MECHANISMS OF TOXICITY OF THE TIRE-WEAR COMPOUND N-(1,3-DIMETHYLBUTYL)-N'-PHENYL-P-PHENYLENEDIAMINE-QUINONE (6PPD-QUINONE) IN FATHEAD MINNOWS (*PIMEPHEALES PROMELAS*)

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DEDICATION

This thesis is dedicated to my parents, who instilled in me a love for learning and have always believed in me.

ABSTRACT

N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-quinone), is an emerging contaminant of concern (ECC) that has been recently identified as the primary cause of an acute lethality event observed in coho salmon (*Oncorhynchus kisutch*), termed Urban Runoff Mortality Syndrome (URMS). Given the widespread occurrence of 6PPD-quinone, there is a need to identify other species of fishes that experience acute lethality and sublethal toxicity following exposure, and to characterize mechanisms of toxicity. It is not known if fathead minnows (*Pimephales promelas*), a native species in North America, and a model species in ecotoxicology, are sensitive to 6PPD-quinone. Aqueous exposure to 6PPD-quinone did not cause mortality or embryo malformations in fathead minnows. Fathead minnow adults showed biochemical signatures of oxidative stress in both the livers and gills, including altered glutathione metabolism and an increase in methionine sulfoxide. This result suggested that fathead minnows could be used as a model species to investigate sublethal effects of 6PPD-quinone. However, fathead minnow embryos microinjected with 6PPD-quinone experienced a suit of cardiovascular abnormalities, including abnormal heart morphology, lack of common cardinal vein development, and hemorrhaging. Fathead minnow embryos that were microinjected with 6PPD-quinone showed a strong transcriptomic response, with alterations in pathways such as vascular endothelial growth factor signalling, endothelial cell-cell adhesion, and inflammation, providing a molecular basis for the cardiotoxicities. This supports the hypothesis that toxicokinetics is a critical determinant of 6PPD-quinone toxicity. This is the first study to demonstrate that sensitivity to 6PPD-quinone can be induced under laboratory conditions in insensitive species of fishes. Overall, this study fills critical knowledge gaps underlying mechanisms of toxicity of 6PPD-quinone to fishes.

USE OF GENERATIVE AI

Microsoft Copilot AI was used to search for academic literature and aided with grammar correction and synonym suggestions in Chapters 3 and 4 of this thesis.

CONTRIBUTION OF AUTHORS

Katherine Anderson-Bain is the primary author of Chapters 1-4. The candidate primarily designed and conducted experiments and analyzed data for Chapters 2-3. Dr. Steve Wiseman contributed to scientific input and guidance of Chapters 1-4. Dr. Markus Brinkmann and Dr. Markus Hecker contributed scientific input and guidance of Chapters 2-3. Tony Montina provided scientific input and guidance for Chapter 2. Dr. Alper J. Alcaraz assisted with data interpretation and figure generation in Chapter 2. Dr. Xiaowen Ji conducted chemical analysis for Chapter 2. Dr. Phillip Ankley conducted chemical analysis for Chapter 3.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

6PPD	N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine
6PPD-quinone	N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone
AHR2	Aryl hydrocarbon receptor 2
AO	Adverse outcome
AON	Adverse outcome network
AOP	Adverse outcome pathway
ARNT	Aryl hydrocarbon receptor nuclear translocator
B[a]P	Benzo[a]pyrene
COX	Cytochrome c oxidase
CYP17A1	Cytochrome P450 17A1
CYP1A	Cytochrome P450 1A
CYP2AD6	Cytochrome P450 2D6
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpf	Days post-fertilization
DPG	1,3-diphenylguanidine
ECC	Emerging contaminant of concern
GPX	Glutathione peroxidase
hpf	Hours post-fertilization
HIS	Hepatosomatic index
IL1B	Interleukin 1β
IRAK3	Interleukin-1 receptor associated with kinase 3
K	Fulton's condition factor
KE	Key event
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LC	Lethal concentration
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MIE	Molecular initiating event
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
MTHFR	Methylenetetrahydrofolate reductase
NMR	Nuclear magnetic resonance
NR5A2	Liver receptor homolog-1
ODC1	Ornithine decarboxylase
OPLS-DA	Orthogonal partial least squares discriminant analysis
PAH	Polycyclic aromatic hydrocarbon
PCP	Personal care product
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PRKCBA	Protein kinase C

RNAseq	RNA sequencing
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SRC	v-src avian sarcoma viral oncogene homolog
TSP	Trimethylsilypropanoic acid
UCP	Uncoupling protein
URMS	Urban Runoff Mortality Syndrome
VEGF	Vascular endothelial growth factor
VIAVC	Variable Importance Analysis based on Random Variable Combination test

*Throughout this thesis, genes are reported is upper case italics, mRNA is reported in lower case italics, and protein is reported in upper case and non-italicized.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Urban Stormwater Runoff as a Source of Pollution

Human activities can result in extensive quantities of natural and anthropogenic chemicals being released into the environment. Environmental contaminants may originate from a variety of sources, including industrial processes, construction, traffic, gardening, and park maintenance (Müller et al., 2020). While people continually engage in production and other pursuits to enhance quality of life, chemical byproducts from these undertakings may enter the air, land, or water and have unintended harmful effects.

Urban stormwater runoff is recognized as a significant source of contaminants to aquatic ecosystems. Surfaces that are integral to urbanization, such as roads and sidewalks, allow for the accumulation of pollutants. Following precipitation events, stormwater runs over the surfaces and mobilizes pollutants that then enter aquatic environments. Urban stormwater contains a complex mixture of chemicals including, but not limited to, plastics, pharmaceuticals, personal care products (PCPs), surfactants, pesticides, metals, and polycyclic aromatic hydrocarbons (PAHs) (Fairbairn, 2018; Grung et al., 2022; Müller et al., 2020). Vehicles can be a major source of some of these contaminants. For example, gasoline, exhaust, tire wear, brake pads, and metal corrosion can be sources of pollutants, such as heavy metals, PAHs, and sulfur oxides (Gupta, 2019; Hwang et al., 2019). In particular, vehicle tires are sources of vulcanizers (chemicals that harden rubbers) such as 1,3-diphenylguanidine (DPG), PAHs such as benzo[a]pyrene (B[a]P), heavy metals such as zinc (Zn) and cadmium (Cd), and antiozonants such as N-(1,3-dimethylbutyl)-N’-phenyl-*p*-phenylenediamine (6PPD), which could pose a risk to the health of aquatic environments (Tamis et al., 2021; Hwang et al., 2016).

1.2 Urban Runoff Mortality Syndrome

Rapid mortality has been observed along western North America when pre-spawn coho salmon (*Oncorhynchus kisutch*) encounter urban stormwater runoff during their annual return from the Pacific Ocean to freshwater creeks for spawning. The phenomenon, called Urban Runoff Mortality Syndrome (URMS) or Urban Stream Syndrome, was first reported to occur in the 1990s following ecological restoration projects in Seattle (Scholz et al., 2011). URMS poses a conservation threat to coho salmon as rates of lethality in urban watersheds have been observed to be as high as 40-90% (Feist et al., 2017). Coho salmon that encounter urban stormwater runoff display a progressive suit of symptoms, including circular surface swimming, gasping, and loss of equilibrium before suffering mortality (Tian et al., 2021). Interestingly, other species of salmon that are found in the same streams as coho salmon, such as chum salmon (*Oncorhynchus keta*), were found not to suffer from URMS when exposed to urban road runoff (McIntyre et al., 2018). Because of the threat that URMS poses to coho salmon, a lot of effort has been directed towards identifying the causative agent(s). Factors such as water temperature, dissolved oxygen, pathogens, and disease were ruled out, but coho salmon mortality had been associated with elevated levels of PAHs and metals (Scholz et al., 2011). Despite this association, it was determined that metals and PAHs were not the cause of URMS in coho salmon (Spromberg et al., 2016). URMS was then associated with urban stormwater runoff that contained tire-wear particles (Peter et al., 2018). In 2021, a landmark study found that N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-quinone) was the causative agent of URMS (Tian et al., 2021). Subsequent laboratory studies showed that the LC_{50} (the concentration that kills 50% of exposed animals) of 6PPD-quinone to coho salmon was 95 ng/L (Tian et al., 2022).

6PPD-quinone is a transformation product of its precursor, 6PPD, which is an antiozonant that is added to motor vehicle tires to prevent the rubber from degrading and cracking (Tian et al., 2021; Evans et al., 1992; Layer and Lattimer, 1990). During vehicle operation, 6PPD reaches the surface of tires and reacts with atmospheric ozone, resulting in the formation of 6PPD-quinone. While the antiozonant properties are the intended use of 6PPD, 6PPD-quinone is an unexpected product of this reaction (Tian et al., 2021; Evans et al., 1992; Layer and Lattimer, 1990). As the vehicle continues to be driven, tire-wear particles with 6PPD-quinone are deposited onto the road. Fish and other aquatic organisms are exposed to 6PPD-quinone during rainfall events, when 6PPD-quinone enters stormwater runoff that enters its receiving water body (Tian et al., 2021).

1.3 Environmental Occurrence of 6PPD-quinone

Identification of 6PPD-quinone as the driver of URMS triggered global efforts to quantify its abundance in the environment. In Canada, 6PPD-quinone in urban runoff has been detected at concentrations up to 2.30 µg/L in the Greater Toronto Area and 1.40 µg/L in Saskatoon (Challis et al., 2021; Johannessen et al., 2021). In the United States, 6PPD-quinone has been detected in urban water samples at concentrations > 1.2 µg/L in Seattle, > 0.2 µg/L in San Francisco, and > 0.4 µg/L in Los Angeles (Tian et al., 2022). Concentrations up to 2.43 µg/L in Hong Kong and 1.56 µg/L in the Pearl River Delta Region of China have been reported (Cao et al., 2022; Zhang et al., 2023). While its presence in urban stormwater runoff poses the biggest threat to aquatic organisms, 6PPD-quinone has also been found in soil, air/dust, river sediment, and wastewater in Australia, China, Japan, and Germany, respectively (Cao et al., 2022; Hiki and Yamamoto, 2022; Rauert et al., 2022; Seiwert et al., 2022).

The potential threat that 6PPD-quinone poses to aquatic environments is highlighted by estimates of mean annual production. Annual global tire rubber emissions are an average of 0.81 kg per person, and it is estimated that up to 45% of tire particles deposited onto roadways can potentially enter receiving waters (Sieber et al., 2020; Wagner et al., 2018). Because the fraction of 6PPD in tires is 0.4 – 2.0%, each individual passenger car is estimated to produce 1.4-500 g of 6PPD-quinone and heavy trucks are estimated to produce 36-10,000 g of 6PPD-quinone (Tian et al., 2021). This is a cause for concern as the use of 6PPD is widespread, and the concentrations of 6PPD-quinone found in receiving waters exceed the LC_{50} for some fish species.

1.4 Acute Lethality Induced by 6PPD-quinone

Since the discovery of 6PPD-quinone as the cause of URMS in coho salmon, there have been numerous studies to identify other species of fish that may be sensitive to 6PPD-quinone induced lethality. To date, sensitivity to 6PPD-quinone is highly species-specific among salmonids. Brook trout (*Salvelinus fontinalis*), rainbow trout (*Oncorhynchus mykiss*), white-spotted char (*Salvelinus leucomaenis pluvius*), and lake trout (*Salvelinus namaycush*) are sensitive to environmentally relevant concentrations of 6PPD-quinone, with LC_{50}s of 0.59 µg/L, 1.00 µg/L, 0.51 µg/L, and 0.50 µg/L, respectively (Roberts et al., 2025; Hiki and Yamamoto, 2022; Brinkmann et al., 2022). Other species of salmonids, including Arctic char (*Salvelinus alpinus*), southern Asian dolly varden (*Salvelinus curilus*), landlocked masu salmon (*Oncorhynchus masou masou*), Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), westslope cutthroat trout (*Onchorhymchus clarkia lewisi*), and pink salmon (*Oncorhynchus gorbuscha*) are insensitive to acute lethality (Foldvik et al., 2024; Montgomery et al., 2023; Hiki and Yamamoto, 2022; Foldvik et al., 2022; Brinkmann et al., 2022). 6PPD-quinone does cause acute lethality of juvenile chinook salmon, although the LC_{50} of 67.31 µg/L is well above environmentally

relevant concentrations (Lo et al., 2023). While few non-salmonid species have been assayed for their sensitivity, laboratory model species, such as zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), as well as more environmentally relevant species such as white sturgeon (*Acipenser transmontanus*) and red drum (*Sciaenops ocellatus*) are insensitive to 6PPD-quinone induced acute lethality (Ackerly et al., 2024; Brinkmann et al., 2022; Hiki et al., 2021). Interestingly, there appears to be no phylogenetic relationship between species that are sensitive and those that are not sensitive to 6PPD-quinone (Table 1.1). This contrasts patterns of species sensitivity to some other classes of contaminants, such as pesticides, where sensitive species have close phylogenetic relationships (Mei, 2024).

Table 1.1. Species of fishes that have been tested for sensitivity to 6PPD-quinone, and if applicable, their LC_{50}.

Salmonids				
Genus	**Species**	**Common name**	**Sensitivity**	**LC_{50} (µg/L)**
Oncorhynchus	*kisutch*	Coho salmon	Sensitive	0.095
Oncorhynchus	*mykiss*	Rainbow trout	Sensitive	1.00
Oncorhynchus	*masou masou*	Landlocked masou salmon	Insensitive	> 3.5
Oncorhynchus	*gorbuscha*	Pink salmon	Insensitive	> 12.8
Oncorhynchus	*tshawytscha*	Chinook salmon	Insensitive at environmentally relevant concentrations	67.31
Salvelinus	*fontinalis*	Brook trout	Sensitive	0.59
Salvelinus	*leucomaenis pluvius*	White-spotted char	Sensitive	0.51
Salvelinus	*namaycush*	Lake trout	Sensitive	0.50
Salvelinus	*curilus*	Southern Asian dolly varden	Insensitive	> 3.8
Salmo	*salar*	Atlantic salmon	Insensitive	> 12.16
Salmo	*trutta*	Brown trout	Insensitive	> 12.16

Non-salmonids				
Genus	**Species**	**Common name**	**Sensitivity**	**LC_{50} (µg/L)**
Acipenser	*transmontanus*	White sturgeon	Insensitive	> 12.7
Sciaenops	*ocellatus*	Arctic char	Insensitive	> 14.2
Sciaenops	*ocellatus*	Red drum	Insensitive	> 449.85
Danio	*rerio*	Zebrafish	Insensitive	> 54
Oryzias	*latipes*	Japanese medaka	Insensitive	> 35

Mechanisms that result in acute lethality remain to be fully elucidated. A study using rainbow trout cell lines demonstrated mitochondria uncoupling in gill cells exposed to 6PPD-quinone (Mahoney et al., 2022). Mitochondria uncoupling can result in inefficient oxygen utilization, and because the gills are the primary respiratory organ in adult fishes, this might explain the gasping behaviour that is observed prior to death. Another proposed cause of 6PPD-quinone induced acute lethality is cardiovascular disruption that leads to plasma leakage of the blood-brain barrier, which is thought to potentially lead to shock and then death (Blair et al., 2021). Additionally, two studies using sublethal chronic exposures to the early life-stages of sensitive species of salmonids further supported that 6PPD-quinone toxicity could include disruption of the cardiovascular system, with coho salmon having gene expression changes that could indicate vascular leakage, and lake trout displaying bleeding in the caudal vein (Roberts et al., 2025; Greer et al., 2023).

The mechanistic basis of species differences in sensitivity to 6PPD-quinone induced acute lethality is not fully known but evidence suggests it is due to toxicokinetic factors – rates of absorption, distribution, metabolism, and excretion (Montgomery et al. 2023). While uncoupling of mitochondrial respiration was observed in a rainbow trout gill cell line, this effect did not occur in liver cells exposed to 6PPD-quinone. Liver cells, but not gills cells, were able to biotransform 6PPD-quinone into a hydroxylated metabolite and did not experience cytotoxicity, which suggests that this hydroxylated metabolite may be important to detoxification (Mahoney et al., 2022). In another study, *in vivo* exposures showed that 6PPD-quinone underwent hydroxylation and glucuronidation with a species-sensitivity relationship, with species that are more sensitive to 6PPD-quinone containing less of these metabolites in their bile than species that are insensitive (Montgomery et al., 2023). Taken together, this suggests that acute lethality to

6PPD-quinone might be due to differences in basal levels of metabolic enzymes; fish that are sensitive are likely unable to metabolize 6PPD-quinone, thus resulting in URMS.

1.5 Sublethal Toxicity Induced by 6PPD-Quinone

While there has been a large focus on 6PPD-quinone induced acute lethality to fishes, there has been much less focus on its sublethal effects. Identified potential sublethal effects of 6PPD-quinone to fishes include increased oxygen consumption, decreased locomotor activity, and altered neurotransmitter profiles (Ricarte et al., 2023; Varshney et al., 2022). Sublethal effects of 6PPD-quinone exposure to fishes require further investigation as they are also cause for concern as many fishes may not experience acute lethality but could still experience other adverse effects due to the widespread environmental occurrence of 6PPD-quinone.

1.6 Fathead Minnows as a Model Organism in Toxicology

Fathead minnows (*Pimephales promelas*) are a species of cyprinid that is commonly utilized as a model organism for aquatic toxicology. Fathead minnows are small (approximately 2-5 g), reach sexual maturity after approximately four months, are easy to breed as they are highly sexually dimorphic but have amenable breeding groupings, and the larvae hatch after 4-5 days at 25ºC (Ankley and Villeneuve, 2006). The embryonic development of fathead minnows is well-defined, and the developing embryo can be seen through their transparent chorion which allows the researcher to view development in-detail (Devlin et al., 1996). Additionally, the genome of fathead minnows is annotated, thereby permitting more reliable genomic analyses in comparison to non-model organisms (Saari et al., 2017). Fathead minnows are the most utilized model organism for regulatory toxicology in North America, for many of the reasons stated previously, in addition to the species being both ecologically important across North America and having reliable experimental extrapolations to field settings (Ankley and Villeneuve, 2006).

1.7 Omics in Ecotoxicology

Omics technologies have advanced the field of ecotoxicology by providing researchers with the ability to look at the comprehensive molecular mechanisms that underly the toxic effects of xenobiotics (Ankley et al., 2006). Omics technologies include transcriptomics, proteomics, and metabolomics. Transcriptomics is the study of the changes in the expression of genes by looking at the entire set of mRNA produced in response to a stimulus. Proteomics is the large-scale study of proteins in a given biological system and thus is the intermediate between alterations in gene expression and functional enzymatic outcomes. mRNA has complex regulatory mechanisms, and thus changes in transcript abundances does not always reflect what is translated into a functional protein (Canzler et al., 2020). Metabolomics is the study of metabolites – low-molecular weight molecules – that are necessary for biological functions, such as energy for growth or signalling cofactors (Ankley et al., 2006). Alterations in metabolites are the products of the biological activity of enzymes and thus is reflective of an organism's phenotype (Ankley et al., 2006). Each of these techniques complements each other, and using multi-omics approaches can provide researchers with the ability to gain a thorough insight into mechanisms of toxicity (Canzler et al., 2020).

1.8 Research Objectives and Hypothesis

Due to the widespread occurrence of 6PPD-quinone in the environment, there is a concern that this emerging contaminant of concern is negatively impacting aquatic organisms. Because 6PPD-quinone was a "true unknown" prior to its discovery, it has only recently linked been to URMS, and thus many of the effects of 6PPD-quinone, and their underlying molecular and biochemical causes, remain to be investigated in-detail (Tian et al., 2021). Many studies to-date have focused on acute lethality and apical effects, with less emphasis on the molecular and

biochemical mechanisms of 6PPD-quinone induced toxicity, that occur in fishes. Fathead minnows are an environmentally relevant species to North America and a model species in ecotoxicology. As such, we wanted to test the fathead minnow for sensitivity to 6PPD-quinone and use multi-omics approaches to investigate potential mechanisms of sublethal toxicity or acute lethality.

The overall goal of this thesis was to elucidate mechanisms of toxicity of 6PPD-quinone in fathead minnows. Specific objectives of this research were to:

1. determine if fathead minnows are a species that is "sensitive" to 6PPD-quinone induced acute lethality.
2. If "insensitive", utilize fathead minnows as a model insensitive species to investigate mechanisms of sublethal 6PPD-quinone toxicity in insensitive species. If "sensitive", utilize fathead minnows as a model species to investigate mechanisms of acute lethality of 6PPD-quinone.

Because species-sensitivity to 6PPD-quinone to-date is highly species-specific to salmonids, it is hypothesized that the fathead minnow, a cyprinid, is an insensitive species. Therefore, if insensitive, many of the biochemical and molecular alterations following exposure to 6PPD-quinone are likely indicative of the sublethal effects of 6PPD-quinone.

CHAPTER 2: APICAL AND MECHANISTIC EFFECTS OF 6PPD-QUINONE ON DIFFERENT LIFE-STAGES OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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2.1 Introduction

N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6PPD-quinone) is an emerging contaminant of concern that is generated through the environmental oxidation of the common rubber tire anti-degradant 6PPD. Widespread occurrence of 6PPD-quinone in stormwater runoff and surface waters have been reported in North America, Australia, Germany, and Japan, with maximal concentrations of 2.4 ug/L, dependent on method of analysis (Tian et al., 2022; Challis et al., 2021; Johannessen et al., 2022; Rauert et al 2022, Seiwert et al., 2022). Concerns about impacts of 6PPD-quinone on aquatic systems have received significant attention since Tian et al. (2021) identified 6PPD-quinone as the cause of urban runoff mortality syndrome (URMS) of coho salmon (*Oncorhynchus kisutch*). In a follow-up study, Tian et al. (2022) determined a half-maximal lethal concentration (LC_{50}) of 6PPD-quinone to coho salmon of <0.10 µg/L. Subsequently, highly species-specific sensitivities to 6PPD-quinone across select fishes have been observed. A study assessing the acute lethality of 6PPD-quinone to four species of commercial, cultural, and ecological importance to North America, reported a 24 h LC_{50} of 0.59 µg/L to brook trout (*Salvelinus fontinalis*), and a 72 h LC_{50} of 1.00 µg/L to rainbow trout (*Oncorhynchus mykiss*), but neither Arctic char (*Salvelinus alpinus*) nor white sturgeon (*Acipenser transmontanus*), suffered mortalities after 96 h of exposure to measured concentrations as high as 14.2 µg/L (Brinkmann et al., 2022). In another study, the 24 h LC_{50} of 6PPD-quinone to white spotted charr (S*alvelinus leucomaenis pluvius*) was 0.51 µg/L, while southern Asian dolly varden (*Salvelinus curilus)* or landlocked masou salmon (*Oncorhynchus masou)* were insensitive to concentrations up to 3.8 µg/L (Hiki and Yamamoto, 2022). Brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) were insensitive to concentrations up to 12.6 µg/L, in a 48 h assay (Foldvik et al., 2022). Concentrations as high as 34 and 54 µg/L, did

not cause acute lethality to zebrafish (*Danio rerio*) or Japanese medaka (*Oryzias latipes*), respectively (Hiki et al., 2021). Using larval zebrafish, Varshney et al. (2022) determined a 96 h LC_{50} of 132.92 μg/L when ethanol was used as the solvent vehicle. Mechanistically, acute lethality might be caused by mitochondrial uncoupling in gill (Mahoney et al., 2022). Formation of DNA adducts has been reported in mammalian cells, fish, and invertebrates exposed to 6PPD-quinone (Wu et al., 2023).

Despite the very great sensitivity of some species of fishes to 6PPD-quinone at environmentally relevant concentrations, little is known about the sublethal effects of 6PPD-quinone. Acute exposures to 54 μg/L of 6PPD-quinone did not impact hatching rates or cause malformations of zebrafish embryos (Hiki et al., 2021). In another study with early life-stage zebrafish, exposure to 6PPD-quinone at 25 μg/L caused developmental effects, including lordosis, kyphosis, and a reduction in size of the eye, while decreased heart rate and intestinal reddening were observed in larvae exposed to 10 and 25 μg/L 6PPD-quinone (Varshney et al., 2022). Larvae exhibited increased oxygen consumption after 96 h of exposure, and this effect increased with increasing exposure concentrations and time (Varshney et al., 2022).

Given the widespread occurrence of 6PPD-quinone in urban aquatic ecosystems, a greater understanding of its sub-lethal effects and the specific mechanism by which it causes toxicity is needed. To begin to develop hypotheses for future studies of sublethal effects of 6PPD-quinone, we employed a multi-omics approach to assess effects of 6PPD-quinone on fathead minnows (*Pimephales promelas*) using EcoToxChips and proton nuclear magnetic resonance (^{1}H NMR) metabolomics. The EcoToxChip system consists of 384-well reduced transcriptomics arrays covering key toxicity pathways of regulatory importance as well as an intuitive data evaluation platform (EcoToxXplorer.ca) (Basu et al., 2019; Soufan et al., 2022).

Specifically, EcoToxChips contain 370 different gene targets and six reference genes that have been manually curated into 21 modules representing five core biological processes (cellular processes, endocrine, immune, metabolism, signalling) (Ewald et al., 2020). These genes were selected based on empirically established linkages between changes in transcript abundance and apical endpoints for select representative chemicals. The genes selected are a snapshot of the whole transcriptome and may or may not be representative of those dysregulated by 6PPD-quinone exposure. Metabolomics, the large-scale study of metabolites in relation to genetic alterations or environmental stimuli, complements genomics, transcriptomics, or proteomics. Quantifying changes in abundance of metabolites and transcripts allows for insight into the activity of biological pathways and physiological performance, and therefore allows mechanisms of toxicity to be elucidated (Ramirez et al., 2013).

2.2 Methodology

2.2.1 Ethics Statement

Studies with adult fathead minnows were approved by the University of Lethbridge Animal Welfare Committee (protocols #1915, #2111) and were in accordance with guidelines from the Canadian Council on Animal Care. Studies with embryos before swim-up are exempt from requiring approval. This exemption was reviewed and approved by the University of Saskatchewan Animal Welfare committee (reference #029Exempt2021).

2.2.2 Chemicals

Native and mass-labeled (d_5) 6PPD-quinone were acquired from Toronto Research Chemicals (Toronto, ON, Canada). Dimethyl sulfoxide was acquired from Fisher Scientific (Ottawa, Ontario, Canada). A working stock of 40 mg/L 6PPD-quinone was prepared in 100%

dimethyl sulfoxide (DMSO) and was used for all adult exposures. Analytical standard solutions of native and mass-labeled 6PPD-quinone were prepared in HPLC-grade methanol.

2.2.3 Embryotoxicity Assays

The fish embryo acute toxicity test followed a modification of OECD Test No. 236 (OECD, 2013). Specifically, embryos were reared until 168 hours post-fertilization so that the assay included hatching and swim-up. Fathead minnow embryos were obtained from brood stock maintained at the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan (original source: Aquatic Research Organisms Inc., NH, USA). Embryos were staged using a Stemi 508 stereo microscope (Zeiss, Oberkochen, Germany), and those at ≤ 32-cell stage were selected and transferred randomly to exposure solutions. For each concentration, 10 to 15 embryos were placed in 150 x 15 mm borosilicate glass Petri dishes containing 50 mL of exposure solution. Exposure solutions consisted of a water control, a positive control of 10 g/L of NaCl, a solvent control containing 0.05% DMSO, and 6PPD-quinone at nominal concentrations of 0.2, 2, 6, 20, and 60 µg/L. All exposures with 6PPD-quinone had DMSO at a final concentration of 0.05%. Exposure solutions were prepared using 24ºC ATRF facility water.

Embryos were maintained in an incubation chamber at 24.0 ± 0.5°C with a photoperiod of 16:8 h light: dark. Water changes of 70% were performed each day, and samples of exposure solution were taken at 0 h and prior to water changes at 24 h and 168 h to confirm concentrations of 6PPD-quinone. Water samples were immediately spiked with 50 µg/L 6PPD-quinone-d_5 and stored in –20 ºC until further analysis. Embryos were checked every 24 h for mortalities, abnormalities (e.g., spinal deformity, pericardial edema, and yolk sac edema), and hatching success. Coagulation, lack of heartbeat, lack of somite formation, and non-tail detachment were

used as lethal endpoints. Any mortalities were removed from the dish each day. The exposure was terminated at 168 h.

2.2.4 Exposure of Sexually Mature Fathead Minnows to 6PPD-quinone

Sexually mature fathead minnows were from a brood stock maintained in the Aquatic Research Facility at the University of Lethbridge (original source: Aquatic Research Organisms Inc., NH, USA). The culture was maintained at 22°C, a 14:10 h light: dark photoperiod, and fed a diet of Sally's bloodworms (San Francisco Bay Brand®, San Francisco, California, USA), hatched brine shrimp (*Artemia salina*, Brine Shrimp Direct, Ogden, Utah, USA) and ground Tetramin (Tetra®, Blacksburg, Virginia, USA) *ad libitum*. Water quality parameters (pH, dissolved O_2, ionized and unionized ammonia, NO_3 and NO_2^-) were tested daily to abide by Canadian Council on Animal Care guidelines.

A modified range finding experiment was performed to assess sensitivity of fathead minnows to 6PPD-quinone. To this end, 20 fish (12 males, 8 females) were transferred from the culture to aerated 45 L glass rectangular aquaria (4 tanks with 3 males and 2 females per tank) at 22 °C and allowed to acclimate for 24 h. After acclimation, fish were exposed in duplicate for 36 h to 0 or 20 µg/L 6PPD-q. The final concentration of DMSO was 0.01% (v/v). Fish were monitored throughout the exposure for characteristic signs of exposure leading to fish becoming moribund (increased ventilation rate, gasping at the water surface, permanent loss of equilibrium, and spiraling motion) as reported previously for brook trout and rainbow trout (Brinkmann et al., 2022).

Because exposure to 20 µg/L of 6PPD-quinone did not cause lethality, sublethal effects of an acute exposure were assessed in a 96 h exposure that followed recommendations of OECD

test No. 208, except we did not expect acute lethality based on results of our preliminary exposure to 6PPD-quinone as 20 μg/L, as described above (OECD 2019). Briefly, fathead minnows (3 males and 3 females per tank) were acclimated for 4 days in 45 L glass aquaria at 22 °C with an 80% daily water renewal. Tanks were fitted with lids and continuous aeration was supplied. Fish were fed ground Tetramin once daily for the first 3 days of acclimation, and food was withheld on the final day. Following acclimation, fathead minnows were exposed for 96 h in duplicate (2 tanks per treatment, 6 fish per tank) to 6PPD-quinone at nominal concentrations of 0, 0.2, 2, or 20 μg/L. A 50% water renewal was conducted daily to replenish concentrations of 6PPD-quinone. A 990 μL sample of surface water was taken before and 1 h after addition of 6PPD-quinone, and immediately before and after each water change. Water samples were taken from control tanks at the same time points. Samples were immediately spiked with 50 μg/L 6PPD-quinone-d_5 and stored at −20°C until they were analyzed. Fish were fed ground Tetramin® at 48 h of exposure. The final concentration of DMSO in all tanks was 0.01% (v/v). Water quality parameters (pH, dissolved O_2, ionized and unionized ammonia, NO_3 and NO_2^-) were tested to abide by Canadian Council on Animal Care guidelines.

At 96 h of exposure, fish were removed from the tank and immediately euthanized in buffered tricaine methanesulfonate (MS-222, Millipore-Sigma). Mass (g) and fork length (mm) were recorded for each fish to determine condition factor (K), and blood was collected in plastic heparinized hematocrit tubes (SafeCrit™ StatSpin™, MarketLab Inc., Caledonia, Michigan, USA) for hematocrit analysis. A sample of whole blood was used for blood glucose analysis (OneTouch® Ultra® 2 meter, LifeScan IP Holdings LLC, Malvern, Pennsylvania, USA). Mass of each liver was recorded to determine hepatosomatic index (HSI). The liver was divided in two parts for metabolomic and EcoToxChip analysis. Left-side gill arches were used for metabolomic

analysis, and right-side gill arches were used for EcoToxChip analysis. Tissues were snap-frozen and stored at -80°C until required.

2.2.5 EcoToxChip Analysis

Total RNA was extracted from individual liver and gill tissues of adult fathead minnows from the control and high (20 µg/L) treatment groups only (n = 6 per treatment group) using a RNeasy Plus Universal Mini kit (Qiagen, Mississauga, ON, Canada), following the manufacturer's protocol. RNA concentration and quality were assessed by spectral profiling in QIAxpert (Qiagen). A mass of 750 ng of total RNA were reverse transcribed to synthesize cDNA using the RT^2 First Strand kit (Qiagen) and then mixed with RT^2 SYBR® Green (Qiagen) to prepare the master mix for EcoToxChip analysis. Individual samples were then run in the FHM v1 EcoToxChip custom RT^2 profiler PCR array (Qiagen CAPU14176E; Table S1) using a QuantStudio 6 Flex instrument, following the manufacturer's protocol.

Reduced transcriptome analysis was conducted in EcoToxXplorer (ecotoxxplorer.ca) using the EcoToxChip *P. promelas* v.1 384-well plate module. All samples passed quality check (6 housekeeping genes (HKG); reverse transcription control - positive PCR control < 5). Ct values > 35 were filtered to remove values with higher uncertainties and variance and were considered as non-detects. All non-detects were imputed by randomly drawing values from the normal distribution that has a mean Ct = 35 (Ct cut-off) and a standard deviation of the data surrounding this cut-off. Datasets were normalized using ΔCt normalization with the 6 built-in HKGs. Differential expression analysis was done using analysis of variance (ANOVA) with a cut-off p-value < 0.05. The EcoToxXplorer groups genes into six general biological processes (Endocrine, Immune, Metabolism, Cellular Processes, Oxidative Stress and DNA Signaling)

informative of outcomes of regulatory concern. These processes are then further divided into 21 EcoToxModules capturing specific types of responses that are described in Ewald et al. (2020).

2.2.6 ^{1}H NMR Metabolomics Sample Processing and Data Acquisition

Effects of 6PPD-quinone on the metabolome of liver and gill of adult fathead minnows was determined according to protocols described by Fujita et al. (2021). After determining the mass of each frozen sample of gill and liver, glass beads were added at 1 mg beads/mg tissue, and 8 µL methanol/mg tissue was added to each sample, after which samples were homogenized in a bullet blender homogenizer. Next, 7.2 µL of ultrapure water/mg tissue and 8 µL of chloroform/mg of tissue were added to each sample, which were then vortexed for 10 s and placed at -80 °C for 10 min. Following this, the samples were centrifuged at 6000 x g for 15 min, and then an 80% fraction of the aqueous layer of each sample was transferred into new 2 mL centrifuge tubes and left in a N_2 gas drying box for 24 – 48 h, until the liquid was evaporated. The dried water-soluble metabolites were re-suspended in 480 µL metabolomics buffer (0.125 M KH_2PO_4, 0.5 M K_2HPO_4, 0.00375 M NaN_3, and 0.375 M KF; pH 7.4 ± 0.02) and 120 µL D_2O with 0.02709% w/v trimethylsilypropanoic acid (TSP) and vortexed for 10 s. Extracted metabolites were stored at -80 °C until needed for ^{1}H NMR processing.

Immediately prior to data acquisition, the samples were thawed at room temperature, centrifuged at 12000 x g for 15 min, and 550 µL of the supernatant was transferred to 5 mm NMR tubes. Spectra were collected on a Bruker Avance III 700 MHz Spectrometer (Bruker, Milton, ON, Canada) using a Bruker triple resonance TBO probe with the outer coil tuned to ^{1}H and the 1-D NOESY H_2O suppression pulse sequence (Bruker noesygppr1d pulse sequence). Liver samples were run for 2048 scans. Gill samples that had a mass greater than 8 mg were run for 1024 scans, and samples with a mass less than 8 mg were run for 2048 scans. Spectra were

automatically phase- and baseline-corrected, as well as referenced to TSP at zero ppm, using TopSpin 4.1.1. The spectra were then exported to MATLAB (MathWorks, Natick, MA, USA) where they first underwent Recursive Segment-Wise Peak Alignment (Veselkov et al., 2009) and then Dynamic Adaptive Binning (Anderson et al., 2011) followed by manual adjustments. Subsequently, each spectrum was normalized to the whole metabolome, excluding the peaks corresponding to the water region and Pareto scaled.

Comparisons of abundance of metabolites in gills and livers from 6PPD-quinone exposed fish to control fish were conducted using MATLAB. A decision tree-algorithm that uses a Shapiro-Wilk's test to determine if the data was normally distributed was used (Goodpaster et al., 2010). The algorithm then selected the Mann Whitney U-test for univariant analysis. Variable Importance Analysis based on Random Variable Combination (VIAVC) was used for multi-variant statistical analysis, which determines the most important variables in the dataset depending on the variable's collective effect on the other variables in the dataset (Yun et al., 2015). Bins were determined to be statistically significant at $p < 0.05$ for either test. The 700 mhZ compound library within Chenomx was used to identify metabolites (Chenomx Inc., Edmonton, AB, Canada). Orthogonal Partial Least Squares Discriminate Analysis (OPLS-DA) figures, heat maps, and ten-fold double cross-validation analysis were generated using the Metaboanalyst R-package (Chong et al., 2018).

2.2.7 Quantification of 6PPD-quinone

Exposure concentrations of 6PPD-quinone were measured using the method outlined in Brinkmann et al. (2022). Briefly, an isotope dilution strategy using 6PPD-quinone-d_5 as the internal standard was applied for quantification of 6PPD-quinone on a Vanquish UHPLC coupled with a Q-Exactive™ HF Quadrupole-Orbitrap™ hybrid mass spectrometer (Thermo-Fisher).

Samples were filtered and injected directly into the UHPLC without prior concentration or extraction.

2.2.8 Statistics

Effects of 6PPD-quinone on gross morphometrics (length, mass, K, HSI), glucose, and hematocrit were determined by use of a one-way ANOVA followed by a Tukey's post-hoc test. Datasets were evaluated for normality by use of a Shapiro-Wilk test and homoscedasticity by use of a Levene's test, and log-transformed if needed. Differences were considered significant at $p < 0.05$.

2.3 Results

2.3.1 Analytical Verification of 6PPD-quinone During Embryo and Adult Exposure

Time-weighted average concentrations of 6PPD-quinone measured over the 168 h embryo exposure ranged from 0.11 to 39.27 µg/L, and approximately 52 to 70% of nominal values. In adult exposures, time-weighted average concentrations of 6PPD-quinone measured over the 96 h exposure were 0.09, 0.85, and 9.65 µg/L, which were approximately 45%, 43%, and 48% of nominal values in the low, medium, and high treatment groups, respectively.

2.3.2 Embryotoxicity

There were no effects of 6PPD-quinone, at any concentration, on survival, developmental malformations, or hatching success of fathead minnow embryos (Data not shown).

2.3.3 Effects on Adults

Exposure to 6PPD-quinone for 96 h did not cause mortality of adult fathead minnows. There were no effects on body length, body mass, or K. However, HSI of minnows exposed to

the greatest concentration of 6PPD was significantly less than HSI of control fish or fish exposed to least concentration of 6PPD-quinone (Table 2.1). Exposure to 6PPD-quinone did not affect blood glucose concentrations or percent hematocrit (Table 2.2). However, because fish were euthanized prior to collection of blood, autolysis of cells might have impacted these measurements.

Table 2.1. Mass, length, condition factor (K) and hepatosomatic index (HSI) of sexually mature fathead minnows after 96 h of exposure to 6PPD-quinone. Values depict the mean ± standard deviation of 12 fish per treatment, rather than tank replicates. Differences were evaluated by use of a one-way ANOVA with a Tukey's pot-hoc test and were considered significant at $p < 0.05$. Different letters indicate significant differences between treatments.

Measured Concentration 6PPD-quinone (μg/L)	Mass (g)	Length (mm)	K	HSI
0.00	1.50 ± 0.47	45.25 ± 5.80	7.12 ± 0.96	1.01 ± 0.60[a]
0.09	1.48 ± 0.41	42.67 ± 2.93	8.12 ± 1.86	0.86 ± 0.31[a]
0.84	1.57 ± 0.44	42.92 ± 4.81	8.36 ± 0.92	0.64 ± 0.37[ab]
9.40	1.48 ± 0.42	42.16 ± 3.88	8.16 ± 0.99	0.55 ± 0.33[b]

Table 2.2. Effects of 6PPD-quinone on concentrations of glucose in blood and percent hematocrit in blood. Values depict the mean ± standard deviation of 11-12 fish per treatment, rather than tank replicates. Differences were evaluated by use of a one-way ANOVA and were considered significant at $p < 0.05$.

Measured Concentration 6PPD-quinone (µg/L)	Blood Glucose (mM)	Hematocrit (%)
0.00	2.05 ± 0.18	32.25 ± 1.86
0.09	2.00 ± 1.12	40.36 ± 5.11
0.84	2.04 ± 0.22	36.53 ± 2.27
9.40	1.78 ± 0.13	32.55 ± 1.93

2.3.4 Liver Metabolome

OPLS-DA plots showed separation between the metabolome of livers from control fish and livers from fish exposed to each concentration of 6PPD-quinone (Figure 2.1A-C). Abundances of 32 unique metabolites were significantly different in livers from fish exposed to at least one concentration of 6PPD-quinone (Table 2.3). There were significant differences in 63/312 bins at 0.2 µg/L that were identified to 21 metabolites, 75/312 bins at 2.0 µg/L that were identified to 20 metabolites, and 122/312 bins at 20 µg/L that were identified to 30 metabolites. Abundances of the phospholipids phosphorylcholine and sn-glycero-3-phosphocholine, as well as the amino acids lysine, alanine, valine, leucine, π-methylhistidine, and phenylalanine were significantly decreased. Abundances of proline, serine, methionine, methionine sulfoxide, glutamine, glutamate, pyroglutamate, homocysteine, isoleucine, arginine, threonine, tyrosine, and aspartate were significantly increased. Additionally, abundances of lactate, glycerate, gluconolactone, choline, cystathionine, S-adenosylhomocysteine (SAH), ethanol, malate, glycylproline, glucose, and glycerol were significantly increased.

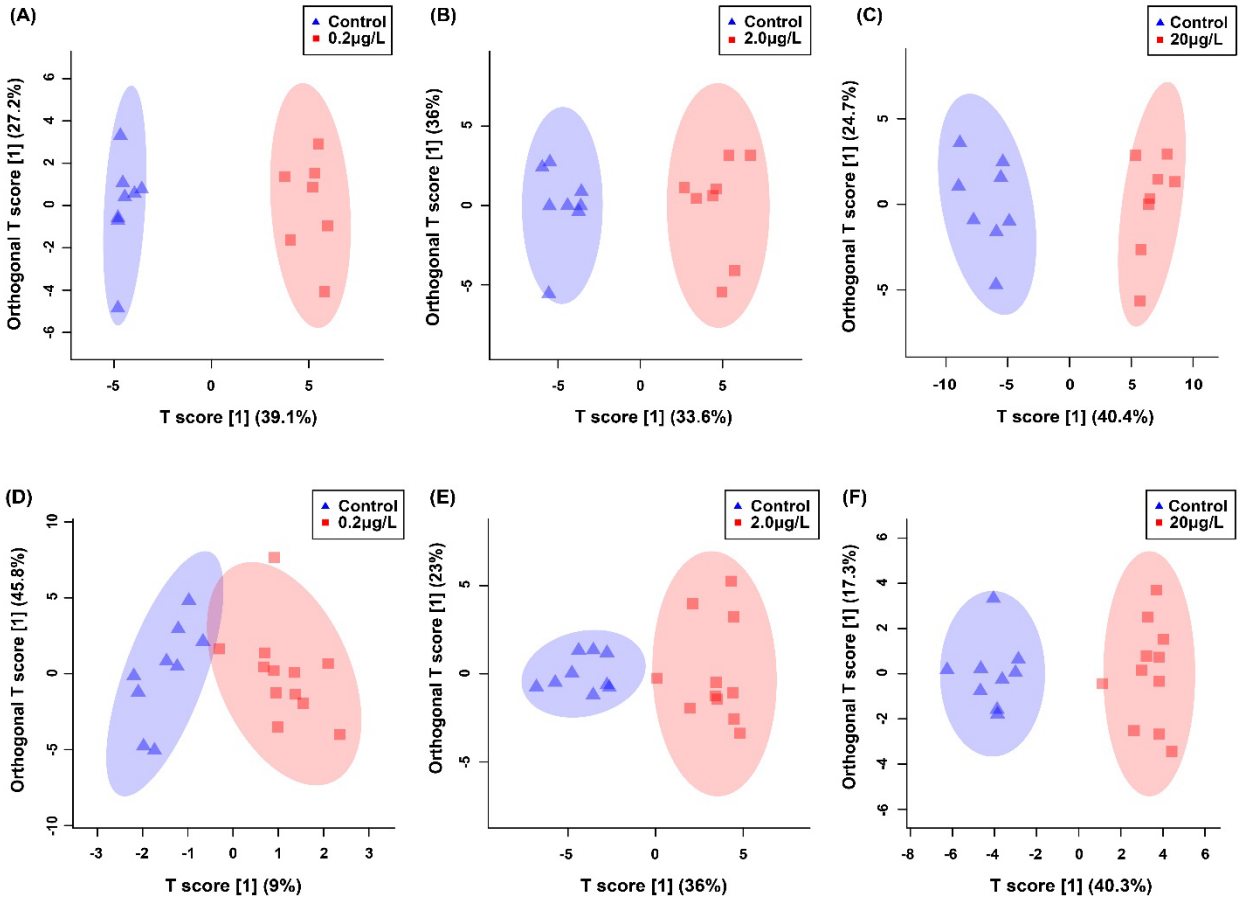

Figure 2.1. Orthogonal partial least squares discriminant analysis (OPLS-DA) scores plots for the metabolome of livers (A-C) and gills (D-F) of fathead minnows that were exposed to 6PPD-quinone at three concentrations versus the control (n = 9), (A) 0.2 µg/L (n = 7; $Q^2 = 0.701$, p = 0.003; $R^2 = 0.983$, p < 0.003), (B) 2.0 µg/L (n = 8; $Q^2 = 0.785$, p <5e-04; $R^2 = 0.951$, p = 0.006), (C) 20 µg/L (n = 8; $Q^2 = 0.676$, p = 0.0025; $R^2 = 0.963$, p = 0.0025), (D) 0.2 µg/L (n = 12; $Q^2 = 0.671$, p < 5e-04; $R^2 = 0.825$, p < 5e-04), (E) 2.0 µg/L (n = 11; $Q^2 = 0.709$, p <5e-04; $R^2 = 0.88$, p = 0.004), (F) 20 µg/L (n = 11; $Q^2 = 0.847$, p < 5e-04; $R^2 = 0.944$, p < 5e-04). Scores plots were generated using bins that were determined to be statistically significant (p < 0.05) by either a Mann-Whitney U-test and/or Variable Importance Analysis based on Random Variable Combination test.

Table 2.3. Changes in abundances of metabolites in livers of adult fathead minnows exposed to 0.2 µg/L, 2.0 µg/L, or 20.0 µg/L of 6PPD-quinone for 96 h. Values are mean percent difference from controls. * Indicates statistically significant by a Mann-Whitney U-test, and + indicates statistically significant by VIAVC Best Subset.

	Mean % difference		
Metabolite	**0.2 µg/L**	**2.0 µg/L**	**20.0 µg/L**
π-methylhistidine	-5.99	-35.72	-45.24*
Phenylalanine	-7.06+	-14.16	-28.01
Threonine	28.15	14.99	41.37*
Proline	38.48*+	35.07*	47.55*+
Lactate	34.01*	34.84*	41.72*
Glycerate	66.55*	64.77*	76.83*
Gluconolactone	42.47*	38.77*	40.32*
Serine	19.23*	25.05*	24.22*
Cystathionine	41.44*	44.05*	49.86*
Leucine	-20.66	-23.38*	-28.82*
sn-glycero-3-phosphocholine	-21.18*	-19.23*	-20.24*
Glycerol	21.01	18.88	29.16*
Glucose	31.14	40.39*	38.07*
Choline	27.94*	28.66	33.06*
Arginine	11.35	22.7*	12.44*
Phosphorylcholine	-79.12*	-1.22	-13.69
Lysine	-19.33*	-17.7	-23.82*
Aspartate	28.13	30.83	45.70*
S-adenosylhomocysteine	31.99*	26.67*	33.67*
Malate	66.96	68.80*	74.06*
Methionine	28.97*	24.09*	29.85*
Glutamine	44.72*+	39.19*	43.22*
Pyroglutamate	39.72*	35.02*	44.44*

Methionine sulfoxide	40.73*	42.35*	47.23*+
Glutamate	37.54*	42.08*	43.62*+
Glycylproline	9.86	6.16	14.56*
Alanine	-26.56*	-25.18*	-35.31*+
Isoleucine	59.02	64.77*	69.47*
Ethanol	26.12*	16.39	27.72*
Valine	-21.04*	-19.64	-25.19*
Homocysteine	34.02*	34.61*	38.95*
Tyrosine	16.00	15.71	31.00*

2.3.5 Gill Metabolome

For gills, OPLS-DA plots showed separation between the control and 0.2 µg/L treatment with minimal overlap, and complete separation between the control and 2.0 µg/L and between the control and 20 µg/L treatments (Figure 2.1D-F). Abundances of 26 unique metabolites were significantly altered across concentrations of 6PPD-quinone (Table 2.4). There were significant differences in 25/268 bins at 0.2 µg/L that were identified to 14 metabolites, 46/268 bins at 2.0 µg/L that were identified to 15 metabolites, and 38/268 bins at 20 µg/L that were identified to 14 metabolites. Abundances of the amino acids arginine, serine, phenylalanine, and cysteine, as well as the metabolites choline, sn-glycero-3-phosphocholine, SAH, ethanol, and methanol were decreased. Abundances of amino acids taurine, methionine, glutamate, leucine, isoleucine, lysine, methionine sulfoxide, valine, and alanine, as well as the metabolites methylguanidine, citrate, malate, acetylphosphate, lactate, and betaine, were significantly increased. The abundance of threonine was significantly decreased in the 0.2 µg/L exposure and increased in the 2.0 µg/L and 20 µg/L exposures. Additionally, abundances of glucose were significantly increased in the 0.2 µg/L exposure and decreased in the 2.0 µg/L and 20 µg/L exposures.

Table 2.4. Changes in abundances of metabolites in gills of adult fathead minnows exposed to 0.2 µg/L, 2.0 µg/L, or 20.0 µg/L of 6PPD-quinone for 96 h. Values are mean percent difference from controls. * Indicates statistically significant by a Mann-Whitney U-test, and + indicates statistically significant by VIAVC Best Subset.

	Mean % difference		
Metabolite	**0.2 µg/L**	**2.0 µg/L**	**20.0 µg/L**
S-adenosylhomocysteine	-6.05*+	-21.05*	-24.50*
Glucose	12.52+	-38.15*	-73.36*
Lactate	2.18+	3.39	0.91
Choline	-2.29+	-17.79*	-23.66*
Cysteine	-3.90	-5.08	-9.83*
Betaine	7.67+	-3.23	0.79
Serine	-0.80	-1.57	-6.63*
Glutamate	4.57+	8.17*+	6.18*
Lysine	9.30	16.2*	3.47
sn-Glycero-3-phosphocholine	-2.97	-12.79*	-8.92*
Ethanol	5.47	-26.38*	-26.04*
Taurine	-5.98+	-10.09	10.28
Methanol	0.42	-12.45	-16.59*
Phenylalanine	-2.75+	-30.69*	-39.11*+
Methylguanidine	23.81*	6.92	15.07
Citrate	8.38+	3.44	-2.05
Malate	11.20+	2.4	-10.35
Methionine	10.24*+	16.41*	12.90*
Acetylphosphate	10.26	20.00*	21.41*
Arginine	-1.84+	-4.74	-6.30
Threonine	-0.35+	16.21*	24.58*
Valine	6.84	18.85*	3.13
Leucine	12.90	29.91*	7.90

Isoleucine	11.09	24.22*	13.94
Methionine sulfoxide	0.95	11.23+	68.54*
Alanine	1.63	12.27	14.40*

2.3.6 EcoTox Chips

Exposure to 6PPD-quinone at 20 μg/L resulted in tissue specific transcriptomic responses (Figure 2.2). A total of 35 and 14 transcripts were significantly dysregulated in the gills and liver, respectively. Of these, 25 and 10 transcripts were up- and down-regulated in gills, respectively, while in the liver, all dysregulated transcripts were up-regulated. Overall, fold-changes observed were mild (gill: up-regulated between 1.5- and 3.2-fold, down-regulated between 0.04- and 0.71-fold; liver: up-regulated between 1.3- and 4.5-fold).

Biological processes (EcoToxProcesses) most affected by exposure to 6PPD-quinone differed between tissues with much greater changes in terms of number of transcripts and magnitude of responses occurring in the gill compared to the liver (Figure 2.3A and B). In gills, 9 out of 21 EcoToxModules had gene sets with median log(2)fold changes >1.5 (Figure 2.4A) while only 3 gene sets out of the 21 modules in liver had median log(2)fold changes of >1.5 (Figure 2.4B). The most dysregulated module with a median log(2)fold gene set change was Corticosteroid module under Endocrine processes, occurring in the gill tissues, with significantly decreased abundance of transcripts of enzymes involved with metabolism of steroid hormones, with *cyp17a1* (log2fold change = -2.62) being the most dysregulated in this module.

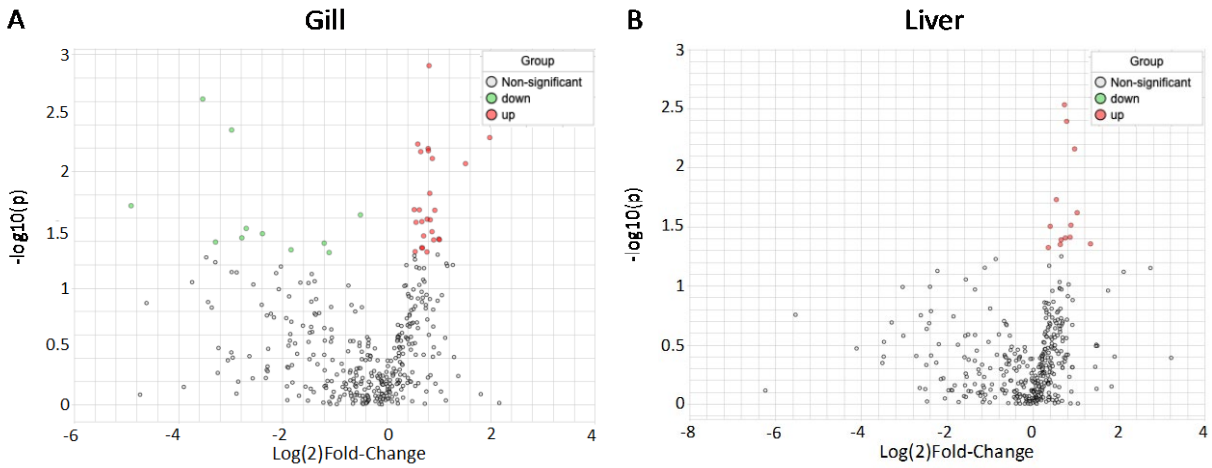

Figure 2.2. Volcano plots of differentially abundant transcripts relative to controls in (A) gills and (B) liver of fathead minnows exposed to 20 µg/L 6PPD-quinone. Significantly ($p < 0.05$) up- and down-regulated transcripts are depicted as red and green data points, respectively.

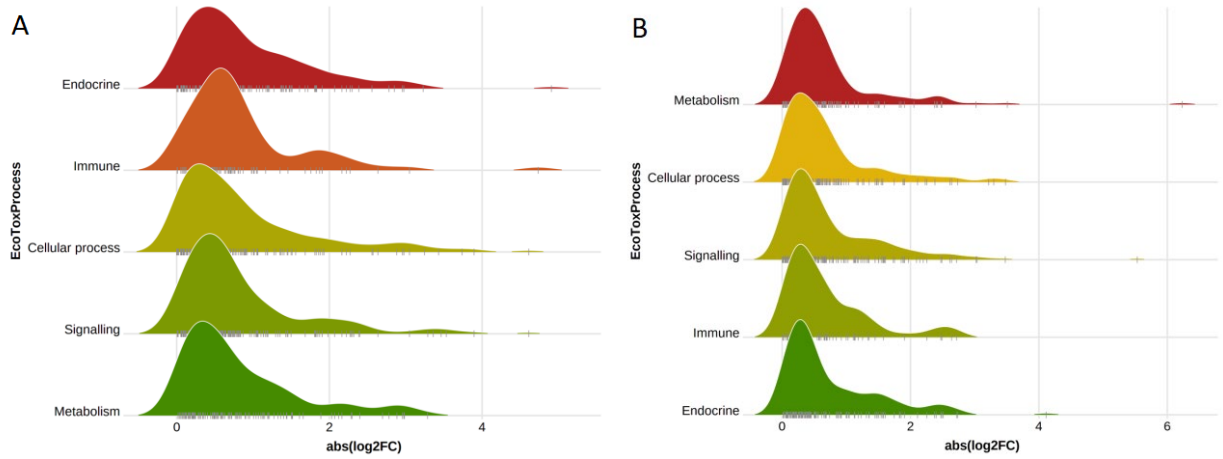

Figure 2.3. EcoToxRidge plots depicting biological processes disrupted in gills (A) and livers (B) of fathead minnows exposed to 20 µg/L 6PPD-quinone. The graph shows the distribution of log(2)fold-changes of genes within each biological process. Processes are sorted by average log(2)fold-change with greatest changes being listed on top.

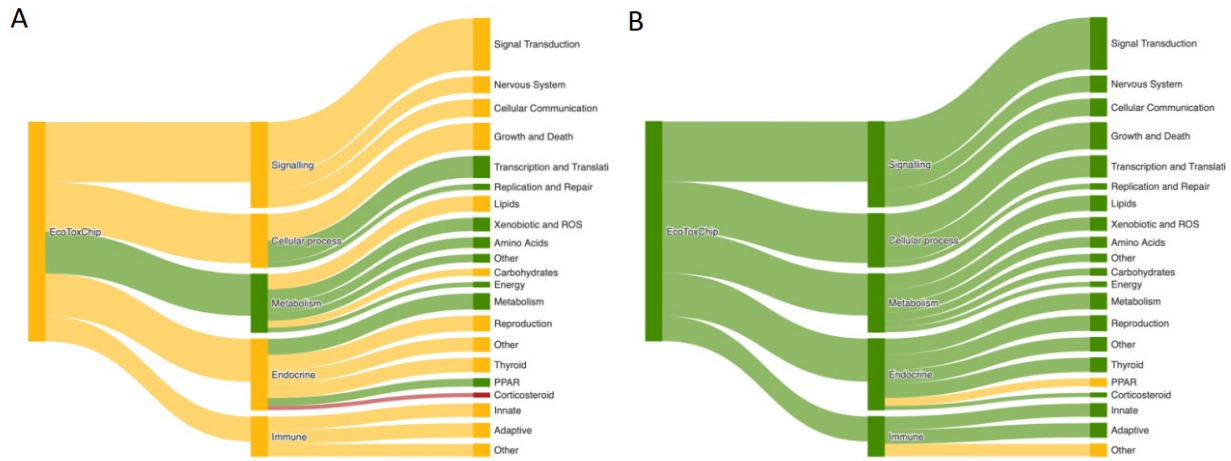

Figure 2.4. Sankey plots showing median log(2)fold changes in genes across the 21 EcoToxChipModules in gills (A) and livers (B) of fathead minnows exposed to 20 µg/L 6PPD-quinone. Different colors depict modules with median log(2)fold transcript abundances greater than 1.5-fold (yellow) and 2.0-fold (red) relative to controls.

2.4 Discussion

2.4.1 Apical Endpoints and General Mechanistic Discussion

The absence of any effect of 6PPD-quinone on survival of fathead minnow embryos exposed to concentrations as great as 39.97 µg/L for 168 h, or adults to concentrations as great as 9.4 µg/L for 96 h suggests this species is insensitive to acute lethality caused by exposure to this chemical. This observation is consistent with effects of acute exposures on survival of other species such as Asian dolly varden and landlocked masou salmon (up to 3.8 µg/L), brown trout and Atlantic salmon (up to 12.6 µg/L), Arctic charr (up to 14.2 µg/L), white sturgeon (up to 12.7 µg/L), Japanese medaka (up to 34 µg/L), and zebrafish (up to 54 µg/L), for which no acute lethality was observed (Hiki and Yamamoto, 2022; Foldvik et al., 2022; Brinkmann et al., 2022; Hiki et al. 2021). Using larval zebrafish, Varshney et al. (2022) determined an LC_{50} of 309 µg/L, which is approximately 32x greater than the maximal concentration used in the present study. Varshney et al. (2022) also reported that exposure to 6PPD-quinone at 25 µg/L caused developmental effects, including lordosis, kyphosis, and a reduction in size of the eye, while decreased heart rate and intestinal reddening were observed in larvae exposed to 10 and 25 µg/L 6PPD-quinone (Varshney et al., 2022).

Adult fathead minnows exposed to the highest concentration of 6PPD-quinone exhibited a decrease in HSI, an indicator of energy status. A decreased HSI might be due to increased glycogenolysis. While concentrations of glycogen in liver were not quantified, stimulation of glycogenolysis is supported by the greater abundance of glucose in livers of minnows exposed to 6PPD-quinone (Table 2.3). Greater concentrations of lactate in livers (Table 2.3) suggest that glucose derived from glycogenolysis might have been used as an energy source within the liver. This is further supported by the lack of any change in concentrations of glucose in blood plasma

from adult fathead minnows (Table 2.2). The lack of any change in blood glucose contrasts with increased concentrations of glucose in blood from rainbow trout and brook trout, both of which are sensitive to acute lethality caused by 6PPD-quinone (Brinkmann et al., 2022). In addition, reduced transcriptome analysis suggested increased metabolism, specifically energy (*cox11*, *cox15*), lipids (*nr1h4*, *nr5a2*), and carbohydrates (*onecut1*) in the liver which might explain the reduced HSI.

Overall, results of both the transcriptomics and metabolomics responses imply a tissue-specific response to 6PPD-quinone. 6PPD-quinone caused significant perturbations in abundances of transcripts of genes associated with the metabolism of xenobiotics, reactive oxygen species (ROS), amino acids, and lipids, as well as those associated with cellular signalling and immune responses. Most responses of the transcriptome occurred at the gills, which is in accordance with previous hypotheses that the gill is the primary target of 6PPD-quinone (Mahoney et al., 2022). The metabolome of gills from 6PPD-quinone exposed minnows displayed a concentration-dependent separation from controls, while at the livers there was a strong response at all three concentrations. Changes in abundance of metabolites associated with one-carbon metabolism, mitochondrial function, and oxidative stress were identified in each tissue, but results suggest that mitochondrial function might have been more impacted in gills and oxidative stress more impacted in livers.

2.4.2 Methionine Cycle and Methylation

Changes in abundances of several metabolites give evidence that exposure to 6PPD-quinone might have disturbed the methionine cycle, in both livers and gills, but that this pathway was more responsive in livers. The methionine cycle, along with the folate cycle and the transsulfuration pathway are three intricately linked pathways that comprise one-carbon

metabolism. In livers, abundances of methionine, SAH, homocysteine, and choline were significantly increased by 27-39% at all exposure concentrations, suggesting that 6PPD-quinone caused a general increase in the methionine cycle. No clear pattern was detected in gills as abundance of methionine was increased and abundances of SAH and choline were decreased at all exposure concentrations. Abundance of betaine increased only at the lowest concentration of 6PPD-quinone and homocysteine was not detected. The decrease in choline in gills suggest that choline might have been used for re-methylation of homocysteine to methionine by betaine homocysteine-S-transferases. Although this process is limited to the livers and kidneys in mammals, betaine homocysteine-S-transferases are expressed in various organs and skeletal tissue in African lungfish (Ong et al., 2015). Transcripts encoding enzymes of one-carbon metabolism are not present on the EcoToxChip, so it is not known whether changes in abundances of intermediates of one-carbon metabolism are due to changes in gene expression. However, enzymes involved in these processes can be allosterically regulated (Ducker and Rabinowitz, 2017; Jeltsch and Jurkowska, 2016; Torres and Fujimori, 2015). For example, methylenetetrahydrofolate reductase (MTHFR), which is the rate-limiting enzyme in one-carbon metabolism and responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate that is a co-substrate for remethylation of homocysteine to methionine, can be allosterically inhibited by S-adenosylmethionine (SAM) (Bezerra et al., 2021).

There are several potential implications of changes in abundances of intermediates of the methionine cycle. One possibility is that cellular methylation reactions are altered by 6PPD-quinone. A critical role of the methionine cycle is regulation of the synthesis of SAM, the universal methyl donor, which is not given in the 700 mhZ Chemomx compound library used for metabolite identification. DNA and histone methylation are important regulators of gene

expression (Moore et al., 2012). Increased SAH, as was determined in livers, is associated with hypomethylation due to inhibition of methylation reactions (James et al., 2002; Ulrey et al., 2005). Compared to livers, SAH abundance was decreased in gills, which could lead to hypermethylation (Ponnaluri et al., 2018). Additionally, increased betaine, as was determined in gills, has been associated with greater DNA and histone methyltransferase activity, which could lead to hypermethylation (Sternback et al., 2021).

2.4.3 Oxidative Stress

Changes in abundances of several metabolites suggest that exposure to 6PPD-quinone might have caused oxidative stress in livers and gills. Abundance of gluconolactone, an oxidation product of glucose that is thought to play a role in oxidative stress (West, 2000), was increased in livers of fathead minnows exposed to all concentrations of 6PPD-quinone. Greater abundance of methionine sulfoxide in livers and gills is also indicative of oxidative stress. Formation of methionine sulfoxide is an antioxidant defense mechanism in which methionine residues scavenge ROS (Moskovitz, 2005; Levine et al., 1999). Although oxidation of methionine can render enzymes inactive, methionine sulfoxide reductases convert methionine sulfoxide back to methionine, preventing this effect (Levine et al., 1999). Consistent with the hypothesis that methionine sulfoxide might be indicative of oxidative stress, abundance of methionine sulfoxide was increased in zebrafish larvae experiencing oxidative stress following mutations to selenoproteins (Cox et al., 2016). Further supporting the hypothesis that 6PPD-quinone might be causing oxidative stress in fathead minnows, abundances of several metabolites of glutathione metabolism, an important antioxidant in the response to oxidative stress, were impacted in livers and gills. As an initial step in synthesis of glutathione, the transsulfuration pathway of one-carbon metabolism includes a condensation reaction of homocysteine and serine to produce

cystathionine. Next, cystathionine is hydrolysed to cysteine that reacts with glutamate to form γ-glutamylcysteine, which in the final step of glutathione synthesis reacts with glycine to form glutathione (Lu, 2001). Among these intermediates in glutathione synthesis, homocysteine and cystathionine, both of which were increased in livers, but not in gills, have been previously shown to increase during oxidative stress (Mosharov et al., 2000). Abundances of serine and glutamate were also increased in livers. In contrast, abundances of serine and cysteine were decreased in gills, whereas abundance of glutamate increased. Γ-glutamylcysteine is not given in the 700 mhZ Chemomx library used for metabolite identification. Changes in abundance of pyroglutamate also support altered glutathione metabolism in fathead minnows exposed to 6PPD-quineone. Abundance of pyroglutamate, an intermediate in glutathione metabolism, was increased in livers of fishes exposed to 6PPD-quinone but was not detected in gills. It has been suggested that concentrations of pyroglutamate increase when glutathione levels are depleted in cells responding to oxidative stress (Gamarra et al., 2019). Greater abundance of pyroglutamte has been reported in Atlantic salmon (*Salmo salar*) exposed to the oxidative disinfectant, peracetic acid (Lazado et al., 2020). Overall, results of the metabolomic analysis provide evidence that 6PPD-quinone might be causing oxidative stress in livers and gills of fathead minnows, and that this effect might be more pronounced in liver.

Induction of oxidative stress is also supported by results of the EcoToxChip analysis. Abundances of three transcripts associated with oxidative stress were significantly increased in the liver: *cox11*, *cox15*, and *nr5a2*. Cytochrome c oxidase (COX) is the last enzyme of the mitochondrial respiratory chain, and its biogenesis is a tightly controlled redox-regulated process (Bourens et al.,2013). Similarly, the orphan nuclear receptor Liver receptor homolog-1 (Lrh-1, Nr5a2) plays a key role in several biological processes, including the regulation of mitochondrial

metabolism and immune response (Michalek & Brunner, 2020). Thus, overexpression of the respective complementary transcripts of these proteins might result into upregulation of metabolic processes, including mitochondrial respiration, leading to excessive amounts of ROS. In gills, most dysregulated genes belong to metabolic, immune, and signal transduction (Table S8) Changes in expression of genes associated with metabolic processes include the induction of *ahr2* and *arnt* which may lead to oxidative stress (Grishanova and Perepechaeva, 2022), while upregulation of *cyp2ad6* and *odc1* have been shown to counter ROS-induced damage (Banerjee et al. 2022; Sato et al. 2020). Interestingly, *cyp1a* was downregulated. Nevertheless, it is worth noting that while the EcoToxChip has a limited number of built-in genes, there is a multitude of dysregulated genes that are related to oxidative stress, and therefore merits further studies, particularly in the gills.

2.4.4 Mitochondrial Dysfunction

Mitochondrial uncoupling in gills, but not livers, has been proposed as a mechanism by which 6PPD-quinone causes acute lethality of rainbow trout (Mahoney et al., 2022). Studies have not investigated whether mitochondrial uncoupling occurs in non-sensitive species. Mechanisms of mitochondria uncoupling include proton leak, electron leak, and electron slip. An in-depth description of these mechanisms can be found elsewhere (Demine et al., 2019; Murphy, 1989). Proton leak occurs when protons diffuse through the mitochondria membrane, which can be mediated by uncoupling proteins (UCPs). In mammals, uncoupling protein 2 (UCP2) is ubiquitously expressed, with greater expression under hypoxic conditions. In tumour cells, which experience a hypoxic microenvironment, cells that over-expressed UCP2 had decreased glucose, increased abundances of citrate and malate that is suggestive a more active citric acid cycle, and increased abundances of alanine, aspartate, and glutamate (Sreedhar et al., 2019). In gills,

abundances of glucose were decreased, and abundance of glutamate was increased at each concentration of 6PPD-quinone. However, abundances of citrate and malate were increased only at the lowest concentration of 6PPD-quinone, and abundance of alanine was increased only at the highest concentration of 6PPD-quinone. Aspartate was not detected in gills. These results suggest that mitochondrial uncoupling due to proton leak was not occurring in gills of fathead minnows exposed to 6PPD-quinone.

2.5 Conclusions

Studies of 6PPD-quinone, to date, have primarily focussed on species-sensitivity to acute lethality. Little is currently known about sublethal effects of this emerging contaminant of concern. In this study, embryos and sexually mature fathead minnows were not sensitive to acute lethality by 6PPD-quinone. Using EcoToxChips and ^{1}H NMR metabolomics, we provide evidence that 6PPD-quinone might disrupt one-carbon metabolism and cause oxidative stress in both the gills and livers of fathead minnows. Profiles of metabolites suggest that 6PPD-quinone does not cause mitochondrial uncoupling in gills of fathead minnows, which has been proposed as the mechanisms of acute lethality in sensitive species (Mahoney et al., 2022). Due to the limited nature of EcoToxChips and ^{1}H NMR, RNAseq and LC-MS/MS based metabolomics would provide greater insight into potential sublethal effects of 6PPD-quinone on insensitive species of fishes. Nevertheless, several hypotheses regarding potential mechanisms of sublethal effects of 6PPD-quinone in non-sensitive species were proposed and should be the focus of future studies

CHAPTER 3: MICROINJECTION OF 6PPD-QUINONE INDUCES DEVELOPMENTAL CARDIOTOXICITY IN FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

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3.1 Introduction

N-(1,3-dimethybutyl)-N'-phenyl-*p*-phenylenediamine-quinone (6PPD-quinone), an environmental oxidation product of the rubber tire antioxidant, 6PPD, is an emerging contaminant of concern that was first identified as the cause of Urban Runoff Mortality Syndrome, an acute lethality phenomenon observed in pre-spawn coho salmon (*Oncorhynchus kisutch*) (Tian et al., 2021). 6PPD-quinone has been detected globally and across various environmental matrices. In North America, concentrations in stormwater discharge ranges from 0.086 to 2.3 µg/L (Challis et al., 2021; Johannessen et al., 2022; Tian et al., 2021).

Acute lethality induced by 6PPD-quinone is highly species-specific. To date, only certain species of salmonids have been characterized as sensitive to acute lethality by 6PPD-quinone. In addition to coho salmon (24 h LC_{50} <0.1µg/L), sub-adult rainbow trout (*Oncorhynchus mykiss*) have a 96 h LC_{50} of 1.0 µg/L, brook trout (*Salvelinus frontalis*) have a 24 h LC_{50} of 0.59 µg/L, and white spotted char (*Salvelinus leucomaenis pluvius*) have a 24 h LC_{50} of 0.51 µg/L (Brinkmann et al., 2022; Hiki and Yamamoto, 2022). Lake trout (*Salvelinus namaycush*) are sensitive to acute lethality, with alevins exposed from hatch until 45 days post-hatch having an LC_{50} of 0.39 µg/L and exogenously feeding fry having a 96 h LC_{50} of 0.50 µg/L. By contrast, other salmonid species, such as westslope cutthroat trout (*Oncorhynchus clarkii lewisi*), chinook salmon (*Oncorhynchus tshawytscha*), non-fry life-stages of Atlantic salmon (*Salmo salar*), Arctic char (*Salvelinus alpinus*), southern Asian dolly varden (*Salvelinus curilus*), sockeye salmon (*Oncorhynchus nerka*), pink salmon (*Oncorhynchus gorbuscha*) and landlocked masu salmon (*Oncorhynchus masou masou*) are insensitive to acute lethality by 6PPD-quinone (Brinkmann et al., 2022; Hiki and Yamamoto, 2022; Greer et al., 2023; Lo et al., 2023; Foldvik et al., 2024; Montogomery et al., 2024). Additionally, a variety of non-salmonids, including white sturgeon

(*Acipenser transmontanus*), zebrafish (*Danio* rerio), Japanese medaka (*Oryzias latipes*), and red drum (*Sciaenops ocellatus*) are insensitive to acute lethality (Ackerly et al., 2024; Brinkmann et al., 2022; Hiki et al., 2021). These findings imply that sensitivity to acute lethality cannot be strictly determined from a phylogenetic perspective.

In addition to acute lethality, various symptoms of early life-stage toxicity have been reported in a variety of fish species exposed to 6PPD-quinone. Lake trout fry displayed blood pooling in the caudal fin and eye, yolk sac edema, and spinal curvature following chronic exposure to 6PPD-quinone (Roberts et al., 2025). No developmental toxicities were reported in zebrafish and Japanese medaka exposed to waterborne 6PPD-quinone exposed up to 54 µg/L and 34 µg/L (Hiki et al., 2021). However, a study by Varshney et al. (2021) reported intestinal inflammation, spinal curvature, reduced swim performance, and increased oxygen consumption in early life-stage zebrafish exposed to waterborne 6PPD-quinone, despite that 25 µg/L was the highest exposure concentration, which is a smaller concentration than used by Hiki et al (2021). Another study reported increased developmental malformations in zebrafish exposed to 6PPD-quinone at concentrations of 1.2 mg/L and above, including enlarged intestines and blood coagulation (Zhang et al., 2023).

Several mechanisms have been proposed to explain the adverse effects of 6PPD-quinone. Acute lethality might be due to mitochondrial uncoupling in the gills, which is consistent with the phenotype of Urban Runoff Mortality Syndrome, including increased ventilation and gasping for air (Mahoney et al., 2022). Increased vascular permeability and leakage of the blood-brain barrier has also been proposed to contribute to acute toxicity, which was supported by transcriptomic responses in early life-stage coho salmon that are suggestive of vascular leakage (Blair et al., 2021, Greer et al., 2023). Species differences in acute lethality might be due to

toxicokinetic differences. This notion is supported by research on the phase I and II transformation product profiles of 6PPD-quinone in sensitive species (coho salmon, rainbow trout, brook trout) compared to species that are insensitive to acute lethality (brown trout, westslope cutthroat trout, non-fry Atlantic salmon, white sturgeon) (Montgomery et al., 2023).

Previously, I identified that fathead minnows (*Pimephales promelas*) are insensitive to 6PPD-quinone induced acute lethality. Adult minnows exposed to a maximum concentration of 9.4 µg 6PPD-quinone/L, or freshly fertilized embryos exposed to a maximum concentration of 39.97 µg 6PPD-quinone/L, did not suffer acute lethality, and there were no incidences of developmental malformations in exposed embryos. However, evidence of sublethal oxidative stress was identified using EcoToxChips transcriptomics and ^{1}H NMR metabolomics (Anderson-Bain et al., 2023). The original objective of the present study was to further investigate the sublethal effects of 6PPD-quinone using fathead minnows as a model organism. However, for the present study, I opted for microinjection of 6PPD-quinone into embryos as an exposure method to ensure consistent and precise delivery of the chemical. Surprisingly, embryos microinjected with 6PPD-quinone displayed a suite of developmental abnormalities that are consistent with those observed in early life-stages of lake trout exposed to this chemical. Therefore, RNAseq was used to investigate the mechanistic basis of this embryotoxicity phenotype.

3.2 Methods

3.2.1 Ethics Statement

All exposures were terminated prior to swim-up and were therefore exempt from requiring approval from the University of Lethbridge Animal Welfare Committee, as per guidelines from the Canadian Council on Animal Care (CCAC).

3.2.2 Fish Culturing and Embryo Collection

A brood stock of fathead minnows was maintained at the University of Lethbridge Aquatic Research Facility (original source: Aquatic Research Organisms, Inc.). Culture tanks were maintained at 25°C with a 14:10 light: dark photoperiod, and water quality was tested daily to abide by CCAC guidelines. Breeding tanks were maintained at 25°C with a 14:10 light:dark photoperiod and consisted of a 2:1 female: male ratio and two breeding tiles that were halved PVC pipe. Fish were fed hatched brine shrimp (*Artemia salina*, Brine Shrimp Direct, Ogden, Utah, USA), ground Tetramin (*Tetra*, Blacksburg, Virginia, USA), and Sally's bloodworms (San Francisco Bay Brand, San Francisco, California, USA) daily. Immediately after collection, embryos from different breeding tanks were pooled and staged using a dissecting microscope. Viable embryos that were ≤ 32-cell stage were used in exposures.

3.3 Exposures to 6PPD-quinone

3.3.1 Microinjection of 6PPD-quinone

6PPD-quinone and a mass-labelled 6PPD-quinone-d_5 were purchased from (Toronto Research Chemicals (Toronto, ON., Canada). DMSO was from Fisher Scientific (Ottawa, ON., Canada). A stock solution of 0.5 mg 6PPD-quinone/mL was prepared in 100% DMSO and used to make solutions for microinjections and waterborne exposures.

For microinjection, 1 mL of the stock solution was serially diluted 3-fold in DMSO to generate five solutions with nominal concentrations of 6,170, 18,500, 55,600, 166,700, and 500,000 µg/L. Full-strength DMSO (6PPD-quinone nominal concentration = 0.0 µg/L) was used as a process and chemical control for microinjection. Approximately 3.0 nL of either 6PPD-quinone dosing solutions or 100% DMSO was microinjected into the yolk sac of fathead minnow

embryos prior to gastrulation (< 6 hpf), using a Narishige IM-400 Electric Microinjector (Narishige, Tokyo, Japan). Embryotoxicity assays were conducted using 30-50 embryos per dose, and exposures were conducted in duplicate using separate batches of embryos. Immediately following microinjection, embryos were placed into 100 mm x 15 mm plastic petri dishes containing dechlorinated City of Lethbridge water and incubated at 26°C for 24 h. To assess embryotoxicity, 24 embryos that were alive at 24 h post-injection were randomly selected and individually placed into plastic 24-well plates (Eppendorf, Canada) containing 1 mL of water and reared in an incubation chamber at 26°C. Embryos that died within the first 24 hours following the microinjection were assumed to have died from the injection process and thus not included in the final mortality data. Approximately 50% of the volume of water in each well was manually exchanged every 24 h. Embryos were monitored daily for mortality and malformations using a dissecting scope. A Zeiss Stereo Discovery V12 microscope (Carl Zeiss Canada, Toronto, ON., Canada), Zeiss Axiocam 105 Color (Carl Zeiss Canada) and ZEN lite imaging software (Carl Zeiss Microscopy, Oberkochen, Germany) was used to capture images and videos. Embryotoxicity assays were terminated at 12 days post-fertilization (dpf).

3.3.2 Waterborne Exposures to 6PPD-quinone

An embryotoxicity assay was conducted with waterborne 6PPD-quinone to verify results of our previous study where fathead minnows were classified as insensitive to acute lethality (Anderson-Bain et al., 2023). Exposure media with nominal concentrations of 0 and 20 µg/L 6PPD-quinone were prepared using 26°C dechlorinated City of Lethbridge water and contained 0.004% DMSO. Embryos that were ≤ 32-cell stage were randomly placed into 100 x 15 mm borosilicate glass petri dishes (Pyrex) containing 50mL of either exposure media, with 15 embryos per treatment. Embryos were incubated at 26°C, and assessments of mortality and

malformations were conducted daily. Approximately 50% of the exposure media was changed every 24 h. Samples of exposure media were taken at 24 h, spiked with standard 50 µg/L 6PPD-quinone-d_5, and stored at -80°C for 6PPD-quinone quantification. The assay was terminated at 5 dpf.

3.3.3 RNAseq

For RNAseq, 50 embryos were microinjected with 3.0 nL of either 100% DMSO or 166,700 µg/L of 6PPD-quinone and placed into 100 mm x 15 mm plastic petri dishes containing dechlorinated City of Lethbridge water and incubated at 26°C for 24 h. After 24 h, any dead embryos were removed from the petri dishes and a 50% water renewal was conducted. At 48 hpf, 10 surviving embryos that were microinjected with either 6PPD-quinone or DMSO were collected, pooled, and frozen at -80°C. This time-point was chosen because it preceded manifestation of developmental toxicities and therefore would allow for detection of changes in gene expression that might be causative of the observed effects. Total RNA was extracted from embryos using TRIzol Reagent (Invitrogen, Carlsbad, California, USA) with a modified protocol. Samples were homogenized, precipitated, and washed 3x to remove as much potential contamination as possible. RNA was solubilized in UltraPure DNase/RNAase-Free Distilled Water (Invitrogen, Grand Island, NY, USA). A260/280 and A260/230 were measured using a NanoDrop One spectrophotometer (Thermo Scientific). Quantification of RNA was conducted with a Qubit 4 Flourometer (Invitrogen) and Qubit RNA BR Assay Kit (ThermoFisher Scientific, Eugene, Oregon) in accordance with the manufacturer's instructions. RNA samples were stored at -80°C.

Sequencing of mRNA was conducted by Novogene Co., Ltd. (Beijing, China). Briefly, sample quantitation, integrity, and purity were measured using an Agilent 5400 Bioanalyzer, and

samples that passed quality check (≥ 100ng of RNA, ≥4.0 RIN, A260/280 = 1.8-2.2, A260/230 ≥ 1.8) proceeded to polyA capture and Illumina sequencing. Six control and six 6PPD-quinone treatment samples were sequenced.

3.3.4 Differential Gene Expression and Pathway Analysis

Paired-end fastq files were uploaded to the Galaxy platform (version 24.1.2.dev0; https:usegalaxy.org) for processing. FastQC (version 1.11; Babraham Bioinformatics, Cambridge, United Kingdom) was used for sample quality check. The first 20 and last 10 bp, polyG sequences, and sequences with a Phred < 30 and length < 20 bp were trimmed using fastp version 0.23.4. Sequences were mapped to the fathead minnow genome (*Pimephales promelas* genome assembly EPA_FHM_2.0 - NCBI RefSeq assembly GCF_016745375.1) using RNA STAR aligner (version 2.7.11a; Dobin et al., 2012). Mapped reads were counted and annotated to a gene symbol (NCBI *Pimephales promelas* Annotation Release 100 – NCBI RefSeq annotation GCF_016745375.1 gtf file) using featureCounts (version 2.0.3; Liao et al. 2014)

Differentially expressed genes were determined using the DESeq2 (version 1.44.0; Love et al., 2014) package in R. The R packages BiocHubsShiny (version 1.4.0; Ramos and Carey, 2024) and AnnotationHub (version 3.12.0; Morgan and Shepherd, 2024) were used to obtain further fathead minnow gene annotation data (HUBID: 114526; org.Pimephales_promelas.eg.sqlite; NCBI genomes; date added to hub 2023-10-20), with the AnnotationDbi package (version 1.66.0; Pagès et al., 2024) used to add gene descriptions and NCBI gene IDs to the DESeq2 output tables. Genes with a missing alias or gene name were manually inserted in the output file containing the significantly dysregulated genes if it could be found following a search on NCBI.

Genes with a p-adjust < 0.05 were uploaded to ShinyGO (version 0.80; http://www.bioinformatics.sdstate.edu/go/; Ge et al., 2019) for pathway analysis using Zebrafish as the pathway species, KEGG database for pathway analysis, FDR cutoff of 0.05, and minimum pathway size of 2.

3.3.5 6PPD-quinone Quantification

For quantification of doses of 6PPD-quinone in embryos, approximately 60 mg of embryos were microinjected with 3.0 nL of either 6PPD-quinone or DMSO, snap frozen, and stored at -80°C. Embryos were extracted using 12 volumes of MeOH per mg of dried mass (dm) and one 5 mm steel bead, and 25 µL of 6PPD-Q d5 dissolved in MeOH were added before complete homogenization in a TissueLyser II (Qiagen, Toronto, Ontario, Canada) at 20 Hz for 3 min. Next, samples were centrifuged at 4000 rpm at 4ºC for 30 min and the supernatant was transferred to a new 2.0 mL microcentrifuge tube with 6 volumes ACN and vortexed for 1 min before storage at –20ºC for 12 h. After salt and protein precipitation, samples were centrifuged at 4000 rpm at 4ºC for 30 min before transferring to a new 2.0 mL glass vial and dried under a gently stream of nitrogen. Samples were then reconstituted in 500 µL of 50:50 MeOH: H2O and filtered using a syringe + PTFE 0.22 µM syringe filter into a new 2 mL glass vial before LC-MS/MS analysis.

Quantification of 6PPD-Q in water samples for the aqueous exposure and in eggs was performed using a Vanquish UHPLC paired with the Q-Exactive™ high-field (HF) hybrid Quadrupole-Orbitrap mass spectrometer (MS) (Thermo-Fisher). LC separation was achieved using a Kinetex® 1.7 µm XB-C18 column (100 x 2.1 mm). The aqueous and organic mobile phases consisted of 5 % methanol in water with 0.1 % formic acid and 100 % methanol with 0.1 % formic acid, respectively. The flow gradient of the mobile phases is found in Table 1. Sample

injection volumes of 5.0 µL were ionized using heated electrospray ionization (HESI) in positive mode with a scan range of 100 – 1000 m/z. Ion source parameters were as follows: sheath gas flow = 35; aux gas flow = 10; sweep gas flow = 1; spray voltage = 4.00 kV; capillary temperature = 350 °C; S-lens RF level = 60; and aux gas heater temperature = 300 °C. A paired full scan and parallel reaction monitoring (PRM) MS method was used, with max injection times = 100 ms, resolutions of 120,000/30,000, and AGC targets of $1.0x10^6/2.0x10^5$, respectively. Transition ions of 6PPD-Q (*m/z* = 215.0818) and 6PPD-Q d5 (*m/z* = 220.1126) were used for quantification.

The instrumental analysis of 6PPD-Q included an 8-point calibration curve, ranging from 0.05 – 750.0 µg/L with an $r^2 = 0.9997$ using a weighting factor of 1/X for quantitation. Instrument blanks were run once after every four samples, and QA/QC standards were run once after the fourth sample to monitor for instrument drift. Data was quantified using TraceFinder (version 4.1). Fathead minnow embryo samples were quantified using isotope dilution from extracted tissue (Table 3.1). Semi-quantification was done using a targeted external calibration method for 6PPD-Q medium samples. The method detection limit ($MDL = \sigma * t_{(n-1,\ 1-\alpha=0.99)}$) (Childress et al., 1999), and limits of detection ($LOD = 3.9 * (\sigma_{pseudo\text{-}blank}/slope)$) and quantitation ($LOQ = 3.3 * LOD$) (Wenzl et al., 2016) were 0.024, 0.0081, and 0.027 µg/L, respectively.

Table 3.1. Flow gradient of mobile phases. Flow rate = 0.2 mL/min, column temperature = 40 °C, solvent A = 95% H2O: 5% MeOH + 0.1% formic acid. Solvent B = 100% MeOH + 0.1% formic acid.

Time (min)	**%B**
0.000	5.0
4.000	100.0
7.500	100.0
7.510	5.0
11.000	5.0

3.3.6 Statistics

Differences in mortality and malformations between controls and each dose of 6PPD-quinone was evaluated using a Fisher's Exact Test in GraphPad Prism 9 (GraphPad Software, San Diego, California, USA). Differences from control were considered statistically significant if $\alpha \leq 0.05$. Raw counts were used for statistical analysis and percentages were used for graphing purposes.

The DeSeq2 R package (version 1.44.0) was used to normalize raw gene counts, generate log2fold changes, conduct a Wald's test to determine differentially expressed genes, and adjust p-values using the False Discovery Rate (FDR) method.

RStudio (version 2024.04.1-748; RStudio Team 2024) operating R (version 4.3.1; The R Foundation for Statistical Computing, 2023) was used to generate all graphs, using ggplot2 (version 3.5.1; Whickham 2016) Adobe Illustrator (version 24.003.20112; San Jose, CA, USA) was used to re-color bar graphs, edit axis titles, and add symbols indicating statistical significance.

3.4 Results

3.4.1 Quantification of 6PPD-quinone

Measured doses of 6PPD-quinone in microinjected embryos were 0.00, 0.53, 1.05, 2.57, 5.26, and 9.55 µg/g. Measured concentrations of 6PPD-quinone in media from waterborne exposures were 0.00 and 11.14 µg/L.

3.4.2 Embryotoxicity

Exposure to waterborne 6PPD-quinone did not cause embryo lethality or developmental abnormalities (data not shown). Microinjection of 6PPD-quinone caused significant

embryotoxicity. At 3 dpf, fathead minnows began to show symptoms of embryotoxicity, including pericardial edema, spinal curvature, heart malformations (flat, not looped), and absence of one or both common cardinal veins (Figure 3.1 and Figure 3.2). Any fish that lacked one or both common cardinal veins were counted as “missing veins”. Hemorrhaging/blood pooling first became apparent at 3 dpf in some embryos while in other embryos it occurred later in development (Figure 3.1). The anatomical location of hemorrhaging/blood pooling was not consistent between individuals within or across 6PPD-quinone doses, occurring in the chest cavity, head, and eyes. (Figure 3.1 and Figure 3.2).

Figure 3.1. Cardiovascular abnormalities observed in fathead minnow embryos exposed to 6PPD-quinone. Embryos that were exposed to 6PPD-quinone were found to have lacked common cardinal veins (B) whereas DMSO microinjected embryos developed both common cardinal veins (A). Arrows in (A) point towards both common cardinal veins, and arrows in (B) point towards missing veins. Fathead minnow controls had a normally developed heart (C) in comparison to the flat, not looped heart found in embryos that were microinjected with 6PPD-quinone (D). Hemorrhaging occurred at random anatomical locations, with (E) and (F) being two examples of a fish with an eye bleed (E) and with hemorrhaging in the chest cavity (F).

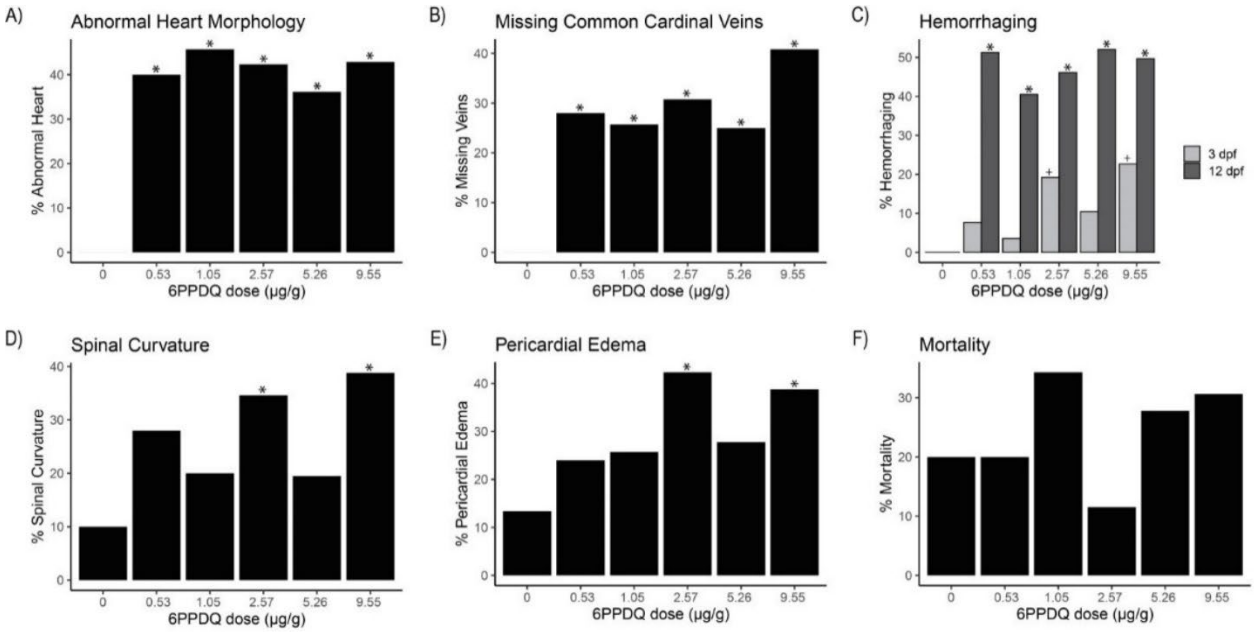

Figure 3.2. Proportion of fathead minnow embryos that presented with abnormal heart morphology (A), missing common cardinal veins (B), hemorrhaging by 3 dpf and 12 dpf (C), spinal curvature (D), pericardial edema (E), and mortality (F) following microinjection with either 0 (DMSO) (n =30), 0.53 (n = 25), 1.05 (n = 26), 2.57 (n = 36), 5.26 (n = 36), and 9.55 (n = 49) µg/g of 6PPD-quinone. Differences from the DMSO control were determined using a Fishers Exact Test. (* and +) indicates significant differences ($p < 0.05$) from the DMSO control.

3.4.3 Transcriptomics and Pathway Overrepresentation Analysis

Reads were mapped to 31828 unique genes. A total of 3873 genes were differentially expressed (p-adjust < 0.05) between embryos microinjected with 5.26 µg/g 6PPD-quinone/L and control embryos (Figure 3.3). Of these, 1521 transcripts were increased in abundance and 2352 transcripts were decreased in abundance (Figure 3.3). Pathway overrepresentation analysis identified 41 KEGG pathways that were dysregulated (FDR < 0.05), several of which are related to cardiovascular development and function, including vascular endothelial growth factor (VEGF) signaling, apelin signaling, tight junctions, focal adhesions, and cell adhesion molecules (Figure 3.4). There was also evidence of oxidative stress and inflammatory responses that might contribute to the observed phenotypes (Table 3.2).

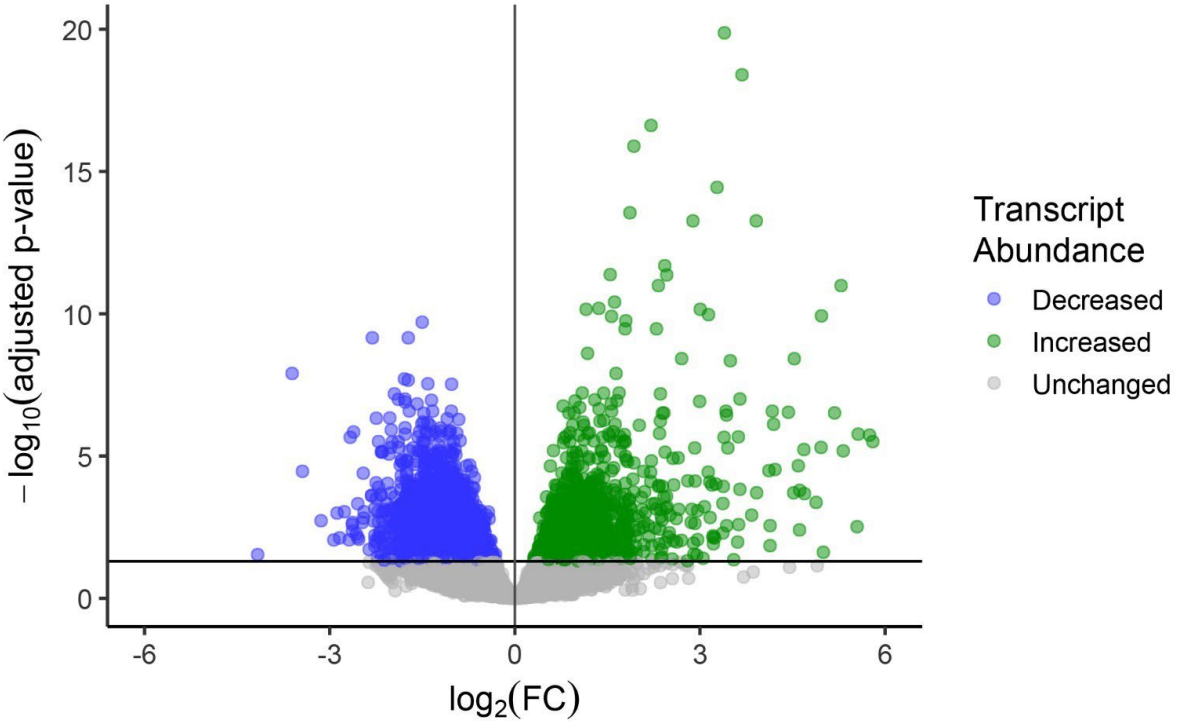

Figure 3.3. Volcano plot of the transcriptome of fathead minnow embryos microinjected with 6PPD-quinone. Transcript directionality refers to gene transcripts that were either significantly (p-adjust < 0.05) increased or decreased in abundance relative to control embryos that were microinjected with DMSO. Each point represents a unique transcript.

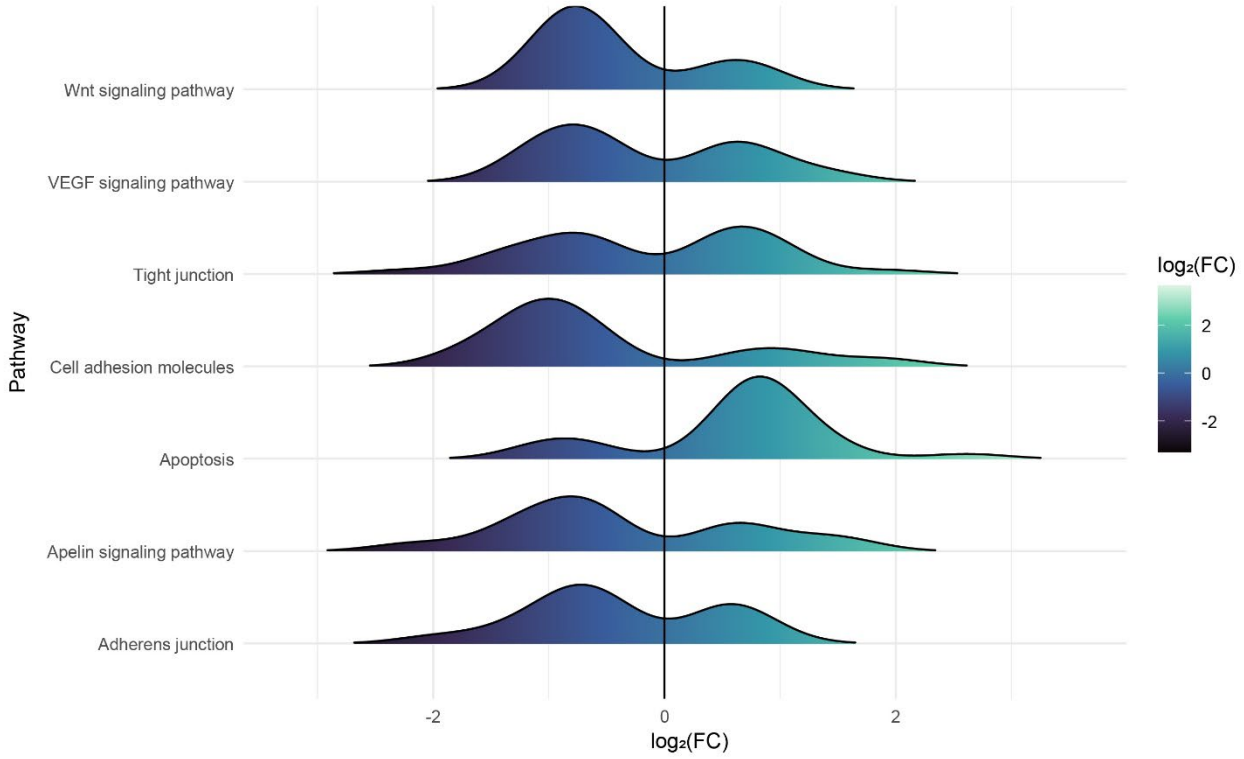

Figure 3.4. Density ridgeline plots showing the distribution of differentially expressed genes based on their $\log_2$(FC) in 7 of the 41 significantly altered KEGG pathways (FDR < 0.05) identified in pathway overrepresentation analysis that could be related cardiovascular development and function.

Table 3.2. A subset of genes that were differentially expressed that may have roles in inflammation or oxidative stress.

Inflammation		
Gene name	**Gene symbol**	**log2(FC)**
interleukin 1β	*il1b*	4.11
interleukin-1 receptor-associated kinase 3	*irak3*	1.79
interleukin 8-like	LOC120469344	3.43
interleukin 8-like	LOC120469346	2.99
C-X-C motif chemokine 9	LOC120479583	4.61
Oxidative Stress		
Gene name	**Gene symbol**	**log2(FC)**
glutathione peroxidase 1b	*gpx1b*	1.85
glutathione peroxidase 4a	*gpx4a*	1.08
glutathione peroxidase 4b	*gpx4b*	0.53
glutathione peroxidase 9	*gpx9*	-0.75
uncoupling protein 1	*ucp1*	0.8
uncoupling protein 2	*ucp2*	1.52
uncoupling protein 3	*ucp3*	2.47

3.5 Discussion

3.5.1 Microinjection of 6PPD-quinone Induced Developmental Toxicity

I previously reported no mortality or malformations in fathead minnow embryos exposed to 6PPD-quinone at concentrations as high as 39.97µg/L in water (Anderson-Bain et al., 2023). These findings were confirmed in the present study as there was no effects on mortality and developmental malformations in fathead minnow embryos that were exposed to 11.14µg 6PPD-quinone/L. However, microinjection of 6PPD-quinone into freshly fertilized embryos caused significant and severe developmental toxicities. There were incidences of spinal curvature and pericardial edema, but these effects were not statistically significant at every dose, likely due to the small number of mild cases in the DMSO-injected controls. Edema and spinal curvatures are not consistent phenotypes of early life-stage exposure to 6PPD-quinone. No gross malformations were observed in coho salmon embryos exposed to 6PPD-quinone up to 7.22 µg/L, including edemas and spinal curvature (Greer et al., 2023). However, exposure of early life-stage lake trout to waterborne 6PPD-quinone up to 13.5µg/L caused yolk sac edema in 100% of individuals in the highest treatment, and occurrence of spinal curvature was as great as 33% in some treatment groups (Roberts et al., 2025). These findings imply that timing, route of exposure, species, and developmental stage could be important factors when considering 6PPD-quinone toxicity.

Embryos microinjected with 6PPD-quinone displayed a suite of cardiovascular abnormalities, including absence of one or both common cardinal veins and abnormal heart morphology that have not been reported previously. Blood pooling/hemorrhaging was also observed and is consistent with bleeding of the caudal vein in lake trout fry chronically exposed to 6PPD-quinone (Roberts et al., 2025). Other studies have also suggested that 6PPD-quinone can cause cardiotoxicity. Expression of genes related to vascular permeability were dysregulated

in coho salmon embryos exposed to 6PPD-quinone (Greer et al., 2023). While the effects cannot be specifically attributed to 6PPD-quinone, disruption of the blood-brain barrier occurred in juvenile coho salmon exposed to urban stormwater runoff (Blair et al., 2021). Fathead minnow embryos injected with 6PPD-quinone exhibited cardiotoxicity, however there was no significant increase in mortality relative to controls. Although the cardiovascular system delivers oxygen to tissues, fish use cutaneous respiration during their early life stages and gradually transition to relying on their gills as they grow and develop (Prescott et al., 2021). I hypothesize that cutaneous respiration allowed embryos exposed to 6PPD-quinone to survive, despite the cardiovascular toxicity.

My results support previous studies which suggest that differences in species-sensitivity to 6PPD-quinone might be due to toxicokinetic factors (Montgomery et al., 2023). In comparison to aqueous exposures, microinjection bypasses the chorion and deposits the chemical directly into the yolk sac of the embryo. The chorion provides a protective barrier for embryos from environmental pollutants, which can help prevent toxicity (Xu et al., 2024). In the present study, fathead minnow embryos were microinjected with 6PPD-quinone prior to gastrulation, which is a developmental window where fish are vulnerable to xenobiotics (Von Westernhagen, 1988). Fish embryos typically have a limited capacity to metabolize chemicals during gastrulation, and the metabolic competency of fishes increases with age (Kühnert et al., 2017; Loerracher, 2021). In a comparative study, species that are insensitive to 6PPD-quinone induced lethality had higher amounts of both a hydroxylated phase I metabolite and an O-glucuronide phase II metabolite compared to sensitive species, suggesting that biotransformation is an important factor for the detoxification of 6PPD-quinone (Montgomery et al., 2023). Therefore, I hypothesize that microinjection of 6PPD-quinone exposed the developing embryo to enough chemical during a

vulnerable period of development where the fish had a limited capacity for xenobiotic metabolism, further supporting the role of toxicokinetic factors in toxicity of 6PPD-quinone.

3.5.2 Microinjection of 6PPD-quinone Altered Expression of Cardiovascular Genes

A suite of genes and pathways that are important for cardiovascular development and function were differentially expressed in embryos microinjected with 6PPD-quinone, providing a molecular understanding for the cardiovascular abnormalities observed in the present study. Angiogenesis is a complex process that utilizes multiple signaling pathways to coordinate the formation of blood vessels (Karamysheva, 2007). A key component of vascular tissue is the inner endothelial barrier that is surrounded by vascular smooth muscle and extracellular matrix (Hungerford and Little, 1999). Normal endothelial barrier function requires regulation of endothelial cell-cell adhesion and endothelial cell shape, and maintenance of endothelial-extracellular matrix adhesion (Claesson-Welsh, 2015). Endothelial barrier function can also be modified by growth factors and inflammation (Claesson-Welsh, 2015).

Endothelial morphogenesis and integrity require tightly regulated cell-cell interactions. These interactions require adherens, tight junctions, and cell adhesion molecules that make up endothelial junctions that are important for maintaining dynamic cell-cell contact, and for cellular communication (Wallez and Huber, 2008). Endothelial junctions are also linked to and modulate the cytoskeleton, which has a role in stabilizing and contracting endothelial junctions, as well as maintaining endothelial cell shape (Wallez and Huber, 2008). Proper endothelial cell-cell interactions prevent gaps between cells, which could cause leakage of contents from blood vessels or disruption of blood vessel patterning (Ellertsdóttir et al., 2010; Dejana et a., 2008). Changes in expression of adherens, cell-adhesion molecules, and tight junctions might have contributed to the hemorrhaging and improper cardinal vein development in embryos

microinjected with 6PPD-quinone. Transcript abundances of nine cadherins that could have roles in endothelial barrier function – N-cadherin/Cadherin 2 (LOC120465020), N-cadherin 2 (*cdh12a*), H-cadherin/cadherin 13 (*cdh13*), cadherin 11 (*cdh11*), protocadherin 1 (*pcdh1a*/*pcdh1b*), protocadherin 7 (*pcdh7a*/*pcdh7b*), protocadherin 10 (LOC120482993), FAT atypical cadherin 1 (*fat1b*), calsyntenin 1 (*clstn1*), and calsyntenin 3 (*clstn3*), were decreased in embryos microinjected with 6PPD-quinone (Colas-Algora and Millan, 2018). Cadherins are the transmembrane component of adherens junctions and are critical for angiogenesis (Colas-Algora and Millan, 2018; Bagatto et al., 2006; Luo and Radice, 2005). Additionally, transcript abundance of intercellular adhesion molecule 3 (*icam3*) - a fathead minnow orthologue for *icam1*, was increased. Activation of intercellular adhesion molecules was shown to decrease expression of endothelial adhesion proteins, such as VE-cadherin, zona occludens 1 (ZO-1), and zona occludens 2 (ZO-2) (Patibandla et al., 2009). Transcript abundance of also known as ZO-1, also known as tight junction protein 1a (*tjp1a*), was decreased. ZO-1 is a key component of tight junctions in endothelial cells, and a decrease in ZO-1 has been associated with disruption of endothelial tight junctions and disrupted endothelial cytoskeleton (Tornavaca et al., 2015). Further, transcript abundance of junctional adhesion molecule 2a (*jam2a*), a component of inter-endothelial junctions, was increased. JAM-2 has been associated with an increase in paracellular permeability (Aurrand-Lions et al., 2001). Overall, these changes in gene expression indicate that alterations in endothelial cell-cell interactions may contribute to 6PPD-quinone induced cardiotoxicity.

Dysregulation of VEGF signaling might have contributed to the cardiotoxicity in embryos microinjected with 6PPD-quinone. VEGF signaling via the VEGF receptor is critical for heart development, angiogenesis, and regulating vascular permeability (Senger et al. 1983;

Zhu et al., 2017). Increased transcript abundance of vascular endothelial growth factor A (*vegfa)* in embryos injected with 6PPD-quinone suggest that hemorrhaging might have been caused by greater expression of the *VEGFA* gene. The original name for VEGFA was "vascular permeability factor" due to its key role and potency in inducing vascular permeability (Senger et al., 1983). The mechanism of increased *vegf* transcript abundance is not known but might have been due to altered Wnt signaling that was evident from the pathway analysis. The Wnt/ß-catenin pathway regulates transcription of VEGF, and mutations in the Wnt signalling pathway have been shown to up-regulate VEGF expression (Easwaren et al., 2003; Farrara et al., 2004). Evidence of altered VEGF signaling also comes from decreased transcript abundances of several genes coding proteins downstream of VEGF receptor, including *prkcba*, *src*, *kras*, and *pik3ca*. Protein kinase C (*prkcba*) is important for increasing endothelial cell proliferation (Takahashi et al., 1999). V-src avian sarcoma viral oncogene homolog (*src*) is involved in phosphorylating cell-junction proteins to maintain endothelial cell stability, promoting cell matrix adhesion assembly, and regulating endothelial cytoskeletal dynamics (Schimmel et al., 2020; Hu et al., 2008). Decreased *src* expression has been shown to potentially reduce blood vessel sprouting (Schimmel et al., 2020). v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*kras*) and phosphatidylinositol-4,5-bisphosphate 3-kinase (*pik3ca*) are components of the Kras/PI3K-Akt signaling pathways, inhibition of which impairs angiogenesis in zebrafish embryos (Liu et al., 2008). Disruption of VEGF signaling can result in loss of regulation of vascular growth and repair, which eventually results in degradation of adherens and tight junctions, endothelial cell apoptosis, vascular rupture, and blood brain barrier leakage (Muneer et al., 2012). VEGF has an anti-apoptotic role, and loss of VEGF signaling can increase apoptosis, leading to severe hemorrhaging in mouse embryos (Dimmeler and Zeiher, 2000). Transcript abundances of several

apoptotic genes, including the Fas receptor (*fas)* and the Fas-associated death domain protein (*fadd*) were increased in embryos microinjected with 6PPD-quinone (Chan et al., 2000). Further supporting the role of loss of VEGF signaling in cardiotoxicity, pharmaceuticals that are designed to inhibit angiogenesis through inhibiting the activity of proteins in the VEGF signaling pathway often present with hemorrhaging complications (Elice and Rodeghiero, 2010). Additionally, disrupted VEGF signaling has been linked to faulty cardiac chamber formation and looping in zebrafish, which are similar to the heart phenotypes in this study (Zhu et al., 2017). Taken together, these changes in transcript abundances indicate that altered VEGF signaling may underpin the cardiovascular abnormalities observed in this study.

Another pathway required for angiogenesis is apelin signaling. This pathway stimulates endothelial cells into a pro-angiogenic state whereby they are activated and acquire migratory and proliferative phenotypes (Helker et al., 2020). Apelin signaling pathway was dysregulated in embryos microinjected with 6PPD-quinone. Transcript abundance of apelin receptor early endogenous ligand (*apela*) was increased and transcript abundance of downstream G-protein coupled receptors, such as *gng5b* were decreased. Dysregulation of this pathway might have contributed to the disrupted cardiovascular phenotype observed in this study.

Exposure to 6PPD-quinone might have triggered an inflammatory response that contributed to cardiotoxicity observed in the present study. Inflammation is an immunovascular response characterized by an increase in vascular permeability as a mechanism to rid the body of pathogens or as a response to tissue damage (Medzhitov, 2010; Sprague and Khalil, 2009). Transcript abundance of interleukin 1β (*il1b*) was increased by 17-fold, making it one of the most differentially expressed transcripts in embryos microinjected with 6PPD-quinone. *Il1b* is an important pro-inflammatory cytokine and immune-regulatory gene (Rauta et al., 2012).

Abundance of *il1b* transcript was also increased in larval coho salmon exposed to 6PPD-quinone (Greer et al., 2023), highlighting it as a potential toxicological factor, irrespective of species. Transcript abundance of two interleukin-8-like genes, LOC120469344 and LOC120469346, were increased by 10- and 7.9-fold in embryos microinjected with 6PPD-quinone. Interleukin 8 is a chemokine produced under inflammatory conditions (Wang et al., 2013; Matsushima and Oppenheim, 1989). Transcript abundance of C-X-C motif chemokine 9 (LOC120479583), which is involved in inflammation (Tokungo et al., 2018), was increased by 24-fold. Because several of the most drastically dysregulated genes in this study are related to inflammation and immune responses, it suggests inflammation and immunological responses played a critical role in 6PPD-quinone mediated developmental cardiotoxicity.

The current study suggests that oxidative stress might have been induced in embryos exposed to 6PPD-quinone, which is consistent with several other studies. In our previous study, abundances of metabolites in the one-carbon metabolism pathway that regulates glutathione synthesis were increased in livers and gills of adult fathead minnows exposed to water-borne 6PPD-quinone (Anderson-Bain et al., 2023). Similarly, abundances of metabolites of one-carbon metabolism and oxidative stress were altered in livers from rainbow trout exposed to 6PPD-quinone (Di et al., 2024). Abundances of four transcripts that code for glutathione peroxidases were altered in the present study - *gpx1b*, *gpx4a*, *gpx4b* were increased in abundance, and *gpx9* was decreased in abundance. Glutathione peroxidases are antioxidant enzymes that use glutathione to reduce hydrogen peroxide and lipid peroxides, thereby preventing damage caused by reactive oxygen species (ROS) (Pei et al., 2023). ROS can originate from multiple cellular processes, including inflammatory responses, which I propose were triggered in embryos exposed to 6PPD-quinone (Papaharalambus and Griendling, 2007). Disruption of mitochondrial

function is another potential source of ROS (Kirkinezos and Moraes, 2001). 6PPD-quinone has been shown to uncouple mitochondrial respiration in the rainbow trout gill cell line, RTG-2 (Mahoney et al., 2022). Although mitochondrial function was not measured in the present study, transcript abundances of the uncoupling proteins – *ucp1, ucp2*, and *ucp3* were increased in embryos exposed to 6PPD-quinone. Expression of uncoupling proteins can increase as an adaptive response to ROS, and these proteins have roles in inducing mitochondria proton leak, which can uncouple mitochondrial respiration (Ledesma et al., 2002). Previous studies did not provide a clear role for oxidative stress in toxicity of 6PPD-quinone (Anderson-Bain et al., 2023; Di et al., 2024). However, increased ROS can cause endothelial dysfunction leading to vascular leakage (Papaharalambus and Griendling, 2007). Therefore, results of the current study suggest that oxidative stress might have contributed to the hemorrhaging in embryos microinjected with 6PPD-quinone. In contrast to our findings, coho salmon larvae exposed to 6PPD-quinone showed transcriptome changes that could indicate vascular leakage, but there were no changes in gene expression that were indicative of mitochondria dysfunction (Greer et al., 2023). Additional studies are needed to better understand the role of oxidative stress in the toxicity of 6PPD-quinone.

3.6 Conclusion

In this study, I intended to explore the sublethal effects of 6PPD-quinone using fathead minnows as a model species as they previously were identified as insensitive to acute lethality and displayed no symptoms of embryotoxicity when exposed to 6PPD-quinone through waterborne exposures. Unexpectedly, I found that microinjection of 6PPD-quinone caused cardiotoxicity, including missing common cardinal veins, hemorrhaging, and abnormal heart morphology. These symptoms resemble those observed in sensitive species of fishes, such as

lake trout (Roberts et al., 2025). Mechanistically, 6PPD-quinone induced gene expression changes indicative of dysregulation of endothelial cell-cell adhesion, inflammation, VEGF signaling, and apelin signaling that are consistent with vascular leakage and disrupted cardiovascular system development. Further, gene expression changes indicative of mitochondrial dysfunction and oxidative stress were observed. These transcriptome responses align with those in coho salmon embryos exposed to 6PPD-quinone (Greer et al., 2023). Overall, this study provides additional support for disruption of the cardiovascular system as a mechanism 6PPD-quinone induced toxicity and provides a molecular basis for this disruption. Additionally, results of this study support the hypothesis that toxicity induced by 6PPD-quinone might be driven by toxicokinetic factors as our microinjection was performed prior to gastrulation, a developmental window where biotransformation capacity is limited.

Although developmental toxicities were induced in the present study, it is unlikely that embryos would be exposed to the doses of 6PPD-quinone administered in the present study. Thus, fathead minnows likely are insensitive to 6PPD-quinone under environmentally realistic exposure scenarios, as suggested previously (Anderson-Bain et al., 2023). However, I propose that microinjection of fathead minnow embryos might be a powerful approach to study mechanisms of toxicity of 6PPD-quinone or, and more importantly, for screening potential replacement antiozonants for 6PPD in tires. Compared to salmonids, fathead minnows are much more amenable to controlled laboratory exposures as breeding cultures can be maintained in basic aquatic facilities. Additionally, utilizing fish embryos reduces the number of adult fishes used in toxicity testing, and embryos are often considered exempt from animal care regulations. However, microinjection is not a commonly used method of exposure, thus limiting the widespread adoption of this approach.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1 Introduction

Anthropogenic environmental pollution can have adverse effects on the health and survival of organisms, including fishes. N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone, commonly known as 6PPD-quinone, is an emerging contaminant of concern that was identified as the causative agent of urban runoff mortality syndrome (UMRS), an acute lethality event that occurs in coho salmon (*Oncorhynchus kisutch*) following exposure to urban stormwater runoff (Tian et al., 2021). Because 6PPD, the parent compound of 6PPD-quinone, is a routinely used tire-additive, there is global concern about adverse effects on other species of fishes. Acute lethality induced by 6PPD-quinone is highly species specific and has only been demonstrated in certain salmonids (Montgomery et al., 2023; Brinkman et al., 2022). Generally, species sensitivity to toxicants occurs in a phylogenetic manner as species that are more closely related have more structurally similar molecular targets and pathways (Spurgeon et al., 2020). However, there is no phylogenetic relationship pattern of species sensitivity to 6PPD-quinone. Evidence suggests that species-sensitivity to 6PPD-quinone might be due to toxicokinetic factors, particularly differences in biotransformation (Montgomery et al., 2023). Proposed mechanisms of acute lethality to 6PPD-quinone include mitochondrial uncoupling at the gills and leakage of the blood-brain barrier (Mahoney et al., 2022; Blair et al., 2021). However, much of the research to-date has focussed on acute lethality and less is known about the potential sublethal effects of 6PPD-quinone to insensitive species of fishes.

There are large knowledge gaps with respect to both which fish species are sensitive to acute lethality and the mechanism(s) that underpin species sensitivity to 6PPD-quinone. Fathead minnows (*Pimephales promelas*) are an important species in toxicology in North America as they

possess many qualities that are desirable to have as a model laboratory organism, including a fully sequenced and annotated genome, and being widely distributed throughout much of the continent (Saari et al., 2017; Ankley and Villenuve, 2006). Because of their small size and ease with which they can be bred in a lab, they are an ideal species for toxicity testing at different life-stages. Therefore, the first objective of this research was to determine if fathead minnows at different life-stages are sensitive to 6PPD-quinone induced acute lethality. The second objective was to use fathead minnows as a model species to investigate mechanisms of sublethal toxicity.

4.2 Summary of Study #1

In this study (Chapter 2), sexually mature fathead minnows were exposed via waterborne exposures to nominal concentrations of either 0 µg/L or 20 µg/L of 6PPD-quinone for 36 h to conduct an initial range-finding experiment. Because there was no mortality from the range-finding experiment, a second exposure was performed in which sexually mature fathead minnows were exposed to measured concentrations of 0 µg/L, 0.09 µg/L, 0.85 µg/L, and 9.65 µg/L for 96 h and samples were collected to investigate sub-lethal effects. 6PPD-quinone had no effect on the condition factor, blood glucose, or percent hematocrit of the fish, although there was a significant decrease in the hepatosomatic index of the fish exposed to the highest concentration of 6PPD-quinone. Livers and gills were used for mechanistic analysis using EcoToxChip transcriptomics and ^{1}H NMR metabolomics. In a parallel embryotoxicity assay, fathead minnow embryos were exposed to 6PPD-quinone at 0.11 µg/L, 1.30 µg/L, 4.32 µg/L, 13.59 µg/L, and 39.27 µg/L for 168 h. 6PPD-quinone had no effect on either embryo malformations or mortality.

The EcoTox Chips showed a tissue-specific response. In the gills, 25 transcripts were increased in abundance and 10 transcripts were decreased in abundance. In the liver, 14 transcripts were dysregulated, with all of them increased in abundance. There was some evidence of oxidative stress, such as increases in *cox11* and *cox15* in the liver (Bourens et al., 2013). Generally, the transcriptomic response was mild.

Using ^{1}H NMR metabolomics, tissue specific responses were observed. In gills, there was a concentration dependent increasing separation of the metabolome of exposed fish compared to control fish. In contrast, there was a large separation at all three 6PPD-quinone concentrations relative to the controls in the livers, but there was no concentration dependent effect. Significant differences were observed in the abundances of 32 metabolites in the livers and 26 metabolites in the gills in at least one concentration of 6PPD-quinone relative to the control. Mechanistically, one-carbon metabolism was altered in both the livers and the gills. One carbon metabolism includes the methionine cycle, which regulates cellular methylation reactions (Friso et al., 2017).

Further, abundances of metabolites indicative of oxidative stress were increased in both tissues. Methionine sulfoxide was increased in abundance in both the livers and gills. There was a concentration-dependent increase in the gills and a strong increase at all three concentrations in the livers. Methionine sulfoxide forms when methionine acts as an antioxidant and reacts with reactive oxygen species (Moskovitz, 2005; Levine et al., 1999). Additionally, there were increases in metabolite intermediates of the transsulfuration pathway, which synthesizes glutathione – a key antioxidant that is critical for the response to oxidative stress. These metabolite intermediates include homocysteine, cystathionine, and pyroglutamate, all of which can increase during oxidative stress (Gamarra et al., 2019; Mosharov et al., 2000). These

alterations in glutathione synthesis were more pronounced in the livers. which indicate that this tissue might be a target of 6PPD-quinone toxicity.

Mitochondrial uncoupling in the gill has been proposed as a mechanism of acute lethality of 6PPD-quinone in rainbow trout (Mahoney et al., 2022). However, inconsistent increases in abundances of citrate, malate, and alanine, and lack of change in abundance of aspartate, suggest that mitochondrial uncoupling did not occur in fathead minnow gills (Sreedhar et al., 2019). This is consistent with the lack of acute lethality that was observed in this study.

4.3 Summary of Study #2

Based on the findings of Chapter 2, the second study (Chapter 3) was designed to identify potential sublethal toxicities of 6PPD-quinone using fathead minnows as a model species. The goal of this study was to use microinjection as an exposure method as it ensures consistent and precise delivery of chemicals to individual eggs. Early life-stages were chosen as they are generally considered to be more sensitive than mature fish (Von Westernhagen, 1988; Loerracher 2021). Whole transcriptome mRNAseq was used for hypothesis generation, as it is more comprehensive than both EcoTox Chips and ^{1}H NMR metabolomics. Fathead minnow embryos were microinjected prior to gastrulation with 0.00, 0.53, 1.05, 2.57, 5.26, or 9.55 µg/g of 6PPD-quinone and observed daily for malformations or mortality. In contrast to the exposure with waterborne 6PPD-quinone (Chapter 2), embryos presented with a suite of developmental toxicities indicative of cardiotoxicity, including missing common cardinal veins, hemorrhaging, and abnormal heart morphology. The hemorrhaging was sporadic, and fish started spontaneously bleeding between 3 dpf until the termination of the embryotoxicity assay at 12 dpf. Hemorrhaging, lack of common cardinal vein development, and abnormal heart morphology are consistent with previous studies showing disruption of the cardiovascular system in species that

are sensitive to 6PPD-quinone (Roberts et al., 2025; Greer, 2023; Blair et al., 2020). Despite the suite of developmental malformation, there was no significant mortality. One explanation for the lack of mortality is that a completed cardiorespiratory system may not be necessary at this life stage as embryos use cutaneous respiration for gas exchange (Prescott et al., 2021). It is hypothesised that lethality would have occurred if fish were reared to life-stages that depend on gills for respiration.

This thesis indicates that microinjection of 6PPD-quinone was able to induce a sensitivity in an otherwise insensitive species. Microinjection involves bypassing the chorion and injecting chemical to the yolk sac of each embryo prior to gastrulation. Therefore, microinjection is a useful tool for ensuring consistent and precise doses of xenobiotics to each embryo. However, gastrulation is also notably a vulnerable period of development for fishes to be exposed to xenobiotics (Von Westernhagen, 1988). Fish embryos have a lower biotransformation capacity during gastrulation, and the metabolic competency of fishes increases with age (Loerracher, 2021). Therefore, I hypothesize that microinjecting 6PPD-quinone potentially permitted a scenario where a large enough dose was administered during a period of development where the fish was unable to metabolize it, resulting in toxicity. This supports the hypothesis that toxicokinetic factors play the most significant role 6PPD-quinone induced toxicity (Montgomery et al., 2023).

To provide a mechanistic basis for the observed developmental toxicities, RNAseq, was performed using fathead minnow embryos that were microinjected with either 0.00 µg/L or 5.26 µg/g 6PPD-quinone and sampled at 48 hpf. The transcriptome showed a large response, with 1521 transcripts increased in abundance and 2352 transcripts decreased in abundance. Pathway analysis showed that alterations in adherens junctions, tight junctions, and cellular adhesion

molecules were dysregulated which could impact endothelial barrier integrity and blood vessel development (Ellertsdóttir et al., 2010; Dejana et a., 2008; Wallez and Huber, 2008). Further, expression of genes indicative of inflammation and VEGF signalling were altered. VEGF signalling is important for cardiovascular system development and regulating vascular permeability (Senger et al., 1986; Zhu et al., 2017). Some of the most drastically increased transcripts were related to inflammation. Inflammation can cause vascular permeability, which could potentially indicate its role in inducing the hemorrhaging in 6PPD-quinone exposed embryos (Medzhitov, 2010). Further, some genes indicative of disrupted mitochondrial function, including several genes encoding uncoupling proteins, were increased in abundance, which could support that mitochondria uncoupling may be a mechanism of toxicity in fathead minnows that have been microinjected with 6PPD-quinone (Ledesma et al., 2002). Lastly, increases in abundances of transcripts of several genes that are important for the response to oxidative stress were increased, indicating that oxidative stress might have contributed to the observed phenotypes (Pei et al., 2023). It is unclear from this study if oxidative stress occurred because of mitochondrial uncoupling, as oxidative stress can also occur following endothelial dysfunction and inflammation (Papaharalambus and Griendling, 2007).

4.4 Future Directions

4.4.1 Using Microinjection as a Tool for Regulatory Testing of 6PPD Alternatives

Fathead minnows are a model species in ecotoxicology, especially in North America. Their small size, ease of breeding, and their well-defined developmental stages mean they can be cultured in even the most basic aquatic facilities. To-date, the only fish species identified as sensitive to 6PPD-quinone are some salmonids. As such, it is likely that toxicity testing with salmonids will be a critical component of efforts to screen potential replacement

chemicals. However, compared to small bodied fished such as fathead minnows, salmonids require more resources, including space and time to use them in toxicity studies. Results of this thesis suggest that microinjection of fathead minnow embryos could be a powerful method to screen potential replacement chemicals for their toxic potential. Future studies could also determine whether microinjection of 6PPD-quinone into embryos of other more broadly used model species, such as zebrafish and Japanese medaka, cause the same adverse effects. However, microinjection is an uncommon exposure method, which may prove to be a barrier to this use.

4.4.2 The Immune System as a Target of 6PPD-quinone

While the understanding of the toxic effects of 6PPD-quinone have increased since its initial discovery, more research is needed to determine the specific toxicodynamic mechanisms that result in its adverse outcomes. This thesis demonstrated that mechanisms of 6PPD-quinone induced early life-stage toxicity are likely very well-evolutionarily conserved. Both the vascular leakage phenotype and the gene expression changes observed in fathead minnow embryos microinjected with 6PPD-quinone were also observed in early life-stages of sensitive species of salmonids (Roberts et al., 2025; Greer et al., 2023; Blair et al., 2021). While the mechanism(s) of this effect are not known, a hypothesis that I generated from this thesis is that 6PPD-quinone targets the innate immune system. The innate immune system acts as a first line of defense for an organism in the event of an infection or injury, producing non-specific responses within minutes to hours (Marshall et al., 2018). The innate immune system is evolutionarily ancient, present to some degree in all multicellular organisms (Suckale et al., 2006). While the innate immune system is important in all life stages of fishes, embryos rely on the innate immune system as they have not yet developed their adaptive immune system (Uribe et al., 2011). In fact, in zebrafish (*Danio rerio*), enzymes and cytokines of the innate immune system are maternally transferred

into embryos (Kamako et al., 2008). Activation of the innate immune system induces inflammation, leading to vascular permeability, both through direct signalling and alterations in the expression of genes in pathways that are similar to what was observed in this thesis, such as VEGF signalling (Fahey and Doyle, 2019). Therefore, if 6PPD-quinone were to induce immunotoxicity through disruption of the innate immune system, it could potentially explain both the cardiovascular malformations and the corresponding gene expression changes that occur in species that are sensitive to 6PPD-quinone. The specific molecular target of 6PPD-quinone that induces this immunotoxicity is not known but should be a topic of future research.

4.4.3 A Putative Adverse Outcome Pathway for Developmental Cardiotoxicity

A major goal of toxicology in the 21st century is to develop and validate new methodologies that reduce the number of animals used in chemical risk assessments and toxicity testing (Lambert et al., 2022). Among these are development of *in vitro* (cell based) and *in silico* (computational) assays to assess and predict chemical toxicity. However, there is a regulatory need to link molecular perturbations to toxicities of regulatory relevance, including survival, growth, and reproduction. Adverse outcome pathways (AOPs) are conceptual frameworks that link molecular initiating events (MIEs) to key cellular- and tissue-level changes, termed key events (KEs) resulting in adverse outcomes (AO) of regulatory relevance (Figure 4.1) (Villeneuve, 2014). AOPs use existing knowledge about mechanisms, but are flexible in that they can be developed, built upon, and modified (Kleinstreuer et al., 2016). AOPs have three development stages: putative, formal, and quantitative (Vinken et al., 2017). AOPs can be expanded to include multiple simultaneous MIEs, KEs, or AOs to generate an adverse outcome network (AON) (Villeneuve, 2014). AOPs and AONs are not chemical-specific; the assumption is that chemicals that have the same MIE will have the same downstream KEs and AOs

(Villeneuve, 2014). Both AOPs and AONs provide utility in predicting environmental consequences of toxicant exposure. The AOP/AON framework should help chemical risk assessors relate results from *in vitro* assays and *in silico* models to apical endpoints of regulatory relevance.

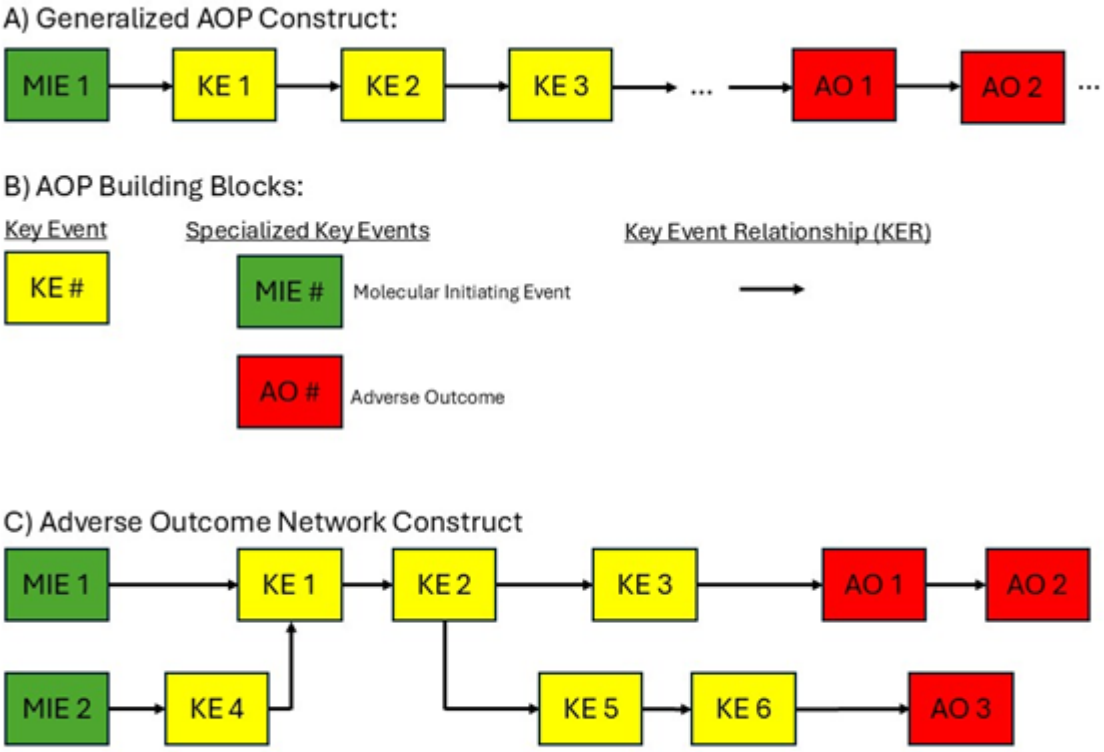

Figure 4.1. A schematic of a generalized adverse outcome pathway (AOP) (A) and adverse outcome network (AON) (C) framework. B) AOPs are composed of key events (KEs), that link molecular initiating events (MIEs) to adverse outcomes (AOs). C) AONs are composed of multiple MIEs and KEs that can be linked to give rise to multiple AOs. Illustration is adapted from Villeneuve et al., (2014).

To-date, there have been no proposed AOPs or AONs that describe early life-stage toxicity induced by 6PPD-quinone. Results of Chapter 3 suggest that dysregulation of multiple pathways or processes could play a role in the embryotoxicity of 6PPD-quinone, including dysregulation of VEGF signaling, inflammation, and disruption of cell-cell adhesion in vascular tissue. It is hypothesized that dysregulation of these pathways/processes might result from activation of an innate immune system by 6PPD-quinone. Based on this, I propose a putative AON that describes embryotoxicity in early life-stages of fish exposed to 6PPD-quinone. Further research is needed to add weight of evidence to this AON, including identification of the MIE.

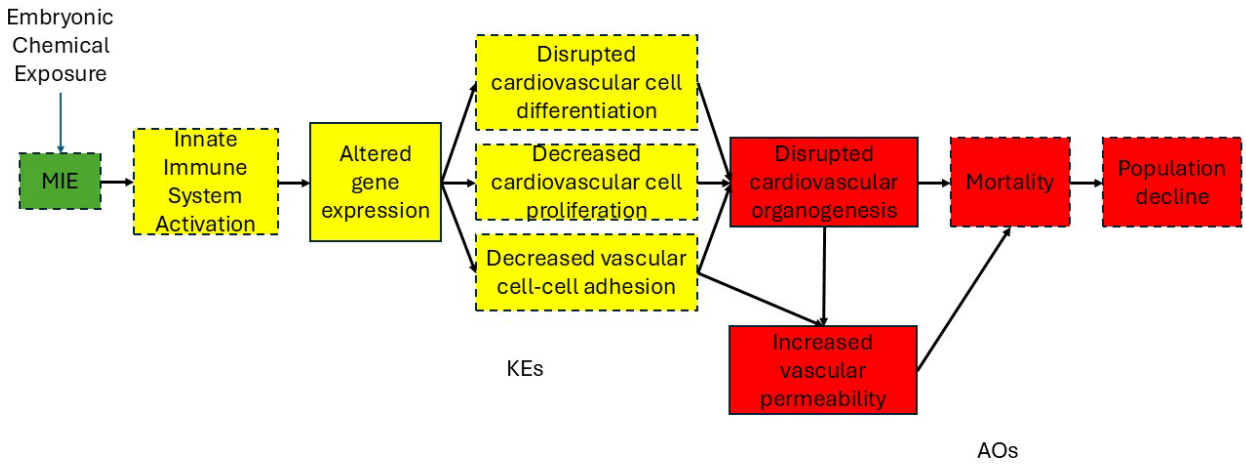

Figure 4.2. A putative adverse outcome network (AON) for the disrupted cardiovascular organogenesis and increased vascular permeability following activation of the innate immune system. Solid boxes indicate components of the pathway that were confirmed in this thesis, whereas dashed boxes indicate components of the pathway that are yet to be determined.

4.5 Conclusion

This thesis advances our understanding of the ecotoxicology of 6PPD-quinone. 6PPD-quinone is commonly found in aquatic environments, and thus mechanisms of both sublethal toxicity and acute lethality are important to understand. This thesis demonstrated that insensitive species of fishes, such as the fathead minnow, may experience sublethal toxicity when exposed to 6PPD-quinone in the environment. Additionally, it was demonstrated that using microinjection as an exposure method was able to induce sensitivity in fathead minnows, a previously identified insensitive species. This suggests that toxicokinetic factors are likely responsible for species-sensitivity to 6PPD-quinone exposure. This also suggests that modifying toxicokinetic factors under laboratory conditions could be useful in future toxicity studies, particularly for testing novel tire wear additives that may replace 6PPD. Future tire wear additives should undergo appropriate testing to ensure vehicle safety while mitigating environmental risks. Developmental toxicity of the cardiovascular system can decrease the fitness of fishes, and therefore it is important to understand the precise mechanistic basis underlying this toxicity. Lastly, a putative AON was constructed to assist with our further understanding these toxic outcomes. Gaining more comprehensive understanding of how toxicants can impact fishes can significantly improve our ability to protect the health of the environment.

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