1A1/1B1 inhibitor (Kawabata and White, 1987; Ladics et al., 1991), providing further evidence of bioactivated metabolites being responsible for PAH immunotoxicity.

1.5.3 AhR-Independent Pathways of Toxicity

While AhR-mediated pathways play a major role in PAH toxicity, they can also be toxic independent of AhR. Incardona et al. (2005) have observed toxicity in zebrafish embryos treated with three-ring PAHs, phenanthrene or dibenzothiophene, after injection of antisense morpholino oligonucleotides to block the AhR pathway, indicating AhR-

independent toxicity of the PAHs. More recently, a more specific mechanism of toxicity was elucidated for phenanthrene by Brette et al. (2017). Phenanthrene is thought to prolong action potential duration in cardiomyocytes through inhibition of K^+ channels. It is also thought to inhibit L-type calcium channels which reduces the release of Ca^{2+} from the sarcoplasmic reticulum in the cardiomyocyte. Altogether, phenanthrene disrupts excitation-contraction (EC) coupling which causes arrhythmia and contractile failure.

Nonpolar narcosis is another AhR-independent toxicity pathway mostly associated with low molecular weight PAHs. This anesthetic effect was thought to occur through a "target lipid model" where certain lipids were targets of the PAHs and differences in these lipid body burdens explained the variation in narcotic effects between species (Di Toro et al., 2000). However, the model used only three compounds, a two, three, and four ringed PAH with the data for the three ringed PAH being obtained from an unpublished source which could not be verified. Furthermore, there have not been any further studies to confirm that PAHs indeed cause narcosis in fish. There have been results indicating that the volatile, monocyclic arenes found in crude oil can contribute to narcosis, but these compounds are rapidly volatilized out of the water column in the first few weeks after an oil spill and generally not considered to pose much risk to fish (Gros et al., 2014).

PAHs can also induce toxicity in fish through oxidative stress (Figure 2). This is mainly due to quinone formation of PAHs, either through photochemical or biological oxidation. Once a quinone is formed, it can undergo redox cycling which results in continuous generation of reactive oxygen species (ROS). This occurs by non-enzymatic

transfer of an electron from a reducing equivalent, such as NAD(P)H, to the quinone, forming a semiquinone. The semiquinone is a free radical which readily transfers the electron to a dioxygen, yielding a superoxide anion while the semiquinone is reverted to the original quinone or a catechol. This cycle can go on indefinitely as long as there is a supply of dioxygen and reducing equivalents and if the quinone is not metabolized into a catechol or dihydrodiol and subsequently detoxified. However, not all PAH quinones are toxic and some may be more stable than others. *para*-Quinones tend to be more stable, as well as more toxic than *ortho*-quinones (Bolton and Dunlap, 2017). Knecht et al. (2013) observed that while 9,10-anthraquinone was not toxic to zebrafish embryos, 1,4-anthraquinone was very toxic. However, the same study also observed similar levels of toxicity between *ortho* and *para*-quinones of phenanthrene and naphthalene which suggests that the trend may be PAH-specific. This indicates that not all PAH quinones may be capable of generating substantial amounts of ROS and cause oxidative stress. The generated superoxide anion, despite being a free radical, is not highly reactive and does not have a high binding affinity to biological molecules (Nordberg and Arner, 2001). It can, however, contribute to the formation of more reactive radicals. Superoxide dismutases (SODs) can catalyze the removal of superoxide anions by transferring one electron from a superoxide anion to another, yielding dioxygen and hydrogen peroxide. Hydrogen peroxide, while not a radical itself, can lead to the formation of radicals. Furthermore, unlike the superoxide anion, hydrogen peroxide is uncharged. This means that it can readily cross lipid membranes and widen the range of oxidative stress. Hydrogen peroxide can generate a hydroxyl radical which is highly reactive. This can be

accomplished through direct reaction of a superoxide anion with hydrogen peroxide which is known as the Haber-Weiss reaction (Haber and Weiss, 1934). However, this reaction, while thermodynamically favorable, has a very low reaction rate (Halliwell and Gutteridge, 2015). The more favorable reaction is the Fenton reaction, where Fe^{2+} acts as a reducing agent instead of a superoxide anion. It should be noted that superoxide anions can reduce Fe^{3+} to Fe^{2+} which supplies reducing agents to the Fenton reaction and further contributes to the formation of hydroxyl radicals, which can indiscriminately bind to many biological molecules, such as DNA, lipids, and proteins which can cause cell damage and death (Davies, 1995; Hemnani and Parihar, 1998; Hensley et al., 2000; Suematsu et al., 2003). Hydroxyl radicals can also set off a chain reaction of lipid peroxidation through proton abstraction from a polyunsaturated fatty acid (PUFA) (Wagner et al., 1994; Porter et al., 1995). This forms a lipid radical which undergoes spontaneous rearrangement followed by the addition of a dioxygen, forming a lipid peroxyl radical. This lipid peroxyl radical can then abstract a proton from another PUFA. The lipid peroxyl radical becomes a lipid peroxide while the PUFA becomes another lipid radical, which undergoes the same reactions as above and continually generates lipid peroxides and radicals. This proliferating peroxidation of lipids can disturb structural integrity of lipid membranes and disrupt signal transduction involving affected lipids. Although they are less studied compared to lipids, proteins are important targets of ROS as well due to possible inactivation of important enzymes and receptors, as well as signaling pathways involving affected proteins. Lastly, DNA can be damaged by ROS and result in abasic sites, base modifications, and single and double strand breaks. This

can affect transcription and replication of DNA, as well as lead to cancer if the damage occurred in oncogenes and to heritable mutations if the damage occurred in gametes.

1.5.4 UV-Activation of PAH Toxicity

While most of the observed toxic phenotypes in fish following several historical oil spills have been sublethal cardiac effects, embryos in shallower intertidal zones have shown higher rates of morality and tissue necrosis compared to deeper areas. This phenomenon was documented particularly after the 2007 Cosco Busan oil spill in the San Francisco Bay (Incardona et al., 2012). This difference in toxicity has been attributed to several uncharacterized chemicals interacting with natural sunlight which increased their toxicities (Incardona et al., 2012; Alloy et al., 2016; Sweet et al., 2017). Barron et al. (2003) have reported increased toxicity of weathered crude oil to pacific herring (*Clupea pallasii*) embryos and larvae following exposure to either sunlight or UV-A radiation. Richard Lee (2003) has further characterized the mechanism of toxicity, suggesting that solar exposure to PAHs after uptake produces free radicals which can react with oxygen to form reactive oxygen species (ROS), leading to oxidative damage to DNA and other cellular components. Crude oil is a complex mixture composed of over 20,000 compounds with PAHs comprising only a small fraction of the mixture (Marshall and Rodgers, 2004). It is likely that compounds other than parent PAHs are significantly contributing to the toxicity of weathered crude oil and further research is warranted to characterize these toxic components.

1.6. Oxy-PAHs

1.6.1 Sources

Oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) are a polar constituent of crude oil and a weathered product of PAHs. Oxy-PAHs can be formed through photochemical and biological oxidation of PAHs in the environment (Saeed et al., 2011; Esbaugh et al., 2016; Sweet et al., 2017). Chemical oxidation can occur through reactions with singlet oxygens or peroxides, as well as peroxyl and hydroxyl radicals (Miller and Olejnik, 2001). Photooxidation can occur directly or indirectly (Jacobs et al., 2008). PAHs can absorb light directly to form a reactive intermediate which then reacts with ground state oxygen to generate oxygenated products (Miller and Olejnik, 2001). They can also be oxidized indirectly by photooxidation of other components in the water column forming peroxyl and hydroxyl radicals which can, in turn, react with PAHs to form oxy-PAHs (Jacobs et al., 2008). Microbial oxidation of parent PAHs can also contribute to oxy-PAH formation and may aid in their removal from the environment as some species are capable of using them as energy sources (Peng et al., 2008; Seo et al., 2009). However, several groups contend that oxy-PAHs are "dead-end products" of PAH degradation and have potential to accumulate in the environment (Cerniglia, 1993; Kochany and Maguire, 1994; Cerniglia, 1997). Advances in analytical methods have also revealed that oxy-PAHs can be present at concentrations equal to or potentially higher than parent PAHs in aquatic environments, posing potential risk to organisms (McKinney et al., 1999; Tidwell et al., 2016).

1.6.2 Effects

While parent PAHs have been studied for several decades and their genotoxic and carcinogenic potentials well characterized (IARC, 1973; Kapitulnik et al., 1977; Conney, 1982; NIEHS, 1998; Arif et al., 1999), little work has been done on weathered products. While there have been few studies comparing toxicities of parent PAHs and oxy-PAHs in fish, several groups have found oxy-PAHs to be more toxic in human-cell cultures compared to parent PAHs (Zhu et al., 1995; Wang et al., 2011). Kawano et al. (2017) observed developmental toxicity in Japanese medaka embryos exposed to several PAH quinones, each eliciting unique malformations. Knecht et al. (2013) have compared the toxicities of 38 different oxy-PAHs in zebrafish embryos and have found a wide range of sublethal developmental effects, as well as acute lethality. They have also suggested that specific toxic moieties, such as adjacent diones or terminal paradiones, may explain the differences in toxicities between oxy-PAHs. Goodale et al. (2015) observed that some oxy-PAHs cause developmental toxicity directly through activation of AhR while others can interact with other transcriptional regulators to indirectly cause toxicity in an AhR-dependent manner. Knecht et al. (2013) have also observed toxicity in oxy-PAHs that are potent AhR agonists, as well as those that are poor agonists. This suggests that direct AhR activation is not required to cause toxicity by oxy-PAHs. The developmental toxicity of oxyPAHs currently cannot be predicted by number of rings, parent PAH bioactivity, nor hydrophobicity (Geier et al., 2018). However, there is increasing evidence

that certain structural components may explain differences in their toxic effects and potencies (Knecht et al., 2013).

1.6.3 Regioselective Toxicity

There has been increasing evidence of isomeric oxy-PAHs causing unique effects with different potencies and magnitudes of response. Knecht et al. (2013) observed that several isomeric oxy-PAHs, such as 9-, 10-, and 12-hydroxybenzo(a)pyrene each caused a unique suite of toxic effects in addition to overlapping effects with variable potencies in developing zebrafish. Recent studies in the Schlenk group have also explored regioselective toxicities of oxy-PAHs using hydroxylated chrysenes as model compounds. Chrysene is a PAH often detected in high concentrations in the environment (Kerr et al., 1999; Tansel et al., 2011). It is also one of the most persistent PAHs in the water column, having a roughly 10-fold higher half-life compared to pyrene, another commonly found four-ringed PAH (Tansel et al., 2011). Photooxidation of chrysene can result in several products, such as 2-hydroxychrysene (2-OHCHR) and 6-hydroxychrysene (6-OHCHR). Diamante et al. (2017) observed that 6-OHCHR caused significant mortality in zebrafish embryos while 2-OHCHR was less toxic. Sublethal cardiovascular toxicity was also observed for both chemicals, with 2-OHCHR being more potent. Previous studies have shown that 2-OHCHR is a more potent agonist of the aryl hydrocarbon receptor (AhR), with roughly four-fold higher affinity compared to 6-OHCHR and 2-fold higher than chrysene (Villeneuve et al., 2002; Lam et al., 2018). As discussed above, several PAHs exert toxicity through AhR induced enzymes, such as

CYP450s. Thus, 2-OHCHR may be metabolized into a more toxic product in medaka embryos compared to 6-OHCHR. However, *in vitro* studies with estrogen receptor alpha have shown that 2-OHCHR is an estrogen receptor agonist, while 6-OHCHR is an antagonist (Tran et al., 1996; van Lipzig et al., 2005; Hayakawa et al., 2007). Estrogen receptors regulate several important developmental genes, such as vascular endothelial growth factor (VEGF) which plays an important role in angiogenesis (Barnabas et al., 2013). Runx1 is another gene that has previously been shown to be upregulated in response to 2-OHCHR exposure (Diamante et al., 2017). This gene plays a major role in hematopoiesis and its product protein has been shown to affect estrogen receptor alpha-mediated gene regulation (Stender et al., 2010). This suggests that 2-OHCHR may cause cardiovascular toxicity through activation of estrogen receptors.

1.7 Overview of research aims and hypotheses

This research aims to better characterize the mechanisms of toxicity of oxy-PAHs and their risks to aquatic organisms. Oxy-PAHs have been shown to be more toxic than parent PAHs and their isomers could have different potencies. Previous studies have shown regioselective toxicities between 2-OHCHR and 6-OHCHR in zebrafish embryos (*Danio rerio*), with 2-OHCHR causing anemia and 6-OHCHR causing mortality (Diamante et al., 2017), but their mechanisms are still largely unknown. Preliminary studies in Japanese medaka embryos (*Oryzias latipes*) have indicated that 2-OHCHR caused both effects while 6-OHCHR causes neither. Furthermore, the longer

developmental time of Japanese medaka made it possible to characterize stage-specific effects and better characterize their mechanisms.

In chapter 2, we characterize the dose-dependent and stage-specific toxicities of 2- and 6-OHCHR in embryonic Japanese medaka. Identification of a sensitive window of development provided insight into the mechanisms of toxicity of these compounds and helped understand their environmental risks. This study builds on the findings of Diamante et al. (2017) and further characterizes 2- and 6-OHCHR toxicity. This chapter was published in *Aquatic Toxicology* (Tanabe et al., 2021).

In chapter 3, we explored the metabolism of 2- and 6-OHCHR following the discovery of a sensitive window of development in chapter 2 which coincided with liver development. Phase I and phase II metabolites of both compounds were extracted from Japanese medaka embryos to assess the percent conversion of hydroxychrysenes to each chemical and to search for a toxic metabolite of 2-OHCHR. Toxicokinetics of both chemicals were also evaluated, as well as the role of CYP and UGT on the toxicity of hydroxychrysenes. This chapter is currently in preparation for publication.

In chapter 4, we evaluated the role of AhR and oxidative stress in 2-OHCHR toxicity to embryonic Japanese medaka. Following the findings in chapter 3 of a potential toxic phase I metabolite formed via induction of cytochrome P450 via AhR, we explored the effects of AhR agonist and antagonist pretreatments, as well as antioxidants to complete a toxicity pathway from 2-OHCHR exposure to anemia and mortality in embryonic Japanese medaka. This chapter is currently in preparation for publication.

Overall, our findings highlight the importance of bioactivation and how it can contribute to regioselective toxicities between isomeric compounds.

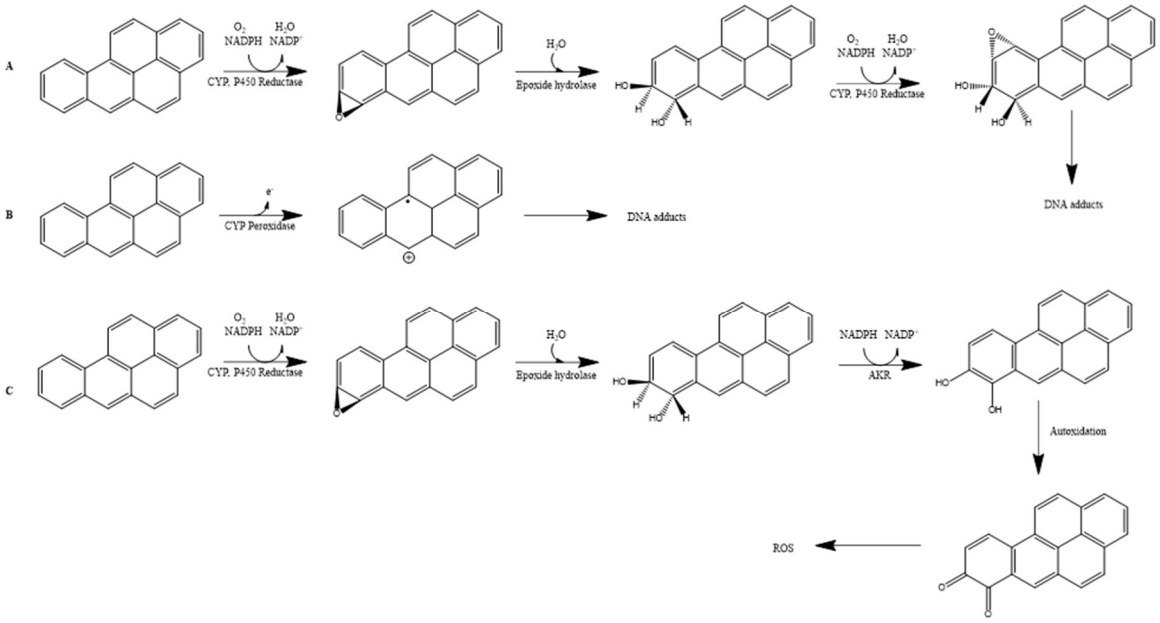

Figure 1-1. The three main activation pathways of PAHs using benzo(a)pyrene as a model PAH. A: cytochrome P450 / epoxide hydrolase (CYP/EH) pathway. B: CYP peroxidase pathway. C: aldo-keto reductase (AKR) pathway. ROS generation is shown in more detail in figure 2.

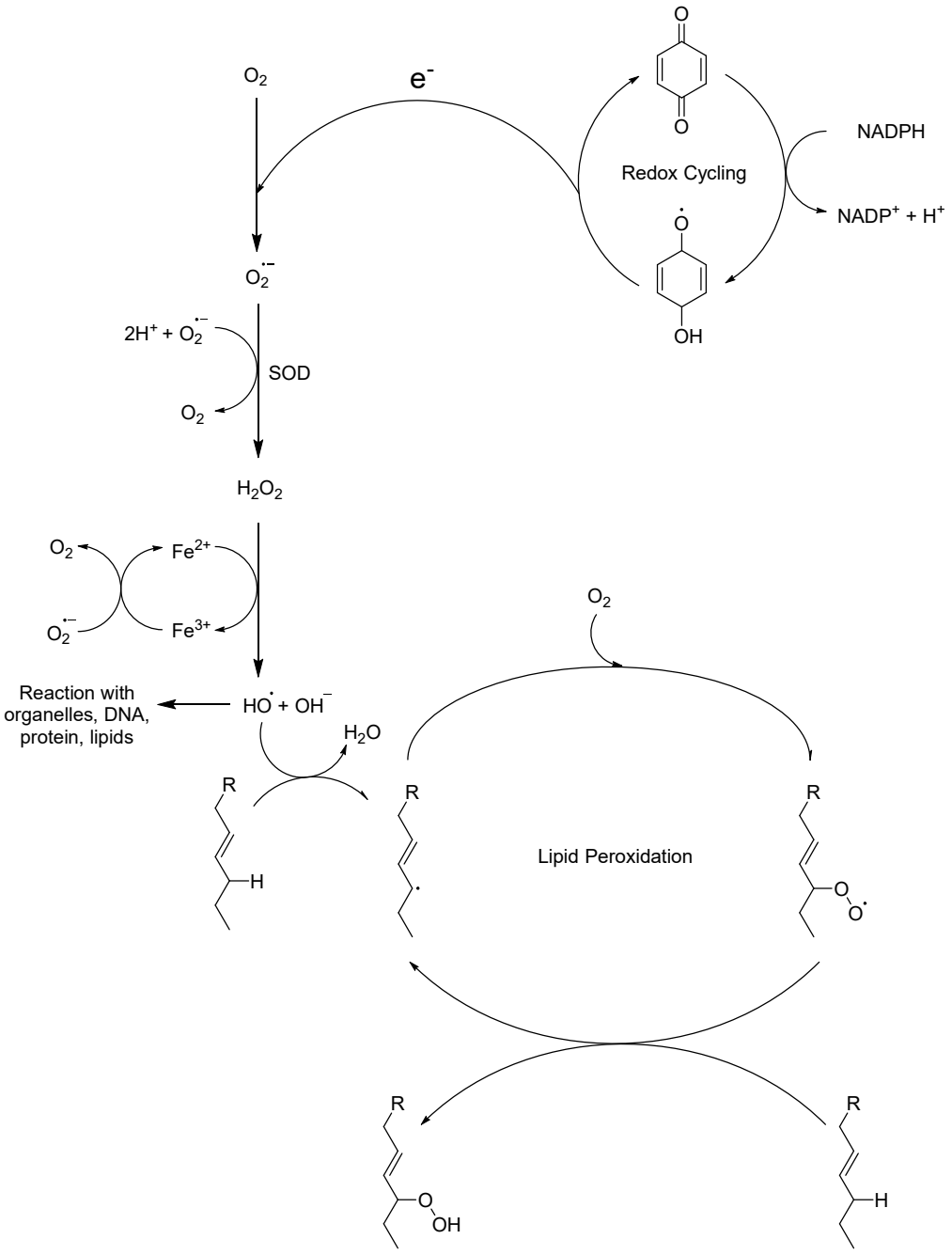

Figure 1-2. A summary of ROS generation by quinone-mediated redox cycling. 1,4-benzoquinone is used as a model quinone but other quinones can undergo the same reactions.

Chapter 2: Stage-dependent and regioselective toxicity of 2- and 6-hydroxychrysene during Japanese medaka embryogenesis

2.1 Abstract

Exposure to oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) at critical developmental time-points in fish models impairs red blood cell concentrations in a regioselective manner, with 2-hydroxychrysene being more potent than 6-hydroxychrysene. To better characterize this phenomenon, embryos of Japanese medaka (*Oryzias latipes*) were exposed to 2- or 6-hydroxychrysene (0.5, 2, or 5 μM) from 4 h-post-fertilization (hpf) to 7 d-post-fertilization. Following exposure, hemoglobin concentrations were quantified by staining fixed embryos with *o*-dianisidine (a hemoglobin-specific dye) and stained embryos were imaged using brightfield microscopy. Exposure to 2-hydroxychrysene resulted in a concentration-dependent decrease in hemoglobin relative to vehicle-exposed embryos, while only the highest concentration of 6-hydroxychrysene resulted in a significant decrease in hemoglobin. All tested concentrations of 2-hydroxychrysene also caused significant mortality (12.2% ± 2.94, 38.9% ± 14.4, 85.6% ± 11.3), whereas mortality was not observed following exposure to 6-hydroxychrysene. Therefore, treatment of embryos with 2-hydroxychrysene at various developmental stages and durations was subsequently conducted to identify key developmental landmarks that may be targeted by 2-hydroxychrysene. A sensitive window of developmental toxicity to 2-hydroxychrysene was found between 52-100 hpf, with a 24 h exposure to 10 μM 2-hydroxychrysene resulting in significant anemia and mortality. Since exposure to 2-

hydroxychrysene from 52-100 hpf, a window that includes liver morphogenesis in medaka, resulted in the highest magnitude of toxicity, liver development and function may have a role in 2-hydroxychrysene developmental toxicity.

2.2 Introduction

The Deepwater Horizon oil spill of 2010 released over 3 million barrels of oil into the Gulf of Mexico (Beyer et al., 2016). This event occurred at the same time and place where several ecologically and economically important pelagic fish species spawn (Arocha et al., 2001; Block et al., 2005; Rooker et al., 2012). Developing fish embryos have been shown to be particularly sensitive to oil exposure, with the heart being the most commonly affected organ (Brette et al., 2014; Incardona et al., 2013, 2014). Exposure to sublethal concentrations of crude oil during early life stages of fish has been shown to result in reduced fitness at later life stages, potentially leading to a decline in fish populations (Claireaux et al., 2013; Incardona et al., 2015; Johansen and Esbaugh, 2017; Magnuson et al., 2018).

Polycyclic aromatic hydrocarbons (PAHs) are toxic components of crude oil and have been primarily responsible for developmental toxicity associated with exposure of early life stage fish to crude oil (Incardona, 2017). PAHs have been shown to cause cardiac, craniofacial, and spinal malformations in developing fish embryos (Incardona et al., 2004, 2014; Sørhus et al., 2016; Vines et al., 2000). These phenotypes have been observed following several historic oil spills, including Deepwater Horizon. However, embryos in shallow intertidal zones have shown higher rates of mortality and tissue necrosis compared

to deeper areas, where sublethal toxicity was due to impairment of heart development and function (Incardona et al., 2012). The difference in toxicity between zones has been attributed to several uncharacterized chemicals interacting with natural sunlight, a transformation that resulted in increased toxicity (Alloy et al., 2016; Incardona et al., 2012; Sweet et al., 2017).

While parent PAHs have been studied for several decades and their genotoxic and carcinogenic potentials are well characterized (Arif et al., 1999; Cooney et al., 1982; IARC, 1973; Kapitulnik et al., 1977; NIEHS, 1998), little work has been done on weathered products of PAHs. Oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) can be formed through sunlight-driven photochemical and biological oxidation of PAHs in the environment (Esbaugh et al., 2016; Saeed et al., 2011; Sweet et al., 2017). Advances in analytical methods have revealed that oxy-PAHs can be present at concentrations equal to or higher than parent PAHs in seawater (Aeppli et al., 2012; Tidwell et al., 2016) and the developmental toxicity of oxy-PAHs currently cannot be predicted by number of rings, parent PAH bioactivity, nor hydrophobicity (Geier et al., 2018).

Chrysene is a four-ring PAH often detected at high concentrations in the aquatic environment (0.1 – 42,710 ng/L) and crude oil compared to other high molecular weight PAHs (Adeniji et al., 2019; Mojiri et al., 2019; Tansel et al., 2011). It is also one of the most persistent PAHs in the water column following oil spills, having a roughly 10-fold higher half-life in the water column compared to pyrene, another commonly observed 4-ring PAH in aquatic environments (Tansel et al., 2011). Photooxidation of chrysene may result in several products, including 2-hydroxychrysene (2-OHCHR) and 6-

hydroxychrysene (6-OHCHR). Biological oxidation to 2-OHCHR has been observed in the marine fungus *C. elegans* (Pothuluri et al., 1995) as well as chrysene dihydrodiols in fish, with the 1,2-dihydrodiol being the major metabolite (Jonsson et al., 2004). Diamante et al. (2017) observed that 2-OHCHR caused circulatory defects in zebrafish (*Danio rerio*) embryos, whereas 6-OHCHR did not induce these effects. In contrast, 6-OHCHR caused significantly higher mortality than 2-OHCHR at similar concentrations.

Therefore, to further explore the mechanisms of toxicity of 2-OHCHR and 6-OHCHR, embryonic exposures using Japanese medaka (*Oryzias latipes*) were conducted and the following hypotheses tested: (1) There is a difference in the proportion of Japanese medaka embryos that present an anemic phenotype following treatment with 2-OHCHR or 6-OHCHR, (2) There is a difference in mortality following treatment with 2-OHCHR or 6-OHCHR, and (3) There is a specific developmental stage and target initiating the anemic phenotype following OHCHR treatment.

2.3 Materials and Methods

2.3.1 Chemicals

2-hydroxychrysene (>99% purity, Toronto Research Chemicals, Ontario, Canada), 6-hydroxychrysene (>99% purity, MRIGlobal, Kansas City, MO), chrysene (≥98% purity, EMD Millipore, Billerica, MA,) and butafenacil (≥98% purity, Millipore-Sigma, St. Louis, MO) were dissolved in 100% DMSO and stored at -20°C in dark conditions. Exposure

solutions were made by diluting stock solutions to 1% DMSO for all controls and treatments.

2.3.2 Maintenance of Medaka Culture

Adult Japanese medaka (*Oryzias latipes*) were maintained under the UCR Institutional Animal Care and Use Committee (IACUC)-approved protocol (AUP#20190017). Embryos were collected 1 h after the lights turned on. Embryos were evaluated for viability under a microscope and sorted for the correct stage following Iwamatsu et al. (2004) for stage-specific morphological characteristics. Medaka were maintained at 28°C on a 12 h:12 h light:dark cycle.

2.3.3 Exposure Regime

Thirty embryos at 4 hpf were selected from those previously sorted and placed in plastic petri dishes (50 x 15 mm) for exposure. Three replicate petri dishes were utilized, each dish containing 30 embryos. Exposure solutions were made fresh daily by dilution of stock solutions to 1% DMSO at appropriate nominal concentrations. The embryos were exposed to 0.5, 2, and 5 μM 2-OHCHR, 6-OHCHR, or chrysene from 4 hpf to 7 dpf. A vehicle control of 1% DMSO water and a positive control of 5 μM butafenacil (Leet et al., 2014, 2015) underwent the same exposure regime. The concentrations of hydroxychrysenes and chrysene were determined based on previous work in zebrafish (Diamante et al., 2017). The 1% DMSO concentration was used to sufficiently solubilize high concentrations (up to 100 μM) of PAHs used for preliminary exposures. This concentration of DMSO did not

affect mortality nor hemoglobin concentrations. Exposures were terminated at 7 dpf for hemoglobin staining and imaging. Exposure water was sampled at 4 hpf and at 7 dpf to determine initial and final water concentrations of 2- or 6-OHCHR.

Embryos were exposed to 5 μM 2-OHCHR during multiple developmental stages based on Iwamatsu et al. (2004). "Stage-to-stage" exposures occurred from 13-15, 38-41, 82-95, or 95-101 hpf, which coincide with stages 13-14 (Early gastrula stage – Pre-mid-gastrula stage), 22-23 (appearance of heart anlage – formation of tubular heart), 30-31 (blood vessel development – gill blood vessel formation stage), and 31-32 (gill blood vessel formation stage – formation of pronephros and air bladder), respectively. "Stage-to-7dpf" exposures occurred from 13-172, 38-172, 82-172, 95-172 hpf, which coincide with stages 13, 22, 30, and 31 to 7 dpf, respectively. Exposures were also conducted from 4-28 (1 d), 4-52 (2 d), 4-76 (2 d), and 4-100 (4 d) hpf which coincide with 1, 2, 3, and 4 d exposures, respectively. Embryos were also exposed to 10 μM 2-OHCHR for 24 h from 4-28, 28-52, 52-76, 76-100, or 100-124 hpf. All exposures were conducted from 4 hpf to 7 dpf with partial exposures of 2-OHCHR for the durations listed above, and at 1% DMSO water for the rest of the duration (Supplemental Figure 1).

Embryos were observed under transmitted light using an Accu-Scope 3000 microscope on a daily basis to quantify number of embryos with anemia or mortality. Embryos with anemia were characterized by no visible moving blood cells. Mortality was characterized by no observable heartbeat.

2.3.4 o-Dianisidine Staining

Following exposure to 0.5, 2, or 5μM 2-OHCHR, 6-OHCHR, chrysene, or butafenacil for 7 d, embryos were fixed, and their hemoglobin stained following a previously established protocol (Leet et al., 2014). Embryos were stained in a solution of 0.6 mg/mL *o*-dianisidine, 0.01 M sodium acetate at pH of 4.5, 0.65% hydrogen peroxide, and 40% ethanol at room temperature in the dark for 30 minutes. Embryos were then transferred to 2 mL microcentrifuge tubes, washed with de-ionized water three times, then fixed in a solution of 4% paraformaldehyde at 4°C for 1 h. Following fixation, embryos were placed in a solution of 0.8% potassium hydroxide, 0.9% hydrogen peroxide, and 0.1% Tween-20 for 30 minutes, and the microcentrifuge tube was shaken every 10 minutes. Embryos were then washed with 1X PBS and fixed overnight in 4% paraformaldehyde at 4°C. The following morning, embryos were transferred to and stored in 1X PBS until imaging.

2.3.5 Imaging

All imaging was conducted using a Stereo Scope 1 (Keyence, Itasca, IL) equipped with a Nikon SLR camera. Embryos were chorionated and positioned in a plate of molded agarose with the heart centered in the image. The proportion of blood coverage was quantified for each embryo in the image using ImageJ. Percent blood coverage was determined by dividing the pixel count of visible blood in the image by the pixel count of the whole embryo. Representative images are shown in Supplementary Figure 2.

2.3.6 Chromatographic Conditions

PAH concentrations were analyzed using a Shimadzu Prominence-i LC-2030 HPLC system with fluorescence detection. A Shiseido Capcell Pak C18 column (4.6 x 150 mm) with a Phenomenex SecurityGuard column was used for separation. All mobile phases were degassed by sonication prior to use. An 11-point standard curve ranging from 5-200 ng/mL was used for quantification of 2- and 6-OHCHR. Separation of PAHs was conducted using the following conditions: a flowrate of 1mL/min, 70% MeOH/H_2O for 7 minutes, then a gradient to 100% MeOH until 11 minutes, then 100% MeOH was maintained until 12 minutes, lastly returning to 70% MeOH/H_2O and maintained from 12-15 minutes to re-equilibrate the column. Excitation and emission wavelengths of 269 nm and 378 nm were used, respectively, based on previously published methods (Wheatley and Sadhra, 1998). The retention times of target analytes were 9.52, 10.57, and 13.00 min for 2-OHCHR, 6-OHCHR, and chrysene, respectively.

2.3.7 Statistical Analysis

All statistical analyses were conducted in Rstudio version 1.2.5019. All data were checked for normality and equal variance assumptions by plotting residuals and quantiles of the data sets. As hemoglobin concentration data did not meet assumptions of normality and equal variance, each treatment was compared using a Kruskal-Wallis test followed by a post-hoc Dunn's Multiple Comparisons Test. Percent mortality and percent anemic phenotype data was compared to the control using the Fisher’s exact test. Statistical tests

were conducted using three replicates. A p-value of 0.05 was used for all statistical tests. All figures were generated using GraphPad Prism version 8.4.3.

2.4 Results

2.4.1 Nominal vs. Measured Concentrations of 2- and 6-hydroxychrysene

Water concentrations of 2- or 6-OHCHR were determined at the beginning (4 hpf) and end (7 dpf) of exposures. Results are summarized in Supplemental Table 1. The average measured concentrations of both 2- and 6-OHCHR between 4 hpf and 7 dpf declined by 12.6%. This decline was likely due to a combination of sorption of chemicals to plastic petri dishes and uptake by the embryos. However, exposure water was replaced daily to maintain a water concentration near the nominal concentration.

2.4.2 Toxicity of 2- vs 6-hydroxychrysene

Exposure to all concentrations of 2-OHCHR resulted in significant mortality relative to vehicle-exposed embryos at 7 dpf ($p<0.05$). The control had 2.22% ± 1.11 mortality following exposures while 0.5, 2, and 5 μM 2-OHCHR resulted in a significant increase in mortality of 12.2% ± 2.94, 38.9% ± 14.4 and 85.6% ± 11.3, respectively. No significant mortality was observed from exposures to 6-OHCHR or chrysene. All concentrations of 2-OHCHR and 5 μM 6-OHCHR resulted in significant reduction in hemoglobin concentrations at 7 dpf compared to controls (Figure 1). The control had a hemoglobin concentration of 12.4% ± 0.28 while 0.5, 2, and 5 μM 2-OHCHR had 8.60% ± 0.26, 3.44% ± 0.38, and 1.49% ± 0.41, respectively, and 5 μM 6-OHCHR had 9.08% ± 0.39 (Figure 1).

No significant decrease in hemoglobin concentrations were observed after treatment with all chrysene concentrations. All concentrations of butafenacil resulted in significant reduction in hemoglobin concentrations; 0.5, 2, and 5 μM butafenacil had hemoglobin concentrations of 0.35% ± 0.09, 0.04% ± 0.01, and 0.01% ± 0.002, respectively (Figure 1).

2.4.3 Stage-dependent Toxicity

To identify a sensitive window of exposure, embryos were exposed to 2-OHCHR for several durations at different developmental stages. Percent mortality and phenotype results at 7 dpf are summarized in Table 2. Exposures at 13-15 and 95-101 hpf resulted in a significant increase in the proportion of embryos with anemia at 7 dpf while 38-41 and 82-95 hpf did not (Table 1). None of these exposures resulted in significant mortality, with survival ranging from 87-94%. Exposures from 13-172, 38-172, 82-172, and 95-172 hpf to 5 μM 2-OHCHR resulted in a significant increase in proportion of embryos with anemia as well as mortality at 7 dpf compared to controls (Figure 3). Exposures from 4-52 hpf and 4-76 hpf resulted in a significantly higher proportion of embryos with anemia and mortality at 7 dpf compared to controls (Figure 4). Exposures from 4-100 hpf resulted in 100% mortality by day 6 but had a proportion of embryos with anemia of 83.3% ± 16.7 at day 5. Exposures from 4-28 hpf did not result in significant anemia nor mortality. Embryos were also exposed for 24 h to 10 μM 2-OHCHR beginning at 4, 28, 52, 76, and 100 hpf and exposed to 1% DMSO water until 7 dpf. A significant proportion of embryos with the anemic phenotype and mortality was observed in 28-52, 52-76, 76-100, and 100-124 hpf exposures (Figure 5).

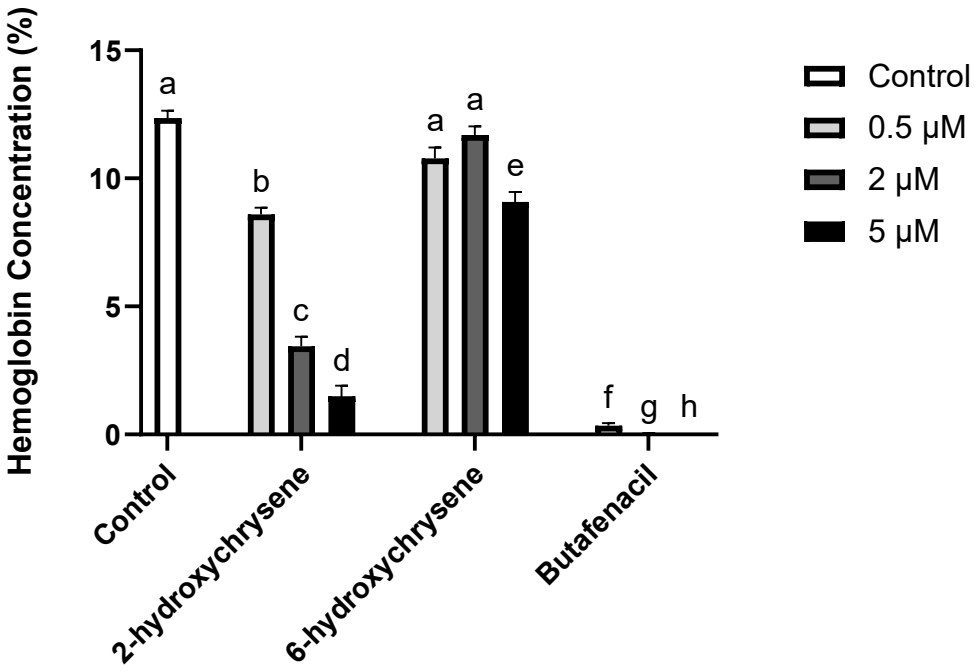

Figure 2-1. Hemoglobin concentrations of medaka embryos stained at 7 dpf after 7 d of exposure to 2-OHCHR. Values represent the mean of three replicates. Letters indicate significance groups revealed by a Kruskal-Wallis test followed by a post-hoc Dunn's Multiple Comparisons Test. Error bars represent + SEM.

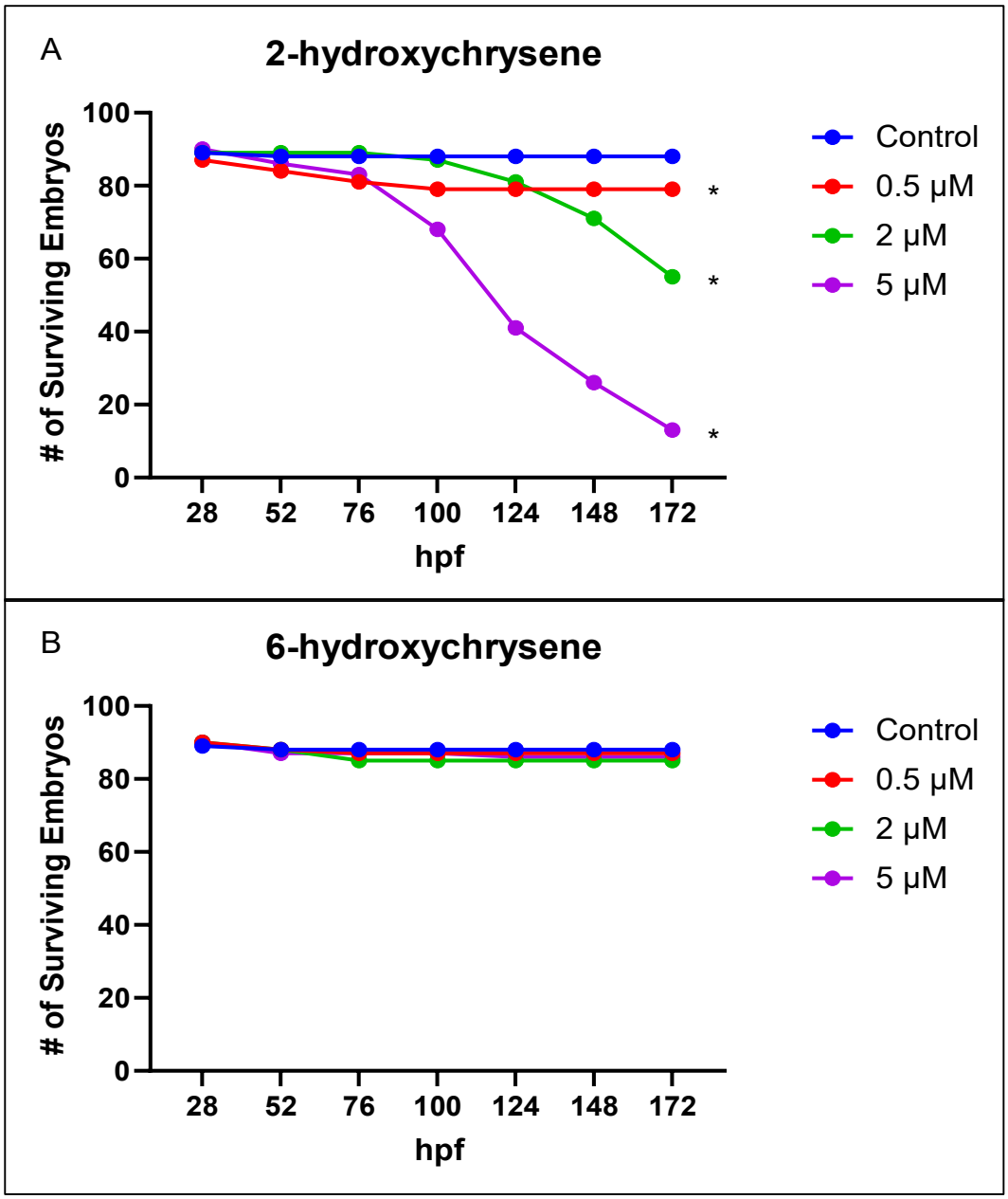

Figure 2-2. Cumulative survival of embryos during exposures to 0.5, 2, and 5 µM 2-OHCHR (A) or 6-OHCHR (B). Asterisks (*) denote significant difference compared to controls at 7 dpf revealed by a Fisher's exact test. Values represent the mean of three replicates.

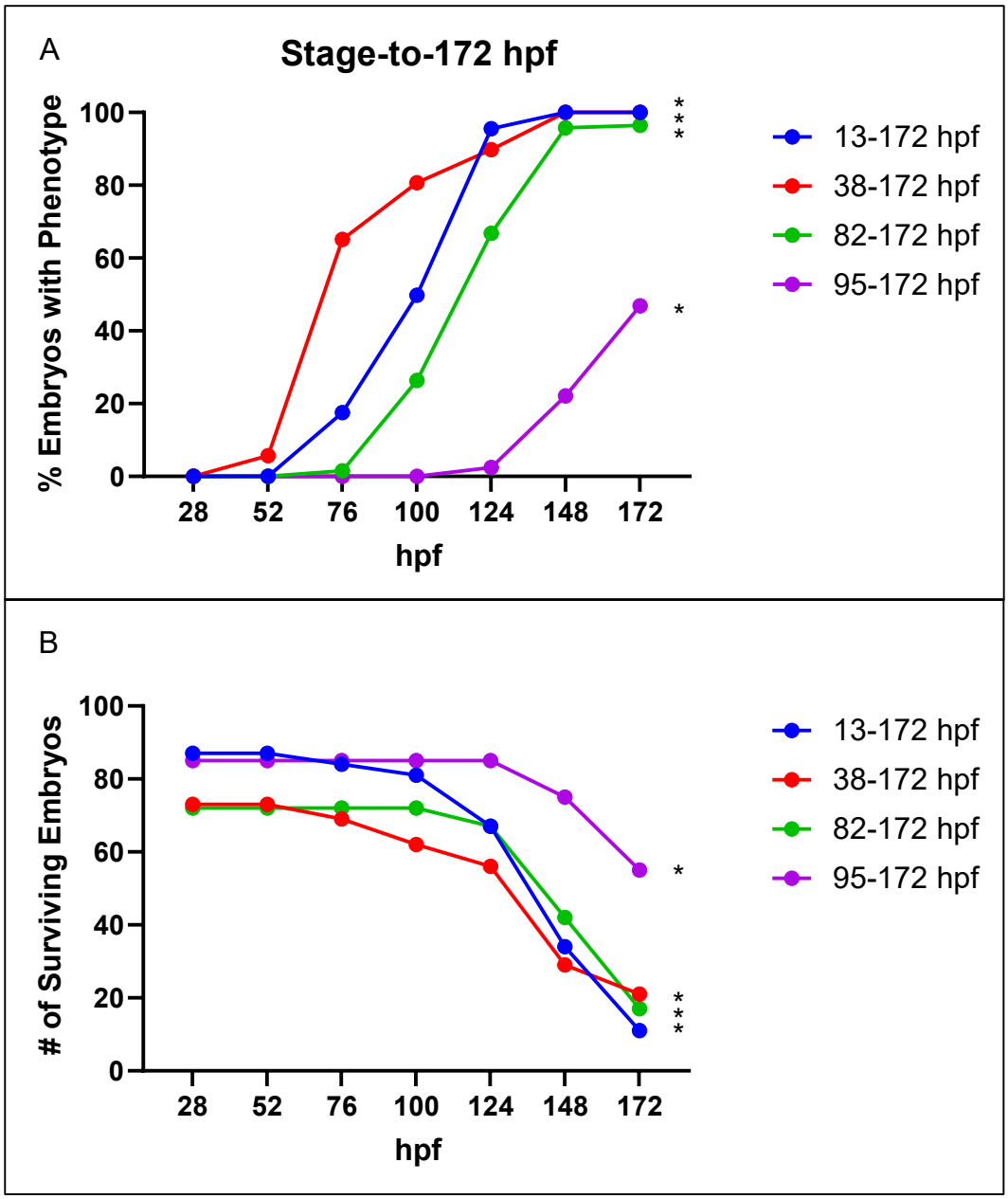

Figure 2-3. (A): Percentage of embryos with anemia during exposure to 5 µM 2-OHCHR from 13-172, 38-172, 82-172, and 95-172 hpf. (B): Cumulative survival of embryos during the same exposures as A. Asterisks (*) denote significant difference compared to controls at 7 dpf revealed by a Fisher's exact test. Values represent the mean of three replicates.

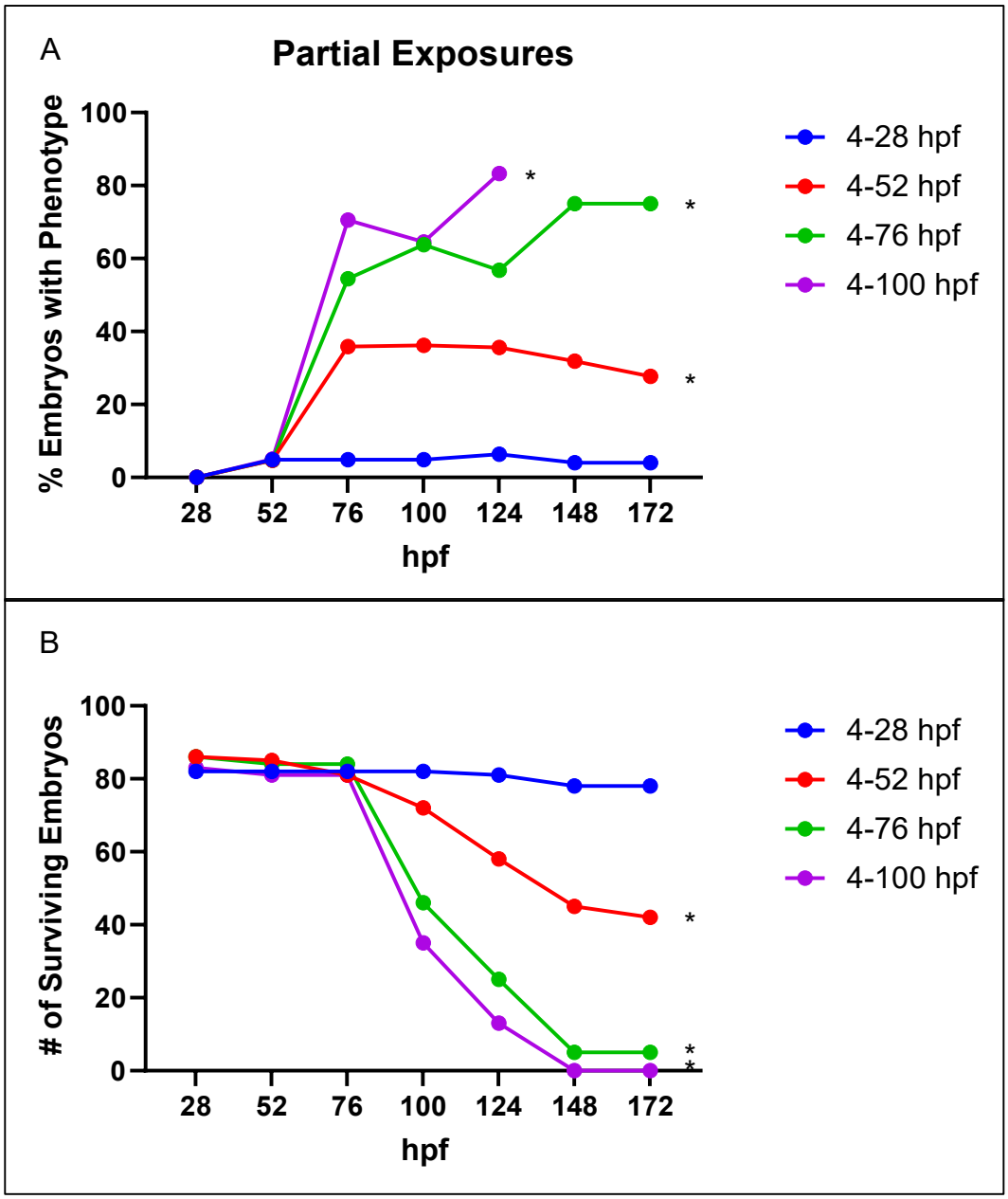

Figure 2-4. (A): Percentage of embryos with anemia during exposure to 5 µM 2-OHCHR from 4-28, 4-52, 4-76, and 4-100 hpf. (B): Cumulative survival of embryos during the same exposures as A. Asterisks (*) denote significant difference compared to controls at 7 dpf revealed by a Fisher's exact test. Values represent the mean of three replicates.

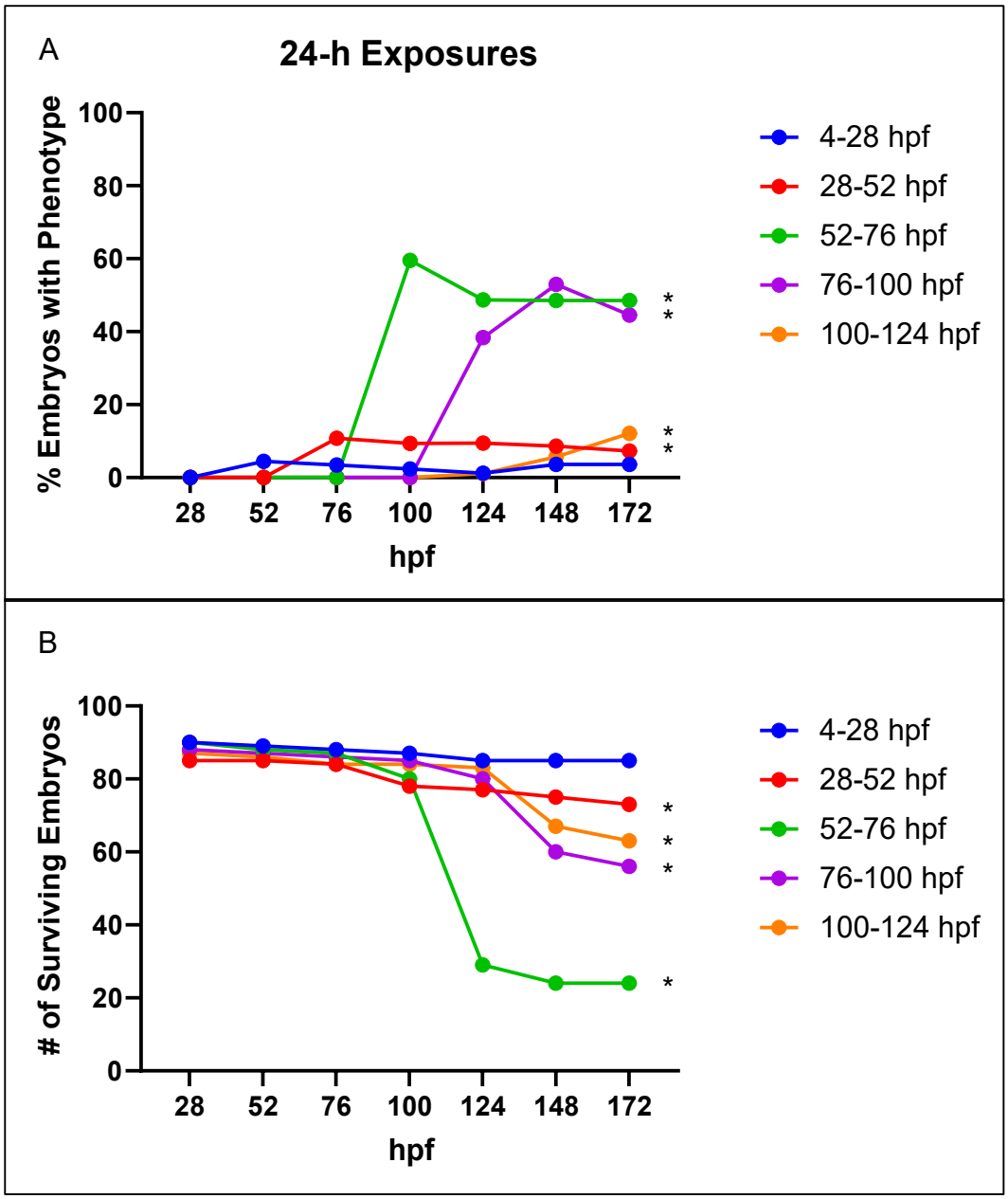

Figure 2-5. (A): Percentage of embryos with the anemic phenotype during exposure to 10 µM 2-OHCHR from 4-28, 28-52, 52-76, 76-100, and 100-124 hpf. (B): Cumulative survival of embryos during the same exposures as A. Asterisks (*) denote significant difference compared to controls at 7 dpf revealed by a Fisher's exact test. Values represent the mean of three replicates.

Table 2-1. Percentage of embryos with anemia during exposure to 5 μM 2-OHCHR from 13-15, 38-41, 82-95, and 95-101 hpf. Asterisks (*) denote significant difference compared to controls at 7 dpf revealed by a Fisher's exact test. Values represent the mean of three replicates ± SEM.

% Embryos with Anemia							
A	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
13-15 hpf	0±0	1.23±1.23	1.23±1.23	3.62±0.04	3.62±0.04	2.38±1.19	4.76±2.38*
38-41 hpf	0±0	1.15±1.15	0±0	2.30±2.30	2.30±2.30	1.19±1.19	1.19±1.19
82-95 hpf	0±0	0±0	0±0	1.19±1.19	1.19±1.19	1.19±1.19	1.19±1.19
95-101 hpf	0±0	0±0	0±0	5.05±1.48	10.66±5.23	7.99±4.34	6.60±2.95*

Table 2. Percent mortality and anemic phenotype of embryos at 7 dpf for all 2-OHCHR exposures. Asterisks (*) denote significant difference compared to controls at 7 dpf revealed by a Fisher's exact test. Values represent the mean of three replicates ± SEM.

	% Mortality	% Phenotype			% Mortality	% Phenotype
13-172hpf	87.8±2.94*	100±0*		13-15hpf	8.89±2.22	4.76±2.38*
38-172hpf	76.7±3.33*	100±0*		38-41hpf	6.67±3.85	1.19±1.19
82-172hpf	81.1±14.2*	96.4±3.57*		82-95hpf	6.67±0	1.19±1.19
95-172hpf	38.9±16.4*	46.9±6.60*		95-101hpf	12.2±4.84	6.60±2.95*
4-28hpf	13.3±3.85	4.06±2.41		4-28hpf	5.56±2.94	3.66±2.14
4-52hpf	53.3±11.5*	27.7±8.89*		28-52hpf	18.9±8.68*	7.30±4.33*
4-76hpf	94.4±4.01*	75.0±25.0*		52-76hpf	73.3±5.09*	48.2±14.9*
4-100hpf	100±0*	83.3±16.7*		76-100hpf	37.8±4.44*	44.6±12.2*
				100-124hpf	7.78±10.7*	12.1±1.38*

2.5 Discussion

Exposure to 2-OHCHR resulted in significant mortality while exposure to 6-OHCHR did not. All concentrations of 2-OHCHR and the highest concentration of 6-OHCHR resulted in a significant decrease in hemoglobin. These results are consistent with previous studies in which 2-OHCHR was more potent than 6-OHCHR at reducing hemoglobin concentrations; however, mortality was higher with 6-OHCHR treatments in zebrafish (Diamante et al., 2017). Jee and Kang (2004) reported that exposure to 1 μM of phenanthrene, a three ring PAH, to adult olive flounder (*Paralichthys olivaceus*) for four weeks similarly resulted in severe anemia. A similar phenotype was reported by Incardona et al. (2004) following exposure of zebrafish embryos to pyrene, a four-ring PAH.

It is unknown why 6-OHCHR caused mortality in zebrafish and not in Japanese medaka. This contradiction suggests the possibility of interspecific differences in oxy-PAH effects, even among teleosts like medaka and zebrafish. While several groups have demonstrated differential toxicity of dioxin-like chemicals to medaka and zebrafish (Elonen et al., 1998; Xu et al., 2018), to our knowledge this is the first study to suggest that there are inter-species, regioselective effects to oxy-PAHs. One possible explanation for the differences in toxicity may be the unique ontogenies of each species. Zebrafish hatch at roughly 48-72 hpf, while medaka hatch at 8-10 dpf (Iwamatsu et al., 2004; Kimmel et al., 1995). Exposure studies for embryonic toxicity assessments are typically terminated at 72 hpf for zebrafish but may go until 7 dpf for medaka.

To better characterize sensitive stages and potential targets involved in anemia, exposures to 2-OHCHR were conducted from various developmental stages until 172 hpf (13, 38, 82, or 95-172 hpf). All treatments resulted in significant anemia and mortality at 172 hpf. Interestingly, anemia was first observed at 52 hpf from 38-172 hpf exposures while 13-172 hpf exposures only resulted in anemia after 76 hpf. It is unknown what could have caused this difference between exposure duration and phenotype which warrants further exploration of sensitive developmental windows.

Embryos were then exposed for short durations (13-15, 38-41, 82-95, and 95-101 hpf). Of these short duration treatments, 13-15 and 95-101 hpf resulted in significant anemia while no mortality was observed. While anemia was observed, the magnitude was much lower compared to other treatment groups. These data suggested that significant uptake of chemical may be required for toxicity to occur. Since limited toxicity was observed, it appears that a longer duration of exposure is required for toxicity. When exposures occurred for 24 h periods from 28-52, 52-76, 76-100, and 100-124 hpf, significant anemia was observed with 3-5-fold higher effects in 52-76 hpf and 76-100 hpf exposures relative to 28-52 hpf and 100-124 hpf. Significant mortality was also observed in all exposures except 4-28 hpf. These results suggest that 52-100 hpf may be a sensitive window of development for 2-OHCHR-induced toxicity. Blood usually appears at 41 hpf and begins circulating at 50 hpf in medaka (Iwamatsu et al., 2004). The observation that exposure after 52 hpf is required for toxicity suggests a target other than initial blood formation is necessary for the phenotype to occur.

One potential target for 2-OHCHR may be the liver which develops in medaka from 52-84 hpf (Iwamatsu et al., 2004). The liver contains PAH-metabolizing enzymes such as cytochrome P450 (CYP). Hepatic CYP1A activity (EROD) increases through embryonic development in medaka, appearing as early as 24-48 hpf and can be induced by the AhR agonist β-naphthoflavone during this period (González-Doncel et al., 2015). CYP1 has been shown to generate oxidatively-active quinones from hydroxylated PAHs which may enhance toxicity of specific compounds (Schlenk et al., 2008). Furthermore, regioselective metabolism has been observed in several cytochrome P450 isozymes (reviewed in Oguri et al., 1994). CYP1A is clearly visible in zebrafish tissue as early as 3 hpf and peaking at 24 hpf (Goldstone et al., 2010). Additional studies are needed to better understand the regioselective differences in uptake and elimination, specifically during these developmentally sensitive periods.

While activation to more toxic metabolites may occur for 2-OHCHR due to liver development, it may also cause toxicity through receptor-mediated mechanisms. Previous *in vitro* studies have determined that 2-OHCHR is a more potent aryl hydrocarbon receptor (AhR) agonist in fish, human, and rat cells, with roughly four-fold higher affinity compared to 6-hydroxychrysene (Lam et al., 2018; Villeneuve et al., 2002). AhRs are found in high concentrations in the liver (Doering et al., 2014) and other AhR ligands have been shown to cause anemia. Belair et al. (2001) reported that 2,3,7,8-tetrachlorodibenzodioxin (TCDD), a potent AhR agonist, disrupted erythropoiesis in zebrafish embryos. Anemia was only found if zebrafish were exposed to TCDD before 96 hpf but after 48 hpf which also corresponds to liver development. Exposures during this window also revealed that while

primitive erythrocytes were unaffected, definitive hematopoiesis was inhibited. This suggests that TCDD disrupts critical processes in erythropoiesis that takes place within this window of development. Wisk and Cooper (1990) found a sensitive window to TCDD toxicity to Japanese medaka at 4-5 dpf which closely coincides with our results. However, treatment with chrysene, which is also an AhR agonist, did not cause abnormal hemoglobin concentrations nor mortality. This suggests that direct AhR activation may not be the sole mechanism of toxicity and that other receptors may contribute to the anemia phenotype.

In addition to AhR, estrogen receptors (ERs) are also prevalent in hepatic tissues and regulate several genes that contribute to blood formation, such as vascular endothelial growth factor (VEGF) which plays an important role in angiogenesis (Barnabas et al., 2013). Runx1 has previously been shown to be upregulated in response to 2-OHCHR exposure (Diamante et al., 2017). This gene plays a major role in hematopoiesis and its product protein been shown to affect ER alpha-mediated gene regulation (Stender et al., 2010). *In vitro* studies have shown that 2-OHCHR is an ER agonist, while 6-OHCHR is an antagonist in yeast, rat, and human cells (Hayakawa et al., 2007; Tran et al., 1996; van Lipzig et al., 2005). This suggests that ERs may also contribute to 2-OHCHR toxicity. While it is possible that distinct mechanisms may be driving 2-OHCHR toxicity, the effects could also be a result of systemic toxicity that is not site-specific. This warrants further studies to evaluate specific mechanisms of 2-OHCHR toxicity.

In summary, regioselective toxicity of hydroxylated chrysenes was observed, with 2-OHCHR being more potent than 6-OHCHR in causing anemia and mortality. While a previous study found that only 2-OHCHR caused reductions in hemoglobin and only 6-

OHCHR caused mortality in zebrafish, we found that 2-OHCHR caused both reductions in hemoglobin and mortality in medaka while 6-OHCHR caused no mortality and anemia at only the highest concentration in medaka. Toxicity of 2-OHCHR was highest at 52-100 hpf, indicating the liver may contribute to the differences in toxicity. These data indicated either transformation to more toxic metabolites or direct interaction with the hepatic targets may play a role in the regioselective toxicity of these compounds. Consequently, further research into the metabolism of 2-OHCHR, as well as other receptor systems are needed to better assess risks posed by oxy-PAHs in the environment.

Chapter 3: Relationships between isomeric metabolism and regioselective toxicity of hydroxychrysenes in embryos of Japanese medaka (Oryzias latipes)

3.1 Abstract

Oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) are ubiquitous contaminants that can be formed through the photochemical or biological oxidation of parent PAHs. The polar properties of oxy-PAHs increase their mobility within the environment which increases the risk of exposure to fauna and flora compared to parent PAHs. Oxy-PAHs can be more toxic compared to their parent compounds and the location and oxidation state of the oxygen on a specific PAH can have dramatic impacts on the toxicity. Our previous studies found 2-hydroxychrysene (2-OHCHR) to be significantly more toxic to embryos of Japanese medaka than 6-hydroxychrysene (6-OHCHR). We have also previously identified a sensitive window of development to 2-OHCHR toxicity in Japanese medaka embryos that closely coincided with liver development, leading us to

hypothesize that differences in metabolism may play a role in the regioselective toxicity. To test this hypothesis, embryos were treated with each isomer for 24 h during liver development (52-76 hpf). Although 6-OHCHR was taken up 97.2% ± 0.18 more rapidly than 2-OHCHR, it was eliminated 57.7% ± 0.36 faster as a glucuronide conjugate. Pretreatment with the general cytochrome P450 inhibitor ketoconazole reduced anemia by 96.8% ± 3.19 and mortality by 95.2% ± 4.76 of 2-OHCHR treatments. In addition, formation of the 1,2-catechol was also reduced by 64.4% ± 2.14. However, while pretreatment with the UGT inhibitor nilotinib reduced glucuronidation of 2-OHCHR by 52.4% ± 2.55 and of 6-OHCHR by 63.7% ± 3.19, it did not alter toxicity for either compound. These results indicate that CYP mediated activation, potentially to the 1,2-catechol, during this stage of development may explain the isomeric differences.

3.2: Introduction

Oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) are ubiquitous contaminants that can be formed through the photochemical or biological oxidation of parent PAHs (Saeed et al., 2011; Esbaugh et al., 2016; Sweet et al., 2017). PAHs enter the aquatic environments through runoff, atmospheric deposition, accidental discharge, and oil spills (Pham and Proulx, 1997; Mackay and Hickie, 2000; Gocht et al., 2007). While few environmental measurements of oxy-PAHs exist, those that have found concentrations to be at similar or even higher than parent PAHs (McKinney et al., 1999; Tidwell et al., 2016). Some groups have suggested that oxy-PAHs are a “dead-end” product that will likely not break down any further which could lead to accumulation as a legacy contaminant

(Cerniglia, 1993; Kochany and Maguire, 1994; Cerniglia, 1997). The polar properties of oxy-PAHs increase their mobility within the environment which increases the risk of exposure to fauna and flora compared to parent PAHs (Wang et al., 2011; Achten and Andersson, 2015). While analytical technologies have improved dramatically over the past few decades, it is still not feasible to measure every oxy-PAH in the environment. Therefore, narrowing the range of oxy-PAHs to monitor to specific compounds would be beneficial for regulators.

In addition, with the potential for enhanced exposure, enhanced lethality, endocrine and developmental effects such as circulatory defects and malformations of the brain, jaw, and eyes have also been shown to be greater in exposures to oxy-PAHs relative to parent PAHs (Knecht et al., 2013; Elie et al., 2015). Given the potential for enhanced formation during remediation events, it is critical to better characterize the toxicity of oxy-PAHs in various environmental media.

Oxy-PAHs have been identified as quinones, carboxylic acids, and hydroxylated compounds with moieties at one or more carbon atoms within the molecule. Interestingly, regioselective toxicity has been observed for multiple isomers of several oxy-PAHs, making it difficult to predict adverse effects (Knecht et al., 2013; Diamante et al., 2017; Tanabe et al., 2021). For example, in zebrafish (*Danio rerio*) 2-hydroxychrysene (2-OHCHR) caused greater anemia in embryos than 6-hydroxychrysene (6-OHCHR), but 6-OHCHR was more lethal (Diamante et al., 2017). However, in Japanese medaka, 2-OHCHR caused both anemia and mortality, but 6-OHCHR caused neither, implying

potential for species-specific effects (Tanabe et al., 2021). In addition, the parent compound chrysene did not cause any toxicity in either species (Diamante et al., 2017; Tanabe et al., 2021).

The toxicity of 2-OHCHR in Japanese medaka embryos closely coincided with liver development (Tanabe et al., 2021). Since the liver contains high concentrations of metabolic enzymes such as cytochrome P450s (CYPs), UDP-glucuronosyltransferases (UGTs), and sulfotransferases (SULTs) (Andreasen et al., 2002; Burkina et al., 2021; Basit et al., 2022), differences in metabolism between the isomers may play a role in the regioselective toxicities of 2- and 6-OHCHR in medaka. Hydroxy-PAHs can be detoxified through conjugation by UGTs and SULTs leading to metabolites that may be readily excreted from the body. Conversely, activation of hydroxylated PAHs may occur through a subsequent hydroxylation of the phenolic compounds by CYP to a catechol which could undergo further oxidation to a quinone, allowing the formation of semi-quinone radicals through one-electron reduction reactions which may generate reactive oxygen species (ROS). In excess concentrations, ROS deplete endogenous antioxidants and cause oxidative stress, potentially leading to damaged proteins, lipids, and DNA (Gant et al., 1988; Guaiquil et al., 2001).

Therefore, to explore the role of metabolism in the toxicity of 2-OHCHR, we investigated the following hypotheses: (1) 2-OHCHR is taken up more rapidly and has a longer embryonic half-life than 6-OHCHR (2); 2-OHCHR undergoes phase I metabolism and activation at a greater rate than 6-OHCHR by CYP; (3); Quinone metabolites of 2-OHCHR

cause greater toxicity than 2-OHCHR or quinone metabolites of 6-OHCHR; and (4); 6-OHCHR undergoes conjugation and detoxification at a greater rate than 2-OHCHR.

3.3 Materials and Methods

3.3.1 Chemicals

2-hydroxychrysene (>99% purity, Toronto Research Chemicals, Ontario, Canada) and 6-hydroxychrysene (>99% purity, MRIGlobal, Kansas City, MO) were dissolved in DMSO and stored at -20°C in a dark environment. Benzo(a)pyrene-3,6-quinone (>99% purity, Toronto Research Chemicals, Ontario, Canada) was dissolved in methanol and stored at -20°C in a dark environment. Exposure solutions were made by dilution of stock solutions to 0.1% DMSO in de-ionized (DI) water. β-Glucuronidase from E. coli (140 U/mg, Millipore-Sigma, St. Louis, MO) and sulfatase from H. pomatia (200 units/mL, Millipore-Sigma, St. Louis, MO) was stored at 4°C in a dark environment. 1,2- and 5,6-quinones of chrysene were synthesized photochemically (ask Kare for method or citation) while the 1,2-catechol of chrysene was synthesized by the reduction of 1,2-quinone using $NaBH_4$ based on the methods of Platt and Oesch (1983). Catechols were identified by fluorescence absorption and mass spectra analyses (see Supplementary Figure 2 for details).

3.3.2 Maintenance of medaka culture

Adult Japanese medaka (*Oryzias latipes*) were maintained under the UCR Institutional Animal Care and Use Committee (IACUC)-approved protocol (AUP#20190017). Collection of embryos occurred one hour after the lights turned on. Embryos were

evaluated for viability under a transmitted light microscope and sorted for the correct stage following staging guidelines from Iwamatsu et al. (2004) for stage-specific morphological characteristics. Medaka were maintained at 28°C on a 14 h:10 h light:dark cycle.

3.3.3 Exposure regime

Thirty embryos at 4 hpf were selected from a previously sorted pool and placed in glass petri dishes (60 x 15 mm) for exposure. Three replicate petri dishes were utilized, each containing 30 embryos. Exposure solutions were made fresh daily by dilution of stock solutions to 0.1% DMSO at appropriate nominal concentrations. The embryos were exposed to 0.5, 3, and 5 μM 1,2-CHQ or 5,6-CHQ from 4 - 172 hpf. A vehicle control of 0.1% DMSO water underwent the same exposure regime. The concentrations of hydroxychrysenes and chrysene-quinones for treatments were determined based upon previous studies (Tanabe et al., 2021).

Ten embryos at 52 hpf were exposed to 5 μM 2- or 6-OHCHR for uptake and depuration analysis. Embryos were exposed for 2, 4, 8, 12, and 24 h for uptake exposures. For depuration exposures, embryos were exposed for 24 h, washed with DI water, then transferred to 0.1% DMSO water in a clean petri dish to allow depuration. Depuration exposures lasted for 4, 8, 12, and 24 h following transfer to water. As mentioned above, the duration and timing of exposures were based on our earlier studies and the timing of liver formation within Japanese medaka (Tanabe et al., 2021).

To explore the role of cytochrome P450s in the toxicity of 2-OHCHR, thirty embryos were exposed to 20 μM ketoconazole, a broad-spectrum cytochrome P450 inhibitor, from 28-52 hpf then to 10 μM 2-OHCHR from 52-76 hpf. Embryos were also pretreated with 10 μM nilotinib, a UDP-glucuronysyl transferase (UGT) inhibitor to assess the effects of glucuronidation on hydroxychrysene toxicity. Embryos (30 pooled individuals) were then transferred to 0.1% DMSO water until 176 hpf. Embryos were washed prior to transfer to new exposure solutions and a new petri dish was used for each exposure to minimize retention of residual chemicals. Ethoxyresorufin-O-deethylase (EROD) activity and measurements of hydroxylated parent compounds within embryos were used to determine the respective efficacy of the inhibitors. To determine if pretreatments with inhibitors provided protection or exacerbated toxicity, anemia and daily mortality were evaluated under transmitted light using an Accu-Scope 3000 microscope as previously described (Tanabe et al., 2021). Anemia was defined by no visible moving blood cells and mortality was identified by no observable heartbeat.

3.3.4 EROD imaging

To measure ethoxyresorufin-O-deethylase (EROD) activity, embryos were exposed to 10 μM 2- or 6-OHCHR and 21 μg/mL 7-ethoxyresorufin (ER) from 52 – 76 hpf. Following exposures, embryos were anesthetized with MS-222, washed with DI water, then transferred to a petri dish with molded agarose for imaging. Resorufin was quantified within embryos using fluorescence microscopy. A Keyence BZ-X710 microscope was used. Embryos were positioned in molded agarose dorsally with the heart centered within

the image. Image analysis was conducted in ImageJ version 1.8.0. The circumferences of the embryos were traced, and the integrated density values of the selection were measured for each embryo. High integrated density values corresponded to high EROD activity.

3.3.5 Sample preparation

To measure metabolism of the hydroxychrysenes in embryos, treatment samples of 30 pooled embryos were extracted by solid phase extraction (SPE). Following exposure from 52-76 hpf, medaka embryos were washed with DI water three times. The water was then removed, and the embryos were dried and weighed before being transferred to a clean 1.5 mL polypropylene conical centrifuge tube. The embryos were flash-frozen in liquid nitrogen then stored at -80°C in a dark environment until extraction.

To identify potential glucuronides or sulfates as putative metabolites, 200 µL DI water was added to the 1.5 mL centrifuge tubes and the embryos were homogenized with a pestle homogenizer for one minute. Glucuronidase (1000 U), sulfatase (1000 U), or 50 µL DI water was then added, and the vial was placed in a temperature-controlled shaker at 37°C for one hour. Methanol (200 µL) and 50 µL of 2 ppm benzo(a)pyrene-3,6-quinone (BPQ) was added as an internal standard to the tube and underwent further homogenization for one minute. The homogenate was then transferred to a glass test tube with the subsequent addition of 500 µL of methanol and 4000 µL of DI water. The final concentration of the homogenate was 15% methanol and 85% DI water.

To enrich putative metabolites from embryonic treatments for chromatographic measurements, Waters Sep-Pak C18 SPE cartridges (3 cc, 1 g sorbent, 55-105 μm particle size) were utilized for extractions on a vacuum manifold. Cartridges were conditioned with 5 mL of methanol then equilibrated with 5 mL of 15% methanol in DI water. Samples were then loaded onto the cartridges then washed with 15% methanol in DI water. Cartridges were then eluted with 10 mL of methanol into 20 mL glass scintillation vials. The eluents were blown down to dryness under a stream of nitrogen gas in a water bath at 40°C. Samples were reconstituted in 500 μL of acetonitrile (ACN), vortexed for 10 seconds, then transferred to autosampler vials for chromatographic analysis. Recovery values ranged from 79-97% based on the total loss of internal standard (BPQ) in each extract.

3.3.6 Chromatographic conditions

A Shimadzu Prominence-i LC-2030 HPLC system with an RF-10AXL fluorometer was used for chromatographic analysis. Samples were injected onto a C18 column (4.6 × 150 mm, Shiseido, Japan) at a flow rate of 1 mL/min. Column temperature was maintained at 40°C. All mobile phases were acidified with formic acid at a concentration of 0.1% by volume. Initial conditions were 30% ACN/H_2O. The chromatographic conditions were as follows: gradient to 60% ACN/H2O from 0 - 25 min, gradient to 95% ACN/H2O from 25 - 27 min, maintain 95% ACN/H2O from 27 - 29 min, gradient to 30% ACN/H2O from 29 - 30 min, maintain 30% ACN/H2O from 30 - 34 min. Retention times of analytes were as follows: 1,2-CAT: 16.575, 5,6-CAT: 17.856, 2-OHCHR: 18.361, 6-OHCHR: 18.842, chrysene: 20.336. Since quinones fluoresce poorly, UV/vis detection at 254 nm was

required for their detection. Their retention times were as follows: 1,2-CHQ: 17.246, 5,6-CHQ: 17.704, BPQ: 18.083. The limit of quantification was 10 ppb for all compounds.

3.3.7 Conversion of 2- and 6-OHCHR

Hydroxychrysenes and quinones were identified based on co-elution with analytical standards using fluorescence, as well as fragmentation patterns using high-resolution mass spectrometry (Supplementary Figure 3-6). Glucuronide and sulfate conjugates were quantified by calculating the difference between parent hydroxychrysene concentrations in extracts treated with glucuronidase or sulfatase and in extracts without deconjugation enzyme treatments.

Percent conversion and mass balance analyses of 2- and 6-OHCHR were conducted after 24 h uptake at the 52 hpf timepoint since liver formation begins to occur at this developmental stage. As noted above, each treatment contained 30 embryos at 52 hpf. Water concentrations after 24 h uptake were compared to initial water concentrations and the difference was assumed to have either entered the embryos or adsorbed to the glass petri dish or to the surface of embryos. To quantify OHCHRs adsorbed to the petri dishes, all water was removed from the dishes, the dishes were dried completely, then washed with 10 mL acetone then 10 mL hexane. The acetone and hexane were collected in a glass scintillation vial, blown to dryness under a stream of nitrogen gas, then reconstituted in 1 mL acetonitrile and transferred to a 2 mL screw thread vial for LC analysis. OHCHRs adsorbed to the surface of embryos were quantified by collecting the water from the

embryo washes which underwent SPE extraction using the same protocol described above. The OHCHRs in the embryos were further divided into parent forms and metabolites, consisting of glucuronides, sulfates, catechols, and quinones based on co-elution with standards.

3.3.8 Uptake and elimination rate constants

Concentrations of 2- and 6-OHCHR were calculated using body burden values from glucuronidase and sulfatase-treated extracts. Due to glucuronides being the major metabolite after 24 h uptake, additional timepoints were sampled for metabolites which allowed the calculations of absorption (K_{ab}) and elimination (K_{el}) rate constants for 2- and 6-OHCHR using body burden values from glucuronidase-treated extracts by linear regression analysis.

3.3.9 Statistical analysis

All statistical analyses were conducted in Rstudio version 1.2.5019 and IBM SPSS Statistics 27. All data were checked for normality and equal variance assumptions by plotting residuals and quantiles of the data sets. Rate constants, body burdens between 2- and 6-OHCHR were compared using two-sample t-tests. Percent mortality and percent anemic phenotype data were compared to the control using a generalized linear model. EROD activities were compared using a two-way ANOVA and a post hoc Tukey HSD test. EROD comparisons utilized thirty replicates while the rest of the statistical tests were

conducted using three replicates. A p-value of 0.05 was used for all statistical tests. All figures were generated using GraphPad Prism version 8.4.3.

3.4 Results

3.4.1 Metabolism of 2- and 6-OHCHR

Approximately 4.22% ± 1.23 of aqueous 2-OHCHR and 9.17% ± 1.03 of aqueous 6-OHCHR was taken up by the embryos after 24 h (Supplementary Table 1). Significant differences in the conversion of parent compound to sulfates, catechols, and quinones were observed between hydroxychrysenes; 68.1% ± 24.1 more 2-OHCHR was converted to sulfate conjugated compared to 6-OHCHR and 370% ± 59.9 more 2-OHCHR was converted to a catechol compared to 6-OHCHR. While 18.0% ± 4.58 of 6-OHCHR was converted to the 5,6-quinone, quinones were not detected in 2-OHCHR extracts. (Table 1.) Figure 1 is a representative chromatogram of an embryonic extract treated with hydroxychrysenes. In addition to catechols and quinones (for 6-OHCHR only), several other metabolites were observed. Based on retention times and mass spectra, these may have been hydroxy-dihydrodiols, triols, or methylated metabolites. They could not be quantified due to limitation of analytical standards.

3.4.2 Toxicokinetics of 2- and 6-OHCHR

No significant differences in body burden were found at 2 or 24 h uptake between 2- and 6-OHCHR (Figure 2). All depuration body burden measurements for 2- or 6-OHCHR were below the limit of detection. However, when samples were treated with glucuronidase or

sulfatase, the resulting hydroxylated compounds allowed the calculation of absorption and elimination rates for the metabolites. Significant differences were found between body burdens of extracts treated with and without glucuronidase or sulfatase at 24 h uptake, as well as during 12 and 24 h depuration (Figure 2). To better estimate rates of uptake and conjugation, glucuronides were measured at additional timepoints (Figure 3). Absorption rates for 2- and 6-OHCHR aglycones were 2.14 ± 0.08 and 4.20 ± 0.29, respectively. Depuration rates for 2- and 6-OHCHR aglycones were 2.16 ± 0.09 and 3.41 ± 0.35, respectively. Significant differences in both absorption and elimination rates were found between 2- and 6-OHCHR ($p < 0.05$) with 6-OHCHR being taken up and eliminated more rapidly than 2-OHCHR.

3.4.3 Toxicity and uptake of 1,2- and 5,6-CHQ

Exposures to 1,2- or 5,6-CHQ did not result in significant anemia or mortality at any tested concentration. Results are summarized in Supplementary Table 1. Body burdens of 1,2- and 5,6-CHQ within embryos following exposure to 3 μM for 24 h from 52-76 hpf were 17.9 ± 1.01 nmol/mg and 9.68 ± 0.77 nmol/mg, respectively.

3.4.4 CYP and UGT inhibitor pretreatments

Significant differences in percent mortality and anemia were found at 100 hpf between 10 μM 2-OHCHR treatments from 52-76 hpf and embryos that were pretreated with 20 μM ketoconazole from 28-52 hpf (Figure 4). Percent anemia and mortality were 96.8% ± 3.19 and 95.2% ± 4.76 lower in ketoconazole pretreatments compared to 2-OHCHR treatments,

respectively. CYP inhibition caused a 64.4% ± 2.14 reduction of 1,2-chrysenediol (catechol), a phase I CYP metabolite of 2-hydroxychrysene (Figure 5). Significant differences in EROD activity were observed between 10 µM 2-OHCHR-treated embryos with and without 20 µM ketoconazole pretreatments, with a 90.8% ± 7.00 reduction in EROD activity in pretreated individuals when normalized to controls (Figure 6).

Pretreatments with 10 µM nilotinib did not affect toxicity of 10 µM 2- or 6-OHCHR treatments (Figure 4) but did significantly reduce glucuronide formation by 52.4% ± 2.55 and 63.7% ± 3.19.

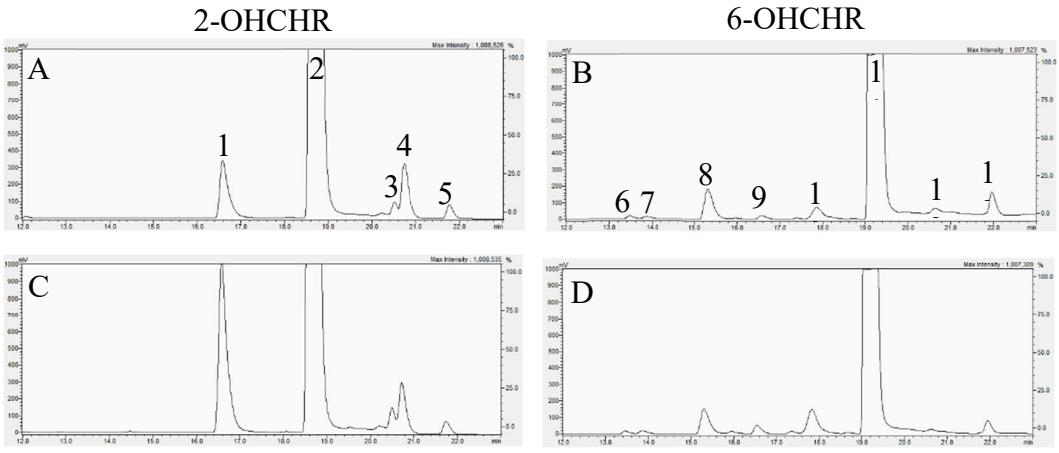

Figure 3-1. Representative chromatograms of tissue extracts following treatment with 2- or 6-OHCHR from 52-76 hpf. A and B were pretreated with 20 µM ketoconazole (CYP inhibitor) from 28-52 hpf while C and D were not. Peaks 1 and 10 indicate a 1,2-catechol and 5,6-catechol metabolite while peaks 2 and 11 indicate the parent hydroxychrysene. All other peaks are unknown metabolites.

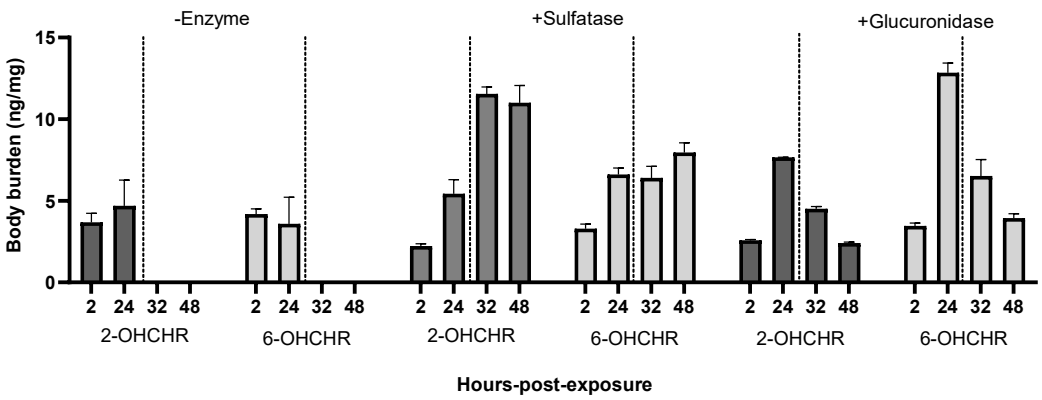

Figure 3-2. Body burdens of OHCHRs in medaka embryos after 2 or 24 hours of uptake and 12 or 24 hours of depuration. Dotted lines indicate the transfer of embryos from exposure solution to water to allow depuration (after 24 h exposure). Depuration body burdens are indicated by 32 and 48 which correspond to 12 and 24 hours of depuration, respectively. Values represent the mean of three replicates. Error bars represent + SEM.

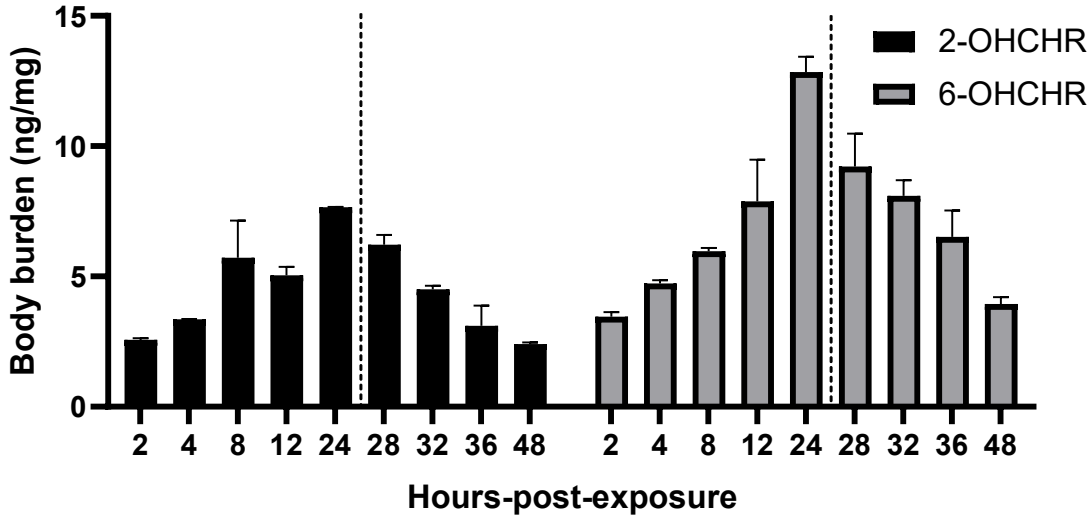

Figure 3-3. Uptake and depuration body burdens of 2- and 6-OHCHR-treated medaka embryos following treatment with glucuronidase. Dotted lines indicate the transfer of embryos from exposure solution to water to allow depuration (after 24 h exposure). Depuration body burdens are indicated by 28, 32, 36, and 48 which correspond to 4, 8, 12, and 24 hours of depuration, respectively. Values represent the mean of three replicates. Error bars represent + SEM.

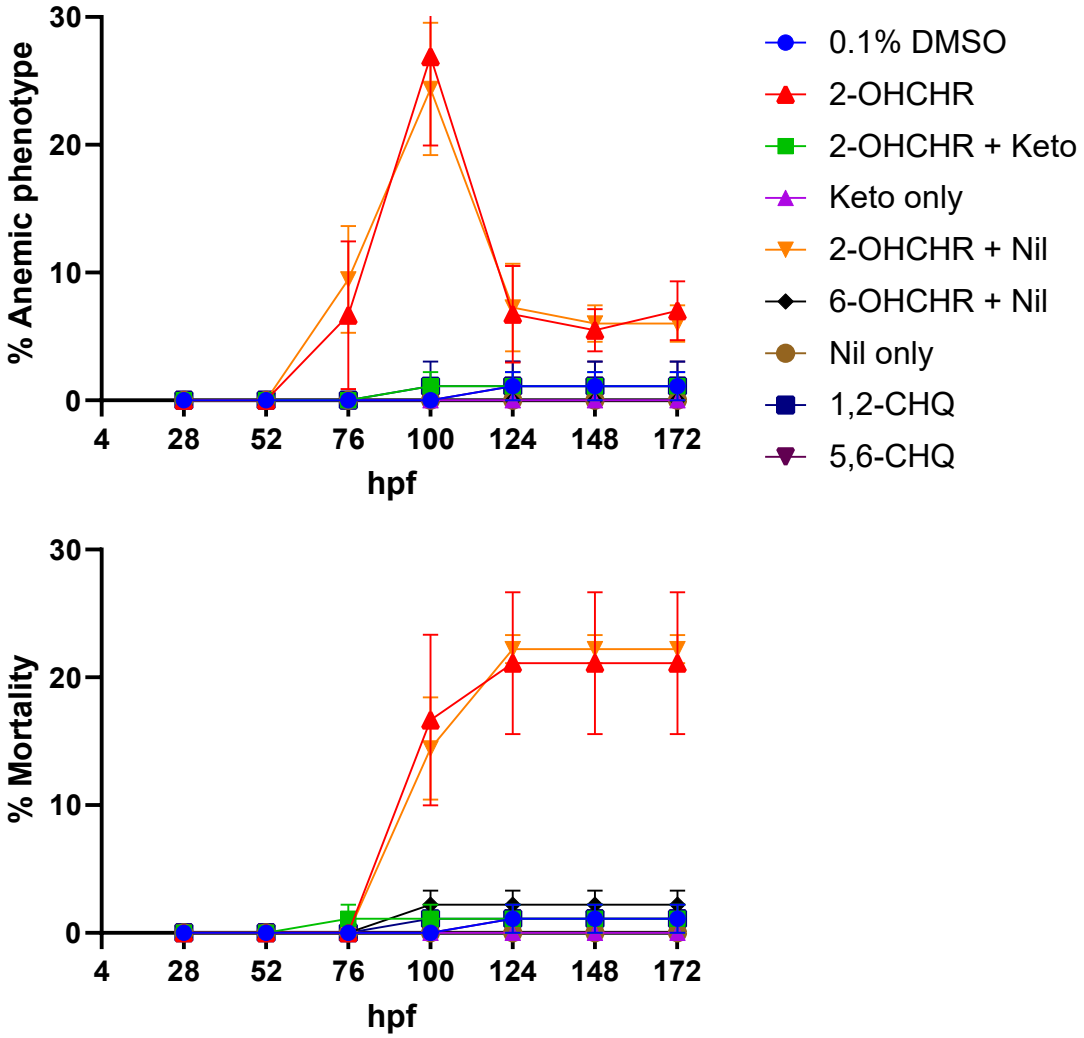

Figure 3-4. Percent anemic phenotype and mortality of medaka embryos pretreated with 20 µM ketoconazole (CYP inhibitor) or 10 µM nilotinib (UGT inhibitor) from 28-52 hpf, exposed to 10 µM 2-OHCHR from 52-76 hpf, then transferred to 0.1 % DMSO until 172 hpf. Values represent the mean of three replicates. Error bars represent ± SEM.

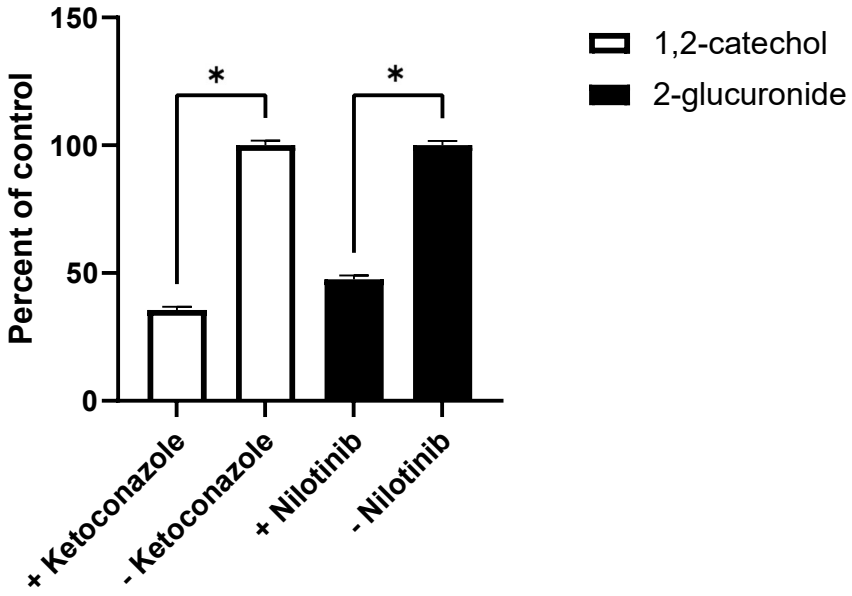

Figure 3-5. Concentrations of 1,2-catechol and 2-glucuronide following pretreatments with the CYP inhibitor (ketoconazole) and UGT inhibitor (nilotinib) compared to controls (no inhibitor). Asterisks (*) represent significant differences in metabolite concentrations with and without the inhibitor. Values represent the mean of three replicates + SEM.

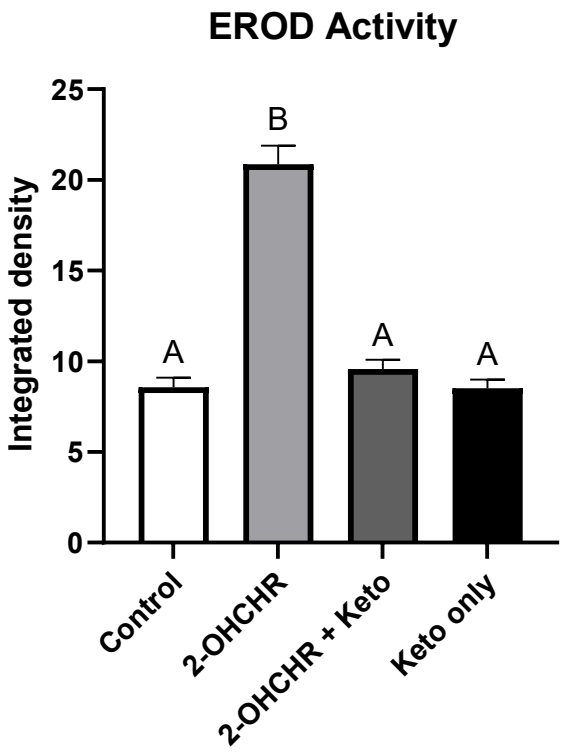

Figure 3-6. EROD activity following treatments with 2-OHCHR from 52-76 hpf, with or without ketoconazole pretreatment from 28-52 hpf. Letters indicate significant differences determined by a two-way ANOVA followed by a post hoc test. Values represent the mean of thirty replicates ± SEM.

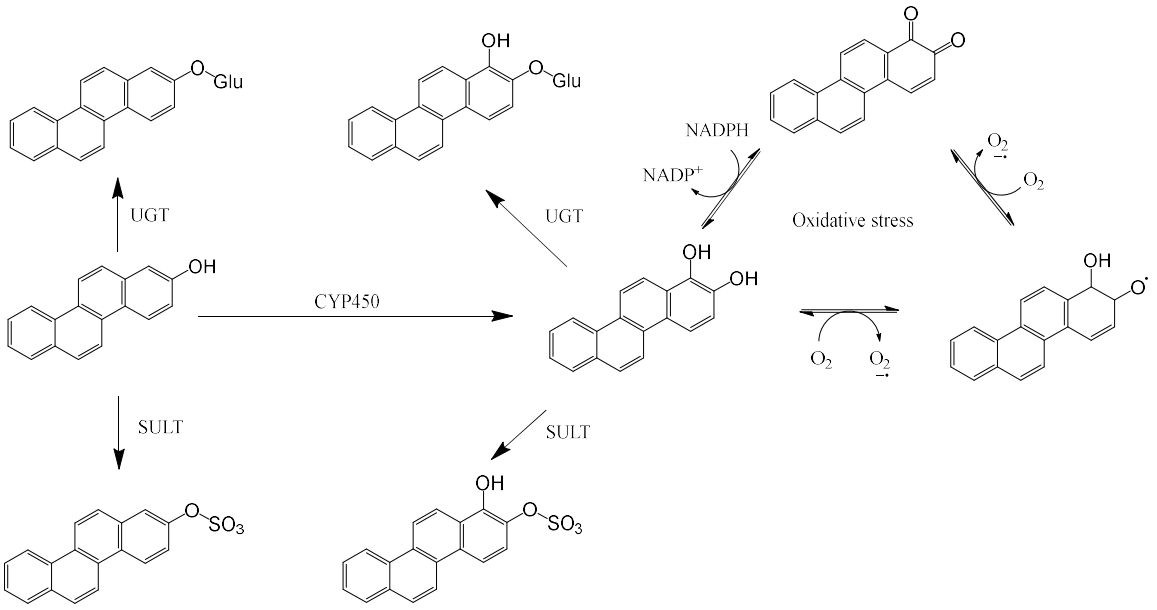

Figure 3-7. Theoretical metabolic pathway of hydroxy-PAHs using 2-OHCHR as a model compound.

Table 3-1. Conversion of 2- and 6-OHCHR metabolites within embryos. Percent parent represents unmetabolized OHCHRs and percent unknown represents uncharacterized metabolites. Asterisk represents a significant difference between 2- and 6-OHCHR. Values represent the mean of three replicates ± SEM.

	2-OHCHR	**6-OHCHR**
% parent	10.8 ± 3.63	4.92 ± 2.26
% glucuronide	31.8 ± 2.92	34.6 ± 3.14
% sulfate*	25.1 ± 2.24	18.7 ± 2.01
% catechol*	20.9 ± 1.68	4.64 ± 1.34
% quinone*	BLD	18.0 ± 4.58
% unknown	11.49 ± 2.22	19.1 ± 7.39

3.5 Discussion

Previous studies in our lab indicated isomeric differences in toxicity between 2- and 6-OHCHR in embryos of Japanese medaka. A sensitive window of development was identified between 52-100 hpf which closely coincided with liver development. The liver contains high concentrations of metabolic enzymes, such as CYP, UGT, and SULT. Therefore, we hypothesized that metabolism may play a role in the regioselective toxicities of 2- and 6-OHCHR.

Conversion of 2- and 6-OHCHR to metabolites differed between compounds after 24 h of treatment at 52 hpf. While formation of glucuronides was similar, sulfates constituted a significantly higher proportion of total metabolites of 2-OHCHR than 6-OHCHR, although there was no significant difference in concentrations. While no significant differences were found in proportions of glucuronide between 2- and 6-OHCHR, it was the major metabolite for both compounds at 24 h with significantly higher concentrations compared to controls. Due to it being the major metabolite, glucuronide formation was further analyzed for toxicokinetic parameters and their effects on hydroxychrysene toxicities.

While uptake was greater with the less toxic 6-OHCHR, conversion and elimination to predominantly conjugated metabolites was also faster, indicating conjugation to metabolites may be a factor explaining the toxicity of either compound (Figure 1). While the concentrations of both OHCHRs in glucuronidase-treated extracts decreased following depuration, the concentrations significantly increased in sulfatase-treated 2-OHCHR

extracts and did not change in 6-OHCHR extracts. This indicates that sulfate-conjugates may not be readily eliminated like glucuronide-conjugates and instead may be retained within the embryos. However, due to the lack of available SULT inhibitors, the effects of inhibiting sulfation could not be assessed. Glucuronides are more hydrophilic compared to sulfates which make them more likely to be eliminated from the body. Schebb et al. (2011) observed that in larval Japanese medaka treated with triclocarban, the glucuronide was the major metabolite and was more rapidly eliminated compared to the sulfate conjugate. However, Van Wijk et al. (2019) observed that zebrafish larvae excreted sulfate conjugates of paracetamol more rapidly compared to glucuronides. It should be noted, however, that paracetamol-sulfate was the major metabolite and was observed at a 10-fold higher concentration within the larvae compared to the glucuronide. In a study with the PAH benzo(a)pyrene, Hornung et al. (2007) observed little to no elimination of metabolites from Japanese medaka embryos between 1-7 dpf but observed elimination post-hatch, contrasting our results. However, this study only quantified a single glucuronide metabolite (BaP-3-glucuronide) and sulfates were not targeted for analysis nor detected which could indicate that other phase II metabolites could have been missed. Additionally, since there are few toxicokinetic studies of PAHs, let alone oxy-PAHs, in fish embryos, the distribution of different PAHs or oxy-PAHs within embryos could vary which may explain differences observed with other studies.

Given the relatively high conversion of 2- and 6-OHCHR to glucuronides, inhibiting conjugation reactions could potentially enhance the toxicity of these compounds. Nilotinib

is a potent UGT1A1 inhibitor which has been characterized in mammalian models but not in fish (Fujita et al., 2011; Liu et al., 2011; Ai et al., 2014). Reductions in overall glucuronides were observed in embryonic treatments, showing that this compound was effective as a UGT inhibitor in Japanese medaka embryos. However, no significant change in toxicity was observed, suggesting diminished glucuronidation of 2- or 6-OHCHR may not have significant influence on the regioselective differences in toxicity.

While conjugation may not be an important pathway of detoxification, sequential oxidation of hydroxy-PAHs to catechols or quinones has been shown to enhance the toxicity of phenolic PAHs (Wislocki et al., 1976; Moorthy et al., 2003; Knecht et al., 2013). Exposures to putative quinone metabolites of 2- and 6-OHCHR did not result in significant anemia or mortality. Quinone metabolites were observed following treatment with 6-OHCHR in medaka embryos (5,6-CHQ), but quinones were not detected following treatments with the more toxic 2-OHCHR. Body burden measurements following 24 h treatment with both quinones were similar to concentrations of 2- and 6-OHCHR, indicating uptake occurred with embryonic concentrations approaching that of 2-OHCHR. Since similar concentrations of quinones and 2-OHCHR were observed within embryos, and toxicity was not observed with quinone treatments, quinone formation may not be a critical step in the isomeric differences in embryotoxicity. While 1,2-CHQ was not detected nor toxic to embryos, other unidentified quinones may play more of a role. *para*-Quinones tend to be more toxic relative to *ortho*-quinones. Knecht et al. (2013) observed that while 9,10-anthraquinone was not toxic to zebrafish embryos, 1,4-anthraquinone was very toxic.

However, the same study also observed similar levels of toxicity between *ortho* and *para*-quinones of phenanthrene and naphthalene which suggests that the trend may be PAH-specific. *para*-Quinones also tend to be more stable than *ortho*-quinones (Bolton and Dunlap, 2017) which may contribute to greater half-lives for *para*-quinones, allowing higher concentrations for potential redox cycling and the generation of reactive oxygen species (ROS). Since several unknown metabolites were present in chromatograms from embryos treated with 2-OHCHR, further spectroscopic analyses may be necessary to determine if other compounds may be more important.

One metabolite that was identified in 2-OHCHR treatments that correlated with the anemic phenotype and mortality was the 1,2-catechol. Catechols are commonly formed via oxygenation of phenolic metabolites by CYP (Jones et al., 1978; Moore et al., 1978; Carmichael and Wong, 2001; Lu et al., 2011). To determine if CYP played a role in 2-OHCHR toxicity, embryos were pretreated with ketoconazole, a broad spectrum CYP inhibitor, prior to 2-OHCHR exposure (Burkina et al., 2021). Formation of the 1,2-catechol was significantly reduced with the CYP inhibitor and pretreatment provided significant protection from both 2-OHCHR-induced anemia and mortality. Previous in vitro studies also indicated that 2-OHCHR has a four-fold higher affinity to AhR compared to 6-OHCHR (Lam et al., 2018; Villeneuve et al., 2002) suggesting 2-OHCHR may induce CYP1 orthologs at a greater rate than 6-OHCHR. Treatment of embryos with 2-OHCHR induced the CYP1 enzyme EROD activity and pretreatment with ketoconazole significantly diminished EROD activity (Figure 6) suggesting CYP1 may be involved in

catechol formation and toxicity. Ketoconazole has also been shown to inhibit CYP1 and CYP3 catalytic activities in rainbow trout, Atlantic cod, and killifish (Hegelund et al., 2004; Hasselberg et al., 2005; Burkina et al., 2021). The 1,2-catechol could presumably be oxidized to a semi-quinone radical which may elicit oxidative stress (Figure 7). Further studies are needed to determine the mechanistic relevance of oxidative stress and its relationship to the anemic phenotype and/or mortality associated with 2-OHCHR.

In summary, metabolism likely plays a significant role in 2-OHCHR toxicity. While uptake and depuration as a glucuronide conjugate were significantly higher for 6-OHCHR than 2-OHCHR, pretreatments with a UGT inhibitor did not affect the toxicity of either compound. However, pretreatments with a CYP inhibitor significantly reduced the toxicity of 2-OHCHR, indicating that a toxic metabolite may contribute to its toxicity. A significantly higher proportion of 2-OHCHR was converted to a catechol metabolite compared to 6-OHCHR, the formation of which was significantly reduced following pretreatments with a CYP inhibitor along with toxicity. These results indicate that the 1,2-catechol may be a toxic metabolite which plays a role in 2-OHCHR toxicity in embryonic Japanese medaka.

Chapter 4: Role of AhR and oxidative stress in the regioselective toxicities of hydroxychrysenes in embryonic Japanese medaka

4.1 Abstract

Oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) are environmental contaminants that can be created through oxidation of the parent PAH, many of which have been found the be more toxic than their parent compounds. Previous studies have found that 2-hydroxychrysene (2-OHCHR) and 6-hydroxychrysene (6-OHCHR) exhibit regioselective toxicities in embryonic Japanese medaka that was prevented by CYP inhibition which reduced the formation of the 1,2-catechol, a potentially toxic metabolite prone to redox cycling and oxidative stress. 2-OHCHR has also been found to be a four-fold more potent aryl hydrocarbon receptor (AhR) agonist compared to 6-OHCHR. These findings led us to hypothesize that AhR activation and oxidative stress play an important role in 2-OHCHR toxicity. While treatments with the AhR agonists PCB126 and 2-methoxychrysene (2-MeOCHR) did not cause significant anemia or mortality, pretreatments with AhR antagonist CH-223191 reduced anemia by 97.2% ± 0.84 and mortality by 96.6% ± 0.69. AhR inhibition was confirmed by a significant reduction (91.0% ± 9.94) in EROD activity. Thiobarbituric acid reactive substances (TBARS) concentrations were 32.9% ± 3.56 higher ($p<0.05$) in 2-OHCHR treatments at 100 hpf compared to controls, indicating oxidative stress. Staining with 2',7'-Dichlorofluorescin diacetate (DCFDA) revealed 42.6% ± 2.69 of embryos exhibiting high concentrations of ROS in caudal tissues, which is a site for embryonic hematopoiesis. Both muscle and

skeletal tissues were affected, as well as some caudal vasculature. These results indicate that AhR may mediate 2-OHCHR toxicity, upregulating CYP and potentially forming the 1,2-catechol that generates ROS in the embryos within caudal tissues, potentially disrupting hematopoiesis leading to anemia and subsequent mortality.

4.2 Introduction

Oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) are environmental contaminants that can be created through oxidation of parent PAHs, which can occur through photochemical or biological means (Saeed et al., 2011; Esbaugh et al., 2016; Sweet et al., 2017). They can have one or more functional groups and include carbonyls, alcohols, and carboxylates (Lundstedt et al., 2017). Due to their increased polarity compared to parent PAHs, they can have increased mobility in aquatic environments, potentially posing a risk to biota (Wang et al., 2011; Achten and Andersson, 2015). Additionally, these compounds are considered to be "dead-end" products, which may lead to accumulation to concentrations that cause adverse effects (Cerniglia, 1993; Kochany and Maguire, 1994; Cerniglia, 1997). While parent PAHs have been extensively studied, little is known about the potencies and modes of action for oxy-PAHs (Lundstedt et al., 2017). A better mechanistic understanding of these compounds can allow regulators to reduce uncertainty when evaluating the risks of oxy-PAHs in the environment.

Aryl hydrocarbon receptors (AhRs) are nuclear receptors that serve as signaling molecules in canonical and non-canonical pathways. The canonical pathway is well studied and

involves the binding of a ligand to AhR which is then translocated into the nucleus. The AhR-ligand complex can then bind to the xenobiotic response element (XRE) which upregulates several enzymes to metabolize the ligand and mitigate its toxic effects (summarized in Shankar et al., 2020). The non-canonical pathways are less studied and can cause effects through several ways, including inhibitory crosstalk, binding to response elements other than XRE, as well as several uncharacterized pathways (Safe et al., 2000; Benamian et al., 2004; Gräns et al., 2010; Vorrink et al., 2014; Wright et al., 2017). PAHs such as pyrene have been observed to cause toxicity through both canonical and non-canonical AhR pathways (Incardona et al., 2006). While little work has been done with oxy-PAHs, several have been shown to be potent AhR agonists. For example, 2-hydroxychrysene was found to have a four-fold greater binding affinity to AhR than its parent compound, chrysene, and the isomeric 6-hydroxychrysene (Villeneuve et al., 2002; Lam et al., 2018). Lille-Langøy et al. (2021) have compared the potencies and efficacies of 31 different substituted PAHs and have found that while many parent PAHs are poor AhR agonists, several of their metabolites were potent and effective agonists, such as 2-methylchrysene and 2-methoxychrysene.

In addition, oxy-PAHs have also been shown to cause oxidative stress. Several PAH-quinones have been shown to cause toxicity through redox cycling or through a reactive intermediate that binds to cellular components, such as protein or DNA (O'brien 1991; Monks et al., 1992; Bolton et al., 2000; Vondráček and Machala, 2021). Redox cycling can be initiated through enzymatic or non-enzymatic reactions which continually produce

reactive oxygen species (ROS) and deplete reducing equivalents (O'brien 1991; Monks et al., 1992; Bolton et al., 2000). However, not all PAH-quinones are oxidatively active, indicating that some may be less reactive and less likely to undergo redox cycling (Rossi et al., 1986).

Previous studies in our lab have demonstrated regioselective toxicities of hydroxychrysenes in Japanese medaka embryos, where 2-hydroxychrysne (2-OHCHR) was found to cause anemia and mortality while chrysene and 6-hydroxychrysene (6-OHCHR) did not (Tanabe et al., 2021). Toxicity coincided with liver development and inhibition of cytochrome P450 (CYP) significantly reduced the toxicity of 2-OHCHR and the formation of the 1,2-catechol metabolite. Quinones were not detected in 2-OHCHR treated embryos but were detected in the non-toxic 6-OHCHR treated embryos (Tanabe et al. in preparation). In addition, exposures of embryonic Japanese medaka to 1,2- and 5,6-quinones of chrysene did not result in toxicity.

Since 2-OHCHR is a potent AhR ligand and inducer of CYP1 enzymes, it is unclear whether the toxicity of 2-OHCHR is dependent on AhR activation or activation through another CYP enzyme to a reactive intermediate. Additionally, it is also unclear whether 1,2-catechol formation elicits oxidative stress, or whether downstream effects from AhR activation are important in the toxicity of 2-OHCHR. Therefore, we investigated the following hypotheses: (1) Canonical AhR signaling contributes to 2-OHCHR toxicity (2) induction of CYP is required for toxicity or (3) 2-OHCHR causes toxicity through oxidative stress in Japanese medaka embryos.

4.3 Materials and Methods

4.3.1 Chemicals

2-hydroxychrysene (purity > 99%) and 6-hydroxychrysene (purity > 99%) were purchased from Toronto Research Chemicals, Ontario, Canada. CH-223191 (purity ≥ 98%), α-Tocopherol (purity ≥ 96%), N-Acetyl-L-cysteine (purity ≥ 99%), 2,6-Di-tert-butyl-4-methylphenol (purity ≥ 99%), 2-Thiobarbituric acid (purity ≥ 98%), 1,1,3,3-Tetramethoxypropane (purity ≥ 99%), and 2',7'-Dichlorofluorescin diacetate (purity ≥ 97%) were purchased from Millipore-Sigma, St. Louis, MO. 2-Thiobarbituric acid was dissolved in 100 mM NaOH and 1,1,3,3-Tetramethoxypropane was dissolved in 1.15% KCl. The rest of the compounds were dissolved in DMSO. Exposure solutions were made fresh daily by dilution of stock solutions to 0.1% DMSO in de-ionized (DI) water.

4.3.2 Maintenance of Medaka Culture

Adult Japanese medaka (*Oryzias latipes*) were maintained under the UCR Institutional Animal Care and Use Committee (IACUC)-approved protocol (AUP#20190017). Embryos were collected one hour after the lights turned on. Embryos were evaluated for viability using a transmitted light microscope and sorted for the correct stage following staging guidelines from Iwamatsu et al. (2004) for stage-specific morphological characteristics. Medaka were maintained at 28°C on a 14 h:10 h light:dark cycle.

4.3.3 Exposure Regime

Thirty embryos at 4 hpf were collected from a sorted pool and transferred to glass petri dishes (60 x 15 mm) for exposure. Three replicate petri dishes were utilized, each containing 20 embryos. Exposure solutions were made fresh daily by dilution of stock solutions to 0.1 % DMSO at nominal concentrations. All exposures after 52 hpf were carried out with dechorionated embryos while pretreatments were conducted with chorionated embryos due to difficulty with dechorionation at early stages. All analyses and statistical comparisons were conducted at 100 hpf due to anemia peaking at this timepoint.

To explore the role of AhR, embryos were treated with the potent AhR agonist PCB126 and a 2-OHCHR metabolite, 2-methoxychrysene, which has a higher AhR affinity compared to 2-OHCHR (Hestermann et al., 2000; Lille-Langøy et al., 2021). Treatments were conducted with 30 nM PCB126 or 10 μM 2-methoxychrysene from 52-76 hpf. Embryos were also pretreated with 10 μM CH223191, a potent AhR inhibitor, from 28-52 hpf then transferred to 10 μM 2-OHCHR from 52-76 hpf. Concentrations and stages for exposure were based on previous studies (Tanabe et al., 2021). Embryos were rinsed with DI water then transferred to 0.1% DMSO water until 176 hpf. Embryos were assessed for anemia and mortality daily under a transmitted light microscope using an Accu-Scope 3000 microscope using the methods described in Tanabe et al., 2021. Anemia was defined by no observable moving blood cells and mortality was defined by a lack of observable heartbeat.

To explore the role of oxidative stress, embryos were pretreated with two antioxidants, 20 µM α-Tocopherol (Vitamin E) or 200 µM N-Acetyl-L-cysteine (NAC) from 28-52 hpf then transferred to 10 µM 2-OHCHR from 52-76 hpf. Embryos were the rinsed, transferred to 0.1% DMSO, and assessed daily for anemia and mortality as described above. Treatment concentrations and exposure duration were determined from range-finding studies.

4.3.4 EROD Imaging

To measure ethoxyresorufin-O-deethylase (EROD) activity, dechorionated embryos were exposed to 10 µM 2- or 6-OHCHR, 10 µM 2-MeOCHR, or 30 nM PCB126 in water containing 21 µg/mL 7-ethoxyresorufin (ER) from 52 – 76 hpf, then transferred to 0.1% DMSO water with the same concentration of ER from 76-100 hpf. Following exposures, embryos were anesthetized with MS-222, washed with DI water, then transferred to a 48 well plate for imaging. Resorufin was quantified within embryos using fluorescence microscopy. A Keyence BZ-X710 microscope was used. Embryos were positioned in molded agarose dorsally with the heart centered within the image. Image analysis was conducted in ImageJ version 1.8.0. The gall bladders of the embryos were traced, and the integrated density values of the selection were measured for each embryo. High integrated density values corresponded to high EROD activity.

4.3.5 TBARS assay

To quantify the formation of malondialdehyde, thiobarbituric acid reactive substance (TBARS) concentrations were measured using the methods described in Kupsco and

Schlenk, 2014. Embryos were exposed to 10 µM 2-OHCHR from 52-76 hpf, then to 0.1% DMSO from 76-100 hpf. They were then rinsed with DI water, dried of excess water, and weighed. Embryos were then transferred to a 1.5 mL centrifuge tube, homogenized in 300 µL 1.15% KCl, then centrifuged at 3000 rpm at 4°C. The supernatant (200 µL) was then transferred to a new centrifuge tube, to which 25 µL 12.6 mM BHT, 200 µL ortho-phosphoric acid, and vortexed for 25 seconds. Next, 25 µL TBA was added, after which the tube vortexed for 25 seconds then incubated at 90°C for 45 minutes. Tubes were then transferred to ice for 5 minutes to stop the reaction. n-Butanol (500 µL) and 50 µL saturated NaCl solution was added, then the tube was shaken to allow the TBARS to partition into the organic phase. The upper butanol layer and transferred to 96 well plates in triplicate (150 µL each). Fluorescence was measured on a (insert name here) plate reader at the following wavelengths: excitation: 530-560, emission: 585-605.

4.3.6 DCFDA staining

To localize potential oxidative stress events, embryos were stained with 2',7'-dichlorofluorescein diacetate (DCFDA) following exposure to 0.1% DMSO or 10 µM 2- or 6-OHCHR from 52-76 hpf and 0.1% DMSO from 76-100 hpf. Times for evaluation and exposure were based on range-finding studies and previous evaluations (Tanabe et al., 2021). After exposure, embryos were rinsed with DI water then transferred to a glass petri dish containing 10 µg/mL DCFDA (10 mL at 0.2% DMSO). The dishes were placed on a shaker in the dark at room temperature for 1 hour. Embryos were then transferred to 10 mL DI water for 10 minutes to allow depuration of DCFDA. This was done twice, then

embryos were anesthetized in 10 mL 300 μg/ml tricaine for 10 minutes. Imaging of embryos was performed using a Leica MZIII Pursuit stereoscope at the following wavelengths: excitation 470/40 nm, emission 525/50 nm. Due to high mortality of dechorionated embryos during the staining protocol, embryos were stained with the chorion intact.

4.3.7 Statistical analysis

All statistical analyses were conducted in Rstudio version 1.2.5019 and GraphPad Prism version 8.4.3. All data were checked for normality and equal variance assumptions and a p-value of 0.05 was used for all statistical tests. Student's t-tests were used to compare percent phenotype and mortality to controls, as well as TBARS concentrations in treatments to controls. EROD activities were compared using a two-way ANOVA and a post hoc Tukey HSD test. All figures were generated using GraphPad Prism.

4.4 Results

4.4.1 AhR agonist treatment

Embryos were treated with the potent AhR agonists, PCB126 and 2-MeOCHR from 52-76 hpf, then transferred to 0.1% DMSO from 76-100 hpf. No significant differences were observed in anemia and mortality at 100 hpf between the treatments and controls. However, cumulative mortality was observed in embryos treated with PCB126 until 172 hpf when toxicity was significantly higher relative to controls (Figure 1). Although anemia was not observed following treatment, significantly greater EROD activity was observed at 100 hpf

between 2-OHCHR (139% ± 0.17), 2-methoxychrysene (222% ± 0.40), PCB126 (433% ± 0.73), and controls. Significant differences in EROD were not observed between CH-223191 pretreatments and controls (Figure 2).

4.4.2 AhR inhibitor pretreatment

Significant differences were observed at 100 hpf between CH-223191 pretreated embryos and non-pretreated embryos exposed to 2-OHCHR, with a 97.2% ± 0.84 reduction in anemia and a 96.6% ± 0.69 in mortality in pretreatments (Figure 1). AhR inhibition was confirmed by a 91.0% ± 9.94 reduction EROD activity of pretreated embryos when normalized for controls. No significant differences were found in toxicity or EROD activity between 6-OHCHR treatments with or without CH-223191 pretreatment.

4.4.3 Oxidative stress measurements

Significant increases were observed in TBARS concentrations at 100 hpf in 2-OHCHR (32.9% ± 3.56) compared to the control (Figure 3). No significant differences were found between 6-OHCHR and controls. A positive control (3% H_2O_2) was used to validate the efficacy of the assay with a 413% ± 31.8 increase in TBARS concentrations compared to controls.

Staining with DCFDA revealed that at 100 hpf, oxidative stress was highest in the tails of embryos, with 42.6% ± 2.69 of the embryos showing intense fluorescence in caudal muscle and skeletal tissue, as well as some fluorescence in the caudal vasculature which indicates a high concentration of ROS (Figure 4). A positive control (3% H_2O_2) was used to validate

the efficacy of the staining with 84.9 % ± 3.28 of embryos showing intense fluorescence in caudal vasculature, as well as muscle and skeletal tissue.

4.4.4 Antioxidant pretreatment

Significant reductions of 2-OHCHR toxicity were observed at 100 hpf between antioxidant pretreated embryos and non-pretreated embryos. NAC-pretreated embryos exhibited an 80.7% ± 1.12 reduction in anemia and a 67.1% ± 1.69 reduction in mortality, while vitamin E caused a 99.1% ± 0.43 reduction in anemia and a 98.9% ± 0.66 reduction in 2-OHCHR-induced mortality in pretreatments (Figure 1).

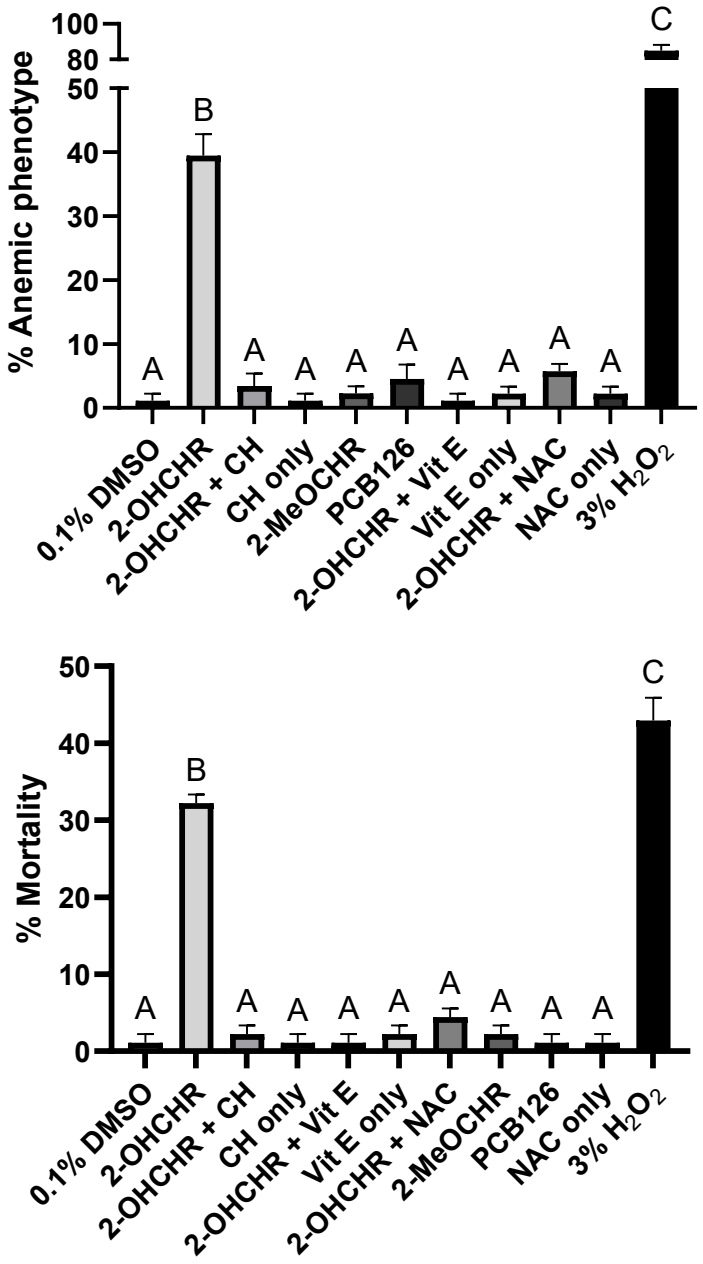

Figure 4-1. Percent anemic phenotype and mortality of medaka embryos at 100 hpf. Pretreatments occurred from 28-52 hpf and exposures took place from 52-76 hpf, then transferred to 0.1 % DMSO until 172 hpf. Values represent the mean of three replicates + SEM.

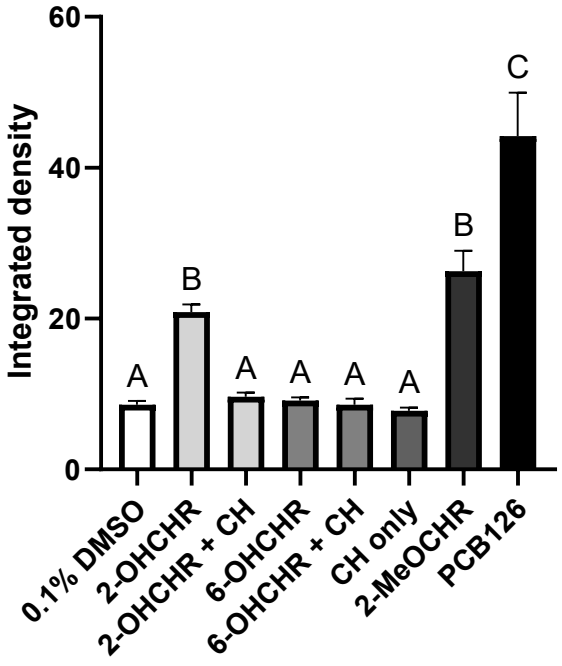

Figure 4-2. EROD activity following treatments with 2- or 6-OHCHR from 52-76 hpf, with or without CH-223191 pretreatment from 28-52 hpf. PCB126 and 2-MeOCHR were used as positive controls for AhR activation. Letters indicate significant differences determined by a two-way ANOVA followed by a post hoc test. Values represent the mean of thirty replicates + SEM.

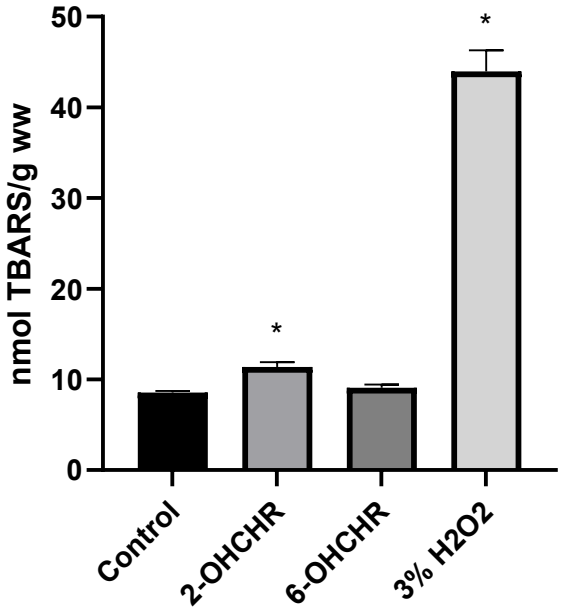

Figure 4-3. TBARS concentrations following treatments with 10 μM 2- or 6-OHCHR from 52-76 hpf. Embryos were also exposed to the positive control, 3% H_2O_2, from 73-76 hpf. Asterisks (*) indicate significant differences from the control. Values represent the mean of thirty replicates + SEM.

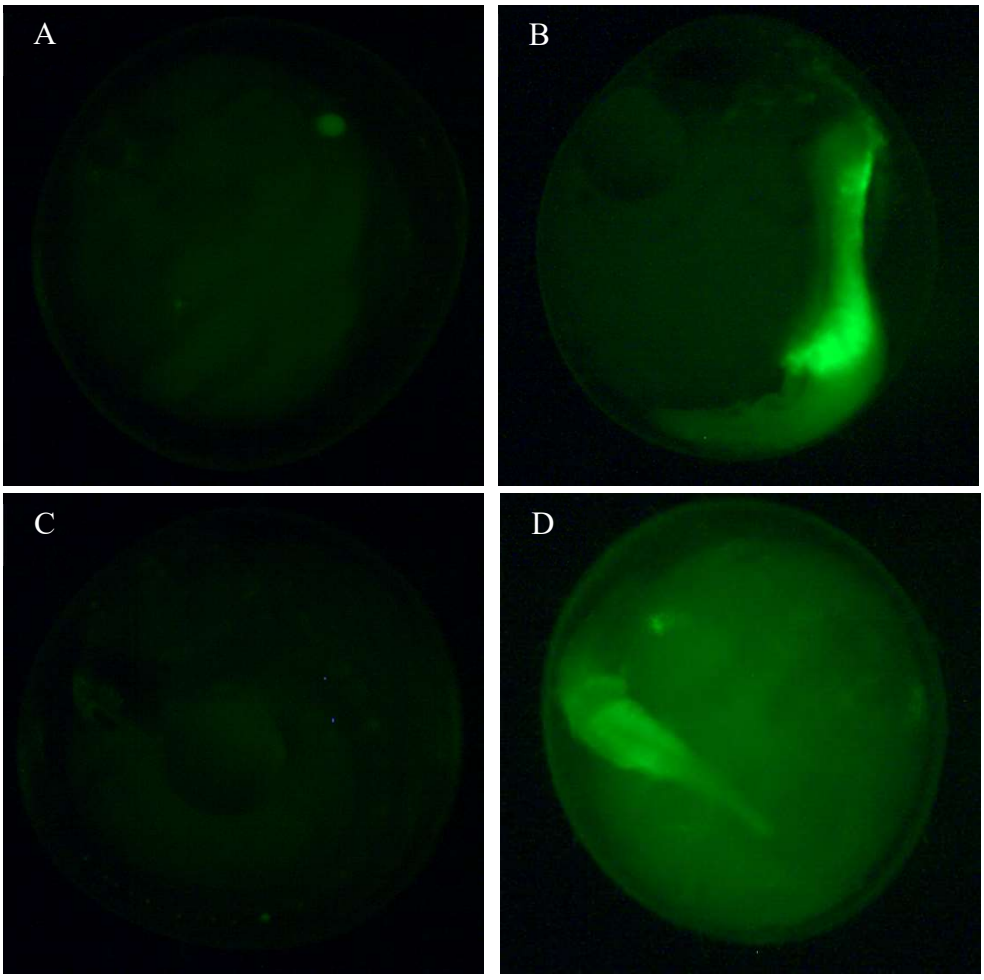

Figure 4-4. DCFDA stained embryos at 100 hpf. A: 0.1% DMSO, B: 2-OHCHR, C: 6-OHCHR, D: H_2O_2 (positive control).

4.5 Discussion

Studies in our lab have indicated that toxicity of 2-OHCHR coincided with liver development in Japanese medaka embryos (Tanabe et al., 2021). Pretreatments with a CYP inhibitor provided significant protection from anemia and mortality at this stage, complementing this observation (Tanabe et al., in preparation). Inhibitor pretreatments also significantly reduced formation of 1,2-catechol in 2-OHCHR treatments, indicating that CYP-mediated metabolism may contribute to 2-OHCHR toxicity. However, 2-OHCHR is also a potent AhR agonist. While AhR can regulate the expression of CYP, it has also been shown to cause toxicity through other signaling pathways. Therefore, canonical pathways of AhR and bioactivation via CYP1 were explored in this study.

Pretreatment with the AhR inhibitor CH-223191 significantly reduced anemia and mortality following 2-OHCHR exposures. AhR inhibition was confirmed by reductions in EROD activity, indicating that AhR plays an important role in the toxicity of 2-OHCHR. However, previous studies have found that while CH-223191 prevented mono-ITP-induced cardiac abnormalities, AHR2 knockdown did not, indicating that CH-223191 may be interacting with other pathways (McGee et al., 2013; Gerlach et al., 2014). Previous studies have shown that pretreatments with ketoconazole, a broad spectrum CYP inhibitor, provided a similar magnitude of protection against 2-OHCHR, indicating that AhR could be upregulating CYP following 2-OHCHR exposure, and that toxicity may occur further downstream. This pathway has been characterized in several other PAHs such as benzo(a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA) (McDougal et al.,

1997; Shimizu et al., 2000; Maayah et al., 2015). PAH binding to AhR results in upregulation of CYP1 which catalyzes the metabolism of these ligands to epoxides, phenols, or dihydrodiols. While DMBA mainly forms mutagenic epoxides, BaP can be subsequently metabolized to acutely toxic catechols, hydroquinones, and quinones which have been shown to be oxidatively active (Miller and Ramos, 2001). BaP toxicity has also been significantly reduced in zebrafish embryos through pretreatments with AhR inhibitor CH-223191 (Huang et al., 2021) and AhR knockdown (Cunha et al., 2020) indicating that AhR plays an important role in PAH-induced toxicity.

To determine the role of AhR signaling in the formation of the anemic phenotype, embryos were treated with the AhR ligands PCB126 and 2-MeOCHR, both of which have been shown to be more potent AhR agonists than 2-OHCHR (Hestermann et al., 2000; Lille-Langøy et al., 2021). However, neither caused significant anemia and PCB did not cause significant mortality until after 124 hpf. AhR activation was confirmed by EROD activities following treatment of the compounds. EROD activity was significantly higher following PCB126 and 2-MeOCHR treatment compared to 2-OHCHR. This result indicates that AhR activation may not contribute to 2-OHCHR induced anemia and mortality. In contrast to our results, TCDD, another potent AhR agonist, has previously been shown to cause anemia and mortality in Japanese medaka; however, this was only in post-hatch animals and indicates a different window of susceptibility (Prince et al., 1990; Tokusumi et al., 2021). Anemia and mortality were also found for PCB126, with minimal toxicity pre-hatch (Vicquelin et al., 2011). Anemia has never been observed with 2-MeOCHR even though

AhR ligand activity has been noted. The observation that TCDD and PCB126 cause anemia in Japanese medaka at later life stages, but not at the 52 hpf stage, again is consistent with liver development being critical in the effects of 2-OHCHR suggesting metabolic activation rather than downstream AhR signaling.

Pretreatment with vitamin E and NAC diminished 2-OHCHR toxicity, indicating oxidative stressmay be a potential mechanism of action for the anemic phenotype and subsequent mortality. Oxy-PAHs elicit cellular toxicity through multiple mechanisms. Oxidative activation to quinones or catechols has been previously observed with BaP and benz(a)anthracene (Wislocki et al., 1976; Seike et al., 2003). In these examples, oxidative stress was shown to be the primary mode of action for acute toxicity. To explore the role of oxidative stress in hydroxychrysene toxicity, embryos were pretreated with the antioxidants, vitamin E and NAC. Both significantly reduced anemia and mortality, further supporting this hypothesis. Previous studies revealed that while quinones were not detected in 2-OHCHR extracts, high concentrations of 1,2-catechol were observed. Interestingly, 6-OHCHR extracts contained high concentrations of 5,6-quinone even though 6-OHCHR was not toxic to Japanese medaka embryos. These results indicate that quinone formation does not equal oxidative stress and that other metabolites may contribute more towards oxidative stress. However, there could be undetected quinones that may be contributing to 2-OHCHR toxicity. Knecht et al. (2013) observed that isomeric quinones from the same parent PAH, such as 9,10- and 1,4-anthraquinone, could have drastically different potencies and effects. It is also interesting to note that many of the quinones that were

observed to not be toxic in this study had low redox potentials, while the more toxic ones had higher potentials (Conant and Louis; 1924; Moriconi et al., 1962). Redox potential is a measure of thermodynamic feasibility of a chemical to undergo a reduction or oxidation reaction and these results indicate that redox potential may be a predictive tool of toxic oxy-PAHs. However, 5,6-CHQ has been shown to have a relatively high redox potential, indicating that other metrics of reactivity may need to be taken into account when considering chemical reactivity. Furthermore, few studies have been done on the toxicities of PAH catechols. This could, however, be due to instability and their tendency to autoxidize in contact with air.

To confirm oxidative stress as a potential mechanism of 2-OHCHR toxicity, TBARS concentrations were measured following treatment with 2-OHCHR, as well as 6-OHCHR and hydrogen peroxide as a negative and positive control, respectively. Significantly higher concentrations were found between 2-OHCHR and the vehicle control (0.1% DMSO) while no significant differences were found between 6-OHCHR and the vehicle control, indicating that 2-OHCHR is indeed causing oxidative stress within treated embryos. Furthermore, exposure to H_2O_2, a potent oxidant, produced a nearly identical anemic phenotype which was followed by mortality soon after which further supports the hypothesis. To visualize where oxidative stress is taking place, embryos were stained with DCFDA, a fluorescent dye used as an indicator for ROS. Staining revealed that oxidative stress is most prevalent in the tails of embryos, including muscle, bone, and some vasculature. This finding is supported by daily observations during exposures where tails

of embryos were found to necrose before the rest of the body followed suit, eventually resulting in mortality. Oxidative stress has been observed in similar regions following treatment with other chemicals in medaka embryos (Kupsco and Schlenk, 2016). While no significant fluorescence was observed within non-caudal blood vessels, this could be explained by interference by the natural fluorescence of the yolk, lowering the signal to noise ratio. Interestingly, early-stage hematopoiesis in teleost embryos occurs within the intermediate cell mass blood islands which are located in the caudal region near the base of the notochord (Iwamatsu et al., 2004). Blood circulation begins at roughly 50 hpf in medaka embryos which closely coincides with the exposure durations in this study. High concentrations of ROS in the caudal region could indicate that 2-OHCHR-induced oxidative stress may be damaging the blood islands which could be causing the anemic phenotype. However, it is not known how long medaka embryos rely on blood islands for hematopoiesis so additional studies measuring other ROS metabolites may be more effective in determining locations within specific cell types.

In summary, AhR played an important role in 2-OHCHR toxicity, presumably through the activation of CYP1 enzymes leading to the formation of oxidatively active metabolites (ex. 1,2-catechol) which were consistent with oxidative stress in embryos treated with 2-OHCHR. While AhR was crucial, strong activation of AhR alone did not cause toxicity, indicating metabolic conversion to a reactive intermediate was needed. Consequently, a potential pathway for the anemic phenotype of 2-OHCHR may be as follows; 2-OHCHR exposure leads to AhR activation, spurring an upregulation of CYP1 which forms the 1,2-

catechol metabolite. The catechol then generates ROS by redox cycling from the semiquinone radical, causing oxidative stress within the embryos which causes anemia and subsequent mortality. Our findings indicate that AhR activation may be involved in the enhanced bioactivation of 2-OHCHR, but that basal metabolism without AhR could also occur. While oxidative stress may be a molecular initiating event eliciting the toxicity of 2-OHCHR, additional key events linking oxidative stress to anemia are needed to better understand the ecological risks of these particular oxy-PAHs.

Chapter 5: Summary and conclusions

5.1 Summary

The overarching goal of this research was to characterize the regioselective mechanisms of toxicity of hydroxychrysenes on Japanese medaka embryos. This work was based on observations of regioselective toxicities between several oxy-PAHs, including studies from out lab utilizing 2- and 6-OHCHR and zebrafish models (Diamante et al., 2017). The experiments in this dissertation were carried out using Japanese medaka due to their long developmental time providing greater insight into sensitive developmental stages, as well as a large library of literature describing the effects of PAHs and their degradates on this species. By characterizing the mechanisms of oxy-PAHs we may be able to better understand the risks that certain classes of oxy-PAHs could pose to the environment.

Chapter 2 presents the toxic effects of 2- and 6-OHCHR on embryonic Japanese medaka. Embryos were exposed to 0.5, 2, or 5 μM 2- or 6-OHCHR from 4-172 hpf and observed

for anemia and mortality daily. We found that 2-OHCHR caused significant anemia and mortality at all doses while 6-OHCHR did not. This contrasts results from Diamante et al. (2017) where only 2-OHCHR caused significant anemia in embryonic zebrafish but only 6-OHCHR caused significant mortality. This indicated not only regioselective effects, but also potential species-specific toxicities for oxy-PAHs. Following confirmation of 2-OHCHR toxicity in embryonic Japanese medaka, we searched for a sensitive window of development. Embryos were exposed to 5 μM 2-OHCHR from 13-15, 38-41, 82-95, or 95-101 hpf. While some toxicity was observed, the magnitudes were much lower compared to previous treatments, indicating that there may not have been enough time for sufficient uptake of the compound. This led to exposures beginning at the same stage as before, but this time ending at 172 hpf. Exposures were also carried out beginning at 4 hpf and lasting for 24, 48, 72, or 96 h. Results from these exposures indicated that significant toxicity was not observed until 76 hpf. To normalize for duration of uptake, embryos were exposed to 10 μM 2-OHCHR from 4-28, 28-52, 52-76, 76-100, and 100-124 hpf. Exposures from 52-76 and 76-100 hpf resulted in significantly higher anemia and mortality compared to other exposures, indicating that there is a window of sensitivity to 2-OHCHR exposure between 52-100 hpf.

Interestingly, this window closely coincided with liver development in Japanese medaka. The liver contains high concentrations of enzymes that are important in the metabolism of several chemicals, including PAHs. Some of these enzymes include CYP, UGT, and SULT which can detoxify chemicals but also activate them in some cases. Therefore, in

chapter 3, we explored the role of metabolism on the toxicities of 2- and 6-OHCHR on embryonic Japanese medaka. First, embryos were exposed to 10 μM 2- or 6-OHCHR from 52-76 hpf, the most sensitive window of exposure elucidated in chapter 2, then metabolites were extracted, identified, and quantified. The chemicals were found to form metabolites in different proportions, with 2-OHCHR forming 68.1% ± 24.1 more sulfates and 370% ± 59.9 more catechols. Although 18.0% ± 4.58 of 6-OHCHR was metabolized to the 5,6-quinone, no quinones were observed in 2-OHCHR extracts. While the percent conversion was similar between both compounds, glucuronides were the major conjugates for both as well. Therefore, we analyzed the toxicokinetics of both compounds and found that 6-OHCHR was not only taken up 97.2% ± 0.18 faster than 2-OHCHR, but also eliminated 57.7% ± 0.36 faster as a glucuronide conjugate. To test if rapid glucuronidation is causing 6-OHCHR to be less toxic, embryos were pretreated with 10 μM nilotinib, a UGT inhibitor, from 28-52 hpf before being treated with 10 μM 2- or 6-OHCHR from 52-76 hpf. While significant reductions in glucuronide concentrations were observed for both compounds, inhibitor pretreatments did not increase the toxicity of either compound. We then focused on phase I metabolism, treating embryos with 0.5, 3, or 5 μM of the putative metabolites 1,2- or 5,6-CHQ from 4-172 hpf. Several groups have observed PAH quinones to be more toxic than parent PAHs (Knecht et al., 2013; Elie et al., 2015), so we hypothesized that these quinones would be as potent, if not more toxic than the hydroxychrysenes. Surprisingly, neither chemical caused anemia or mortality. Uptake of both compounds was confirmed by tissue extractions and chromatographic analysis. However, pretreatments with ketoconazole, a broad spectrum

CYP inhibitor, significantly reduced anemia and mortality following 2-OHCHR treatments. Inhibitor pretreatment also reduced 1,2-catechol formation in 2-OHCHR treatments by 64.4% ± 2.14. These results indicated that a CYP metabolite, potentially the 1,2-catechol, may be causing the toxic effects of 2-OHCHR.

The AhR is an important nuclear receptor that can not only regulate CYP expression but can also cause toxicity through several signaling pathways (Safe et al., 2000; Benamian et al., 2004; Gräns et al., 2010; Vorrink et al., 2014; Wright et al., 2017). Some PAH toxicities have been reported to be AhR-dependent while others are toxic independent of AhR. Since CYP was shown to be critical to 2-OHCHR toxicity in chapter 3, we explored its role in toxicity in Chapter 4. Because we showed that 1,2-catechol was associated with toxicity, and it is an oxidatively active metabolite, we also explored the role of oxidative stress in 2-OHCHR toxicity. To explore the role of AhR in 2-OHCHR toxicity, embryos were exposed to potent AhR agonists, PCB126 and 2-methoxychrysene, as well as the AhR antagonist CH-223191. While PCB126 and 2-methoxychrysene did not cause significant anemia or mortality at 100 hpf, pretreatment with CH-223191 reduced anemia by 97.2% ± 0.84 and mortality by 96.6% ± 0.69. AhR inhibition was confirmed by a 91.0% ± 9.94 reduction in EROD activity. The magnitude of protection, as well as reduction in EROD activity were both very similar to that which was observed with ketoconazole, the CYP inhibitor. Pretreatment with antioxidants also prevented toxicity in embryos treated with 2-OHCHR. In addition, concentrations of thiobarbituric acid reactive substances (TBARS) were significantly higher (32.9% ± 0.04)

in 2-OHCHR exposures at 100 hpf compared to controls, indicating oxidative stress. Staining with DCFDA revealed that the highest concentration of ROS was in the tails of the embryos, which has been observed with chemicals (Kupsco and Schlenk, 2016). These results confirm the importance of AhR to 2-OHCHR toxicity while also indicating that AhR activation alone does not cause the toxicity. The role of CYP is also highlighted, with an oxidatively active metabolite like 1,2-catechol likely causing the observed toxicity through redox cycling between itself and a semiquinone radical, generating ROS.

5.2 Conclusions

In conclusion, the hypotheses and results of each chapter build upon and complement each other to piece together the puzzle of the regioselective toxicities of 2- and 6-OHCHR. Our results allow us to draw a hypothetical pathway of 2-OHCHR as follows; 2-OHCHR exposure leads to AhR activation, causing an upregulation of CYP which forms the 1,2-catechol metabolite. The catechol then generates ROS by redox cycling from the semiquinone radical, causing oxidative stress within the embryos which causes anemia and subsequent mortality. We hope that this work can be used to further investigate the risks of oxy-PAHs and to help narrow the species to focus risk assessments and monitoring. While there are hundreds of oxy-PAHs in the environment, many of which causing unique effects, a better understanding of their toxicities, particularly mechanisms of action can help group these compounds into chemicals of concern and reduce uncertainty in ecological risk assessments.

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Appendix A – Chapter 2 supplemental information

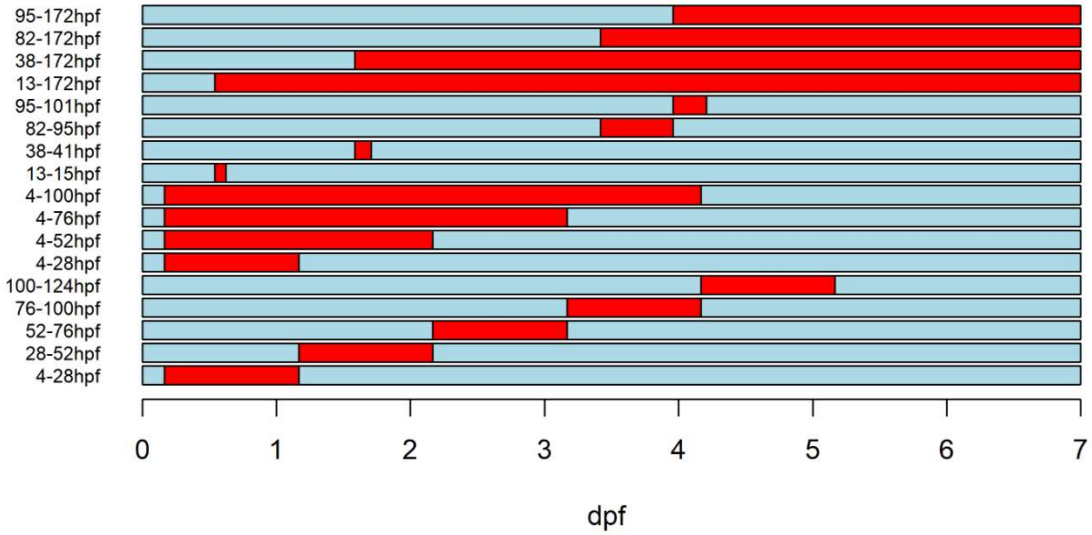

Figure A-1. Exposure regime. Blue = 1% DMSO vehicle control, red = 2-OHCHR. 10 µM 2-OHCHR was used for 4-28, 28-52, 52-76, 76-100, and 100-124 hpf exposures (bottom five) and 5 µM for all other exposures listed above.

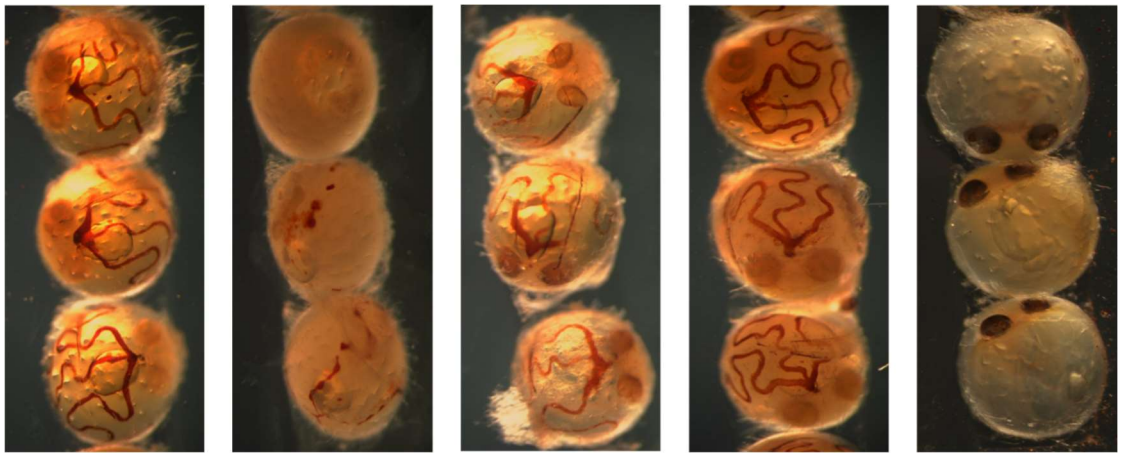

Figure A-2. Medaka embryos stained with o-dianisidine at 7 dpf. From left to right; 1% DMSO vehicle control, 5 µM 2-OHCHR, 5 µM 6-OHCHR, 5 µM chrysene, 5 µM butafenacil.

Table A-1. Measured concentrations of 2- or 6-OHCHR in exposure solutions. Values represent the mean of three replicates ± SEM. ND = not detected.

	Nominal Concentration (µM)	Measured Initial Concentration (ng/mL)	Percent of Expected Initial Concentration (%)	Measured Final (172 hpf) Concentration (ng/mL)
	0	ND	-	ND
2-OHCHR	0.5	105.33±10.01	86.23±8.19	88.03±10.84
2-OHCHR	2	381.8±66.76	78.14±13.66	351.53±42.68
2-OHCHR	5	1130±50.09	92.51±4.1	979±83.22
6-OHCHR	0.5	105.97±10.49	86.75±8.59	94.7±12.22
6-OHCHR	2	372.2±82.16	76.18±16.82	329.1±34.92
6-OHCHR	5	1100.33±44.11	90.08±3.61	928±143.47

Appendix B – Chapter 3 supplemental information

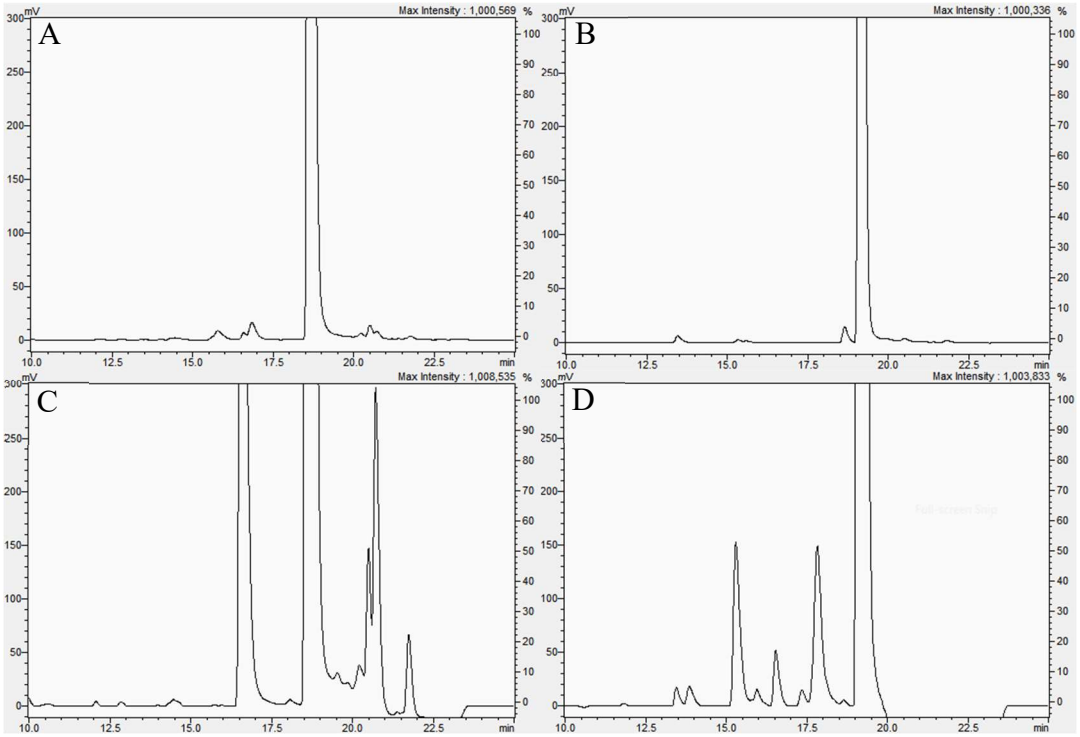

Figure B-1. Chromatograms of tissue extracts of embryos exposed from 52-76 hpf 2- or 6-OHCHR. A and B are 2- and 6-OHCHR extracts after 24 h uptake without glucuronidase or sulfatase treatment. C and D are the same treatments but with glucuronidase and sulfatase.

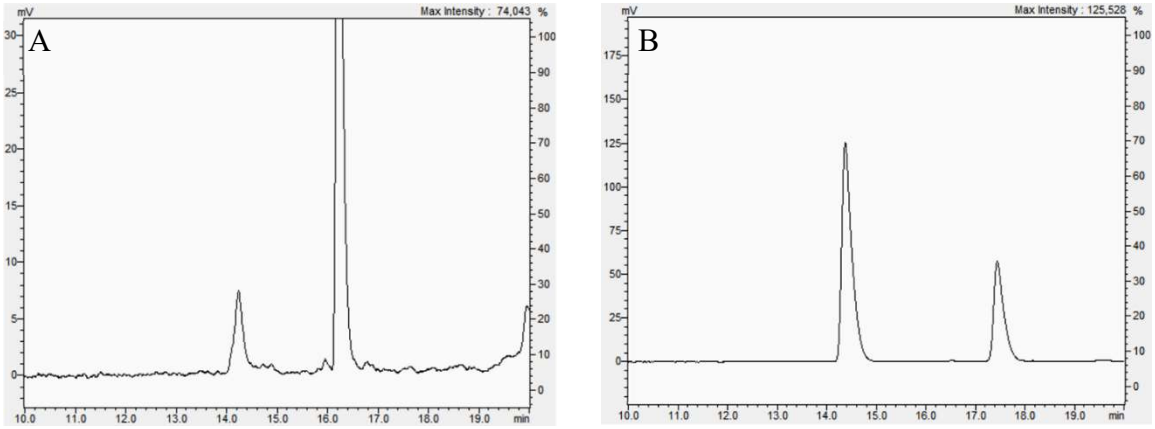

Figure B-2. Fluorescence chromatograms of sodium borohydride reductions of 1,2- (A) and 5,6-chrysenequinone (B). The right peak is the catechol while the left is suspected to be a dihydrodiol.

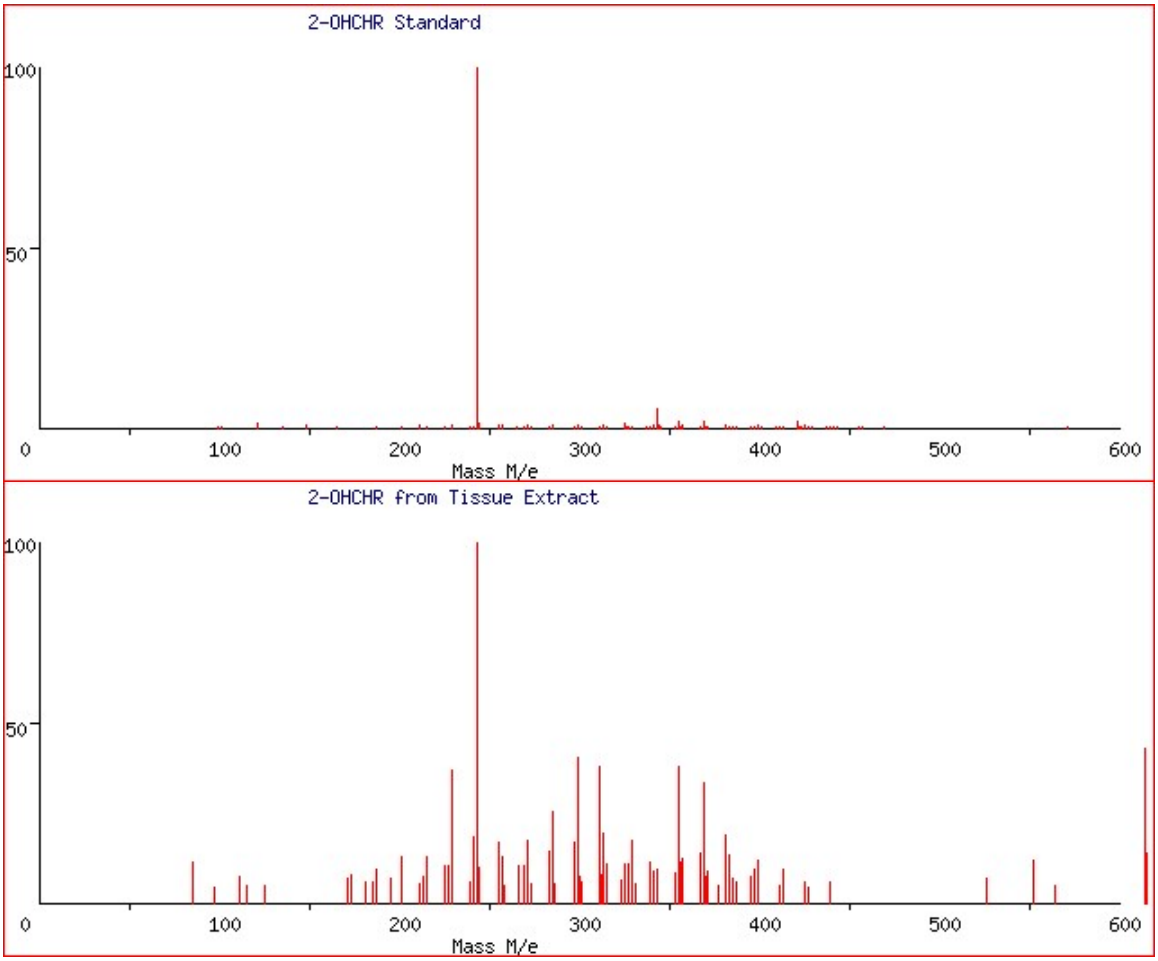

Figure B-3. High resolution mass spectra of 2-OHCHR from analytical standards compared to tissue extracts after exposure to 2-OHCHR from 52-76 hpf. Samples were run in negative ion mode.

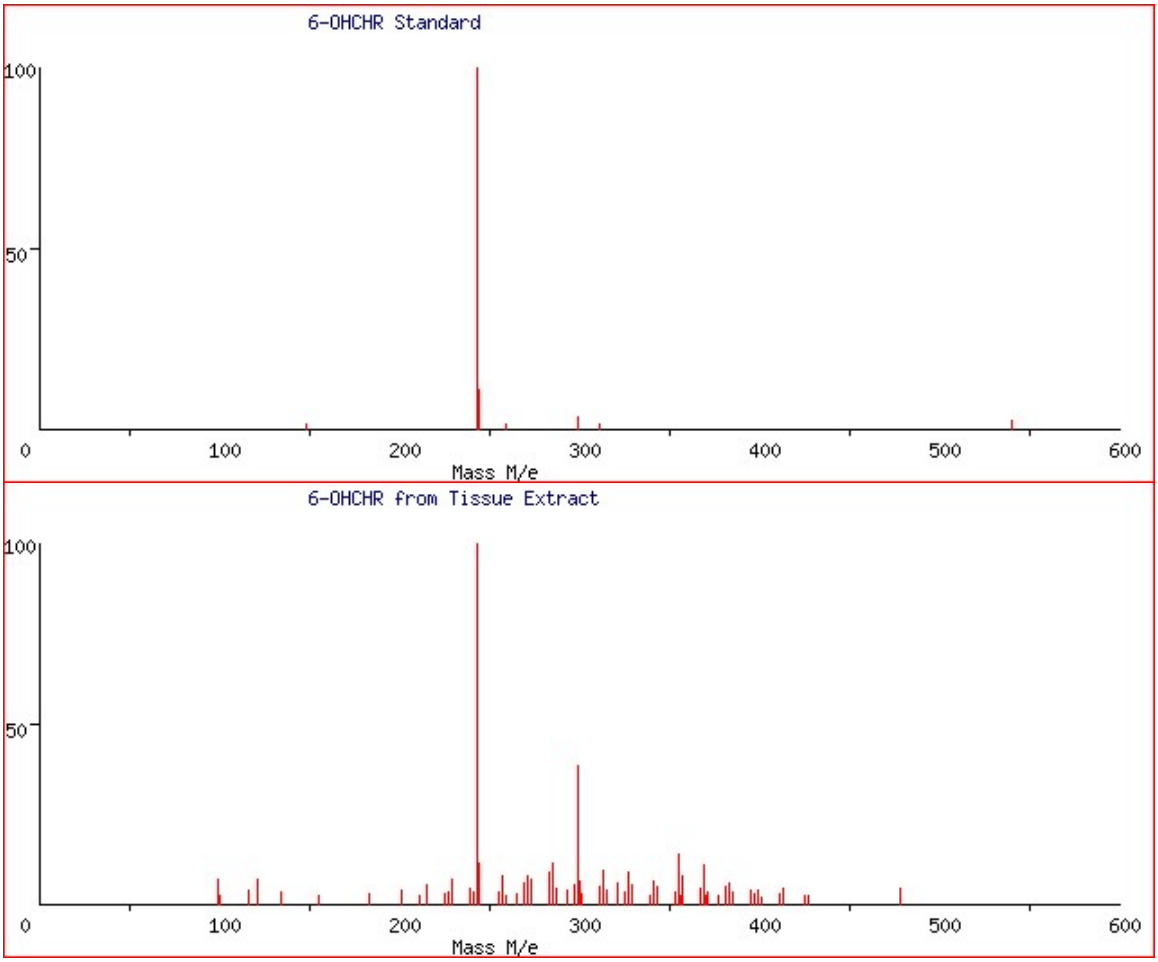

Figure B-4. High resolution mass spectra of 6-OHCHR from analytical standards compared to tissue extracts after exposure to 6-OHCHR from 52-76 hpf. Samples were run in negative ion mode.

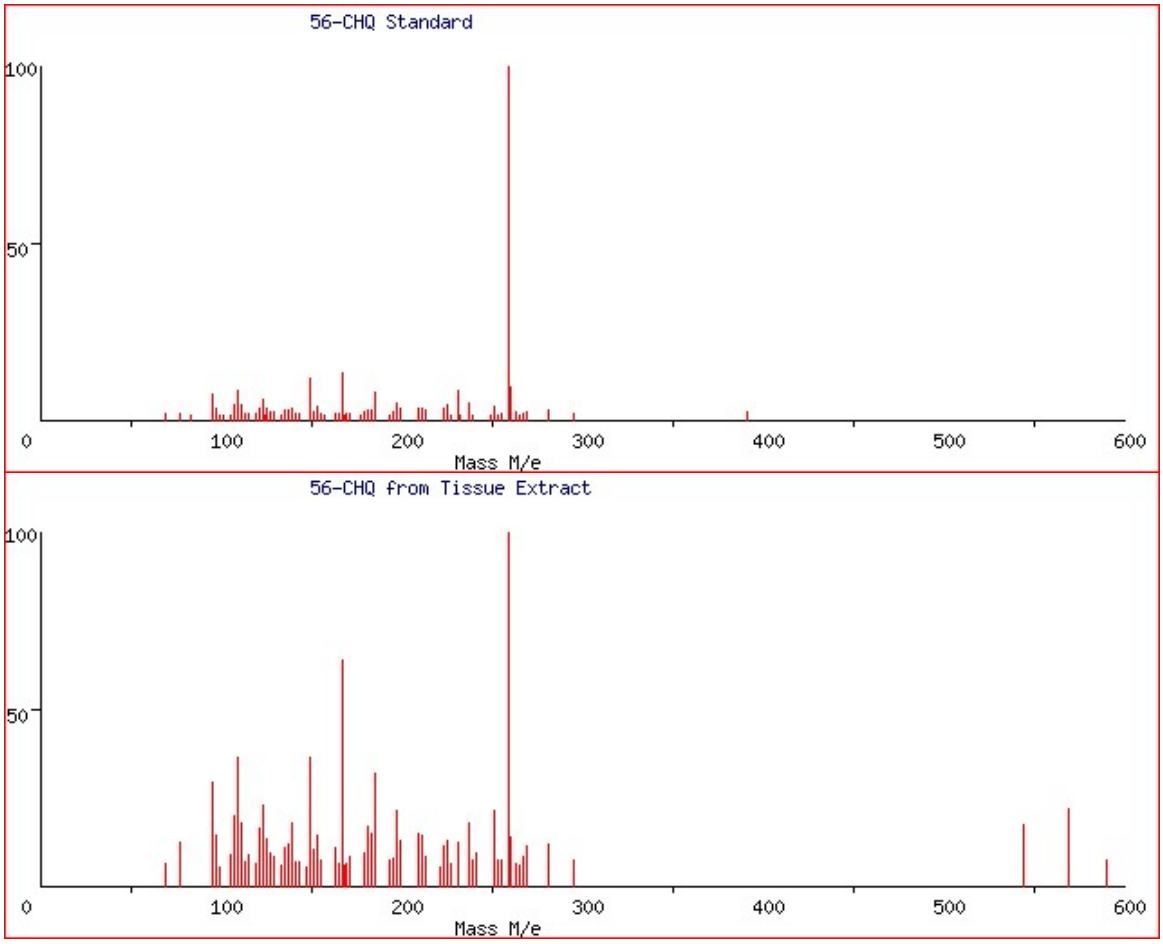

Figure B-5. High resolution mass spectra of 5,6-CHQ from analytical standards compared to tissue extracts after exposure to 6-OHCHR from 52-76 hpf. Samples were run in positive ion mode.

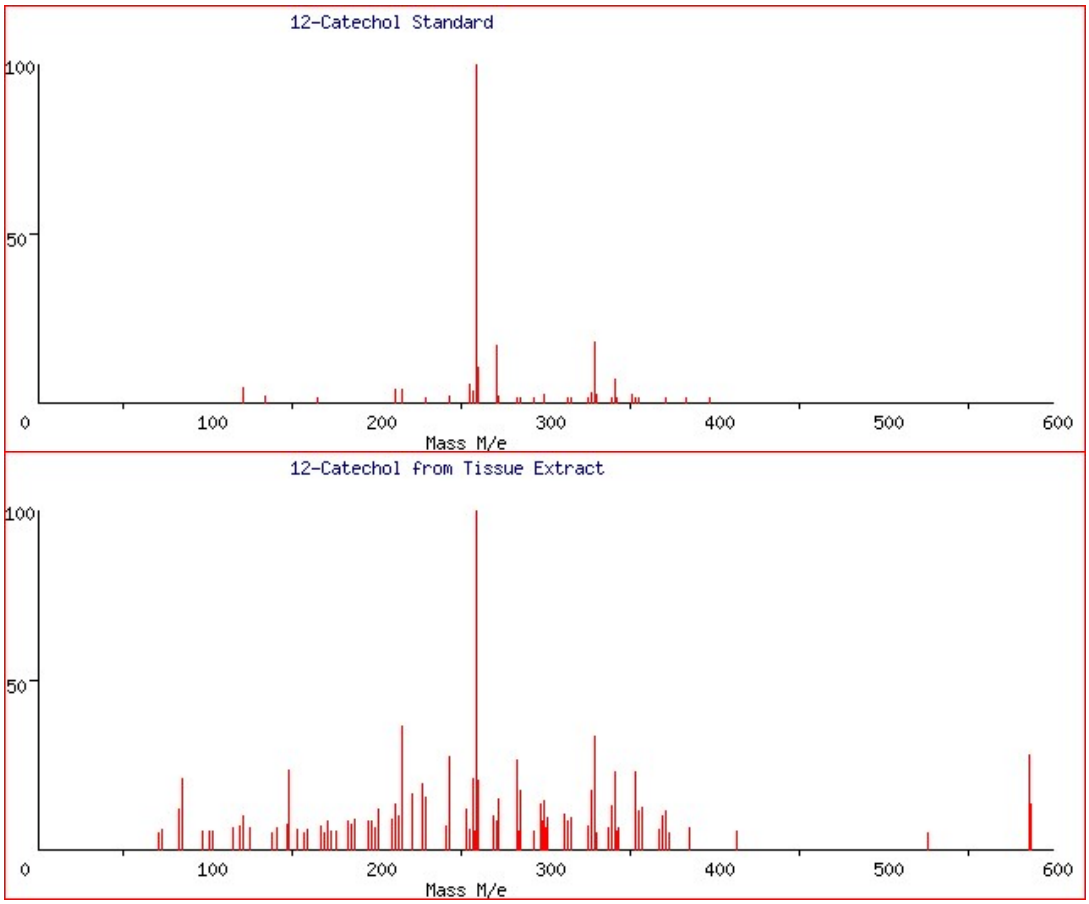

Figure B-6. High resolution mass spectra of the 1,2-Catechol of chrysene from sodium borohydride reductions of 1,2-CHQ compared to tissue extracts after exposure to 2-OHCHR from 52-76 hpf. Samples were run in negative ion mode.

Table B-1. Mass balance of 2- and 6-OHCHR adsorbed to the exposure dish (glass) and onto embryos, taken up by the embryos, and how much remained within the water. Values represent the mean of three replicates ± SEM.

	2-OHCHR	**6-OHCHR**
% in water	76.5 ± 0.948	74.5 ± 0.921
% on glass	16.9 ± 1.03	11.5 ± 1.439
% on embryo	2.29 ± 1.317	4.76 ± 1.657
% uptake	4.22 ± 1.23	9.17 ± 1.03