

**THE CARDIORESPIRATORY AND METABOLIC TOXICITY OF
ACUTE NAPHTHALENE AND PYRENE EXPOSURE IN ADULT
ZEBRAFISH (*DANIO RERIO*): RELATIONSHIP TO ARYL
HYDROCARBON RECEPTOR ACTIVATION.**

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ABSTRACT

Benzo-a-pyrene (BaP), a representative polycyclic aromatic hydrocarbon (PAH), causes adverse cardiorespiratory and metabolic effects in adult zebrafish (*Danio rerio*), while other petrogenic PAHs e.g. naphthalene (NAP) or pyrene (PYR), are not well characterized. To investigate the hypothesis that acute exposure (48h) to NAP and PYR causes sublethal cardiorespiratory and metabolic impairment similar to BaP, adult zebrafish were aqueously exposed to NAP (0, 37, 370, and 3700 µg/L) or PYR (0, 0.25, 2.5 and 25 µg/L). Both PAHs had no major effects on metabolic rate or swimming endurance. Cardiac effects of acute NAP exposure were to increase filling of the heart during diastole and increase stroke volume, while PYR lowered heart rate. Overall, both NAP and PYR tended to increase glycogen and triglyceride levels in heart, liver and skeletal muscle, along with increased mRNA expression of CS and HOAD (both rate-limiting enzymes). However, PYR caused hepatic HOAD expression to decrease. One potential mechanism of toxicity for NAP and PYR could be through AhR activation, agreeing with observations that CYP1A mRNA expression was increased in cardiac and skeletal muscle, but disagreeing with observed decrease in liver after NAP exposure. In contrast, PYR had no effect on CYP1A mRNA expression in any tissues examined. Both NAP and PYR tended to increase SOD and GSR mRNA in the three tissues examined, suggesting activation of the antioxidant response. NAP and PYR may exert toxicity through three different mechanisms that could be related or functionally independent: 1) AhR agonist, 2) increased expression of cytochrome P450 1A (CYP1A) and/or 3) increased oxidative stress. To investigate mechanisms of toxicity, acute aqueous exposures (48h) to NAP or PYR were conducted in adult zebrafish alone or in the presence of an AhR antagonist

(CH-223191), CYP1A inhibitor (fluoranthene), or antioxidant scavenger (tempol). NAP or PYR alone failed to significantly alter cardiovascular function or most metabolic endpoint examined at the moderate exposure levels examined in this study. In contrast, co-exposure of each pharmacological antagonist with either PAH produced a number of effects that were not merely reversing the effects of the PAHs, but instead appeared to be independent of or distinct from the PAH effect by itself. In order to determine response patterns and relationships among the end-points, principal components analysis (PCA) was performed. PCA revealed two components that explained 71% of the variance in the data using 17 of the end-points. The first component was the one that best distinguished NAP and PYR alone groups from controls. This component was positively correlated with GSR, SOD and HOAD in cardiac and skeletal muscle as well as muscle CYP1A and a higher ratio of atrial-ventricular rates. The second component distinguished among groups treated with the three inhibitors, being positively correlated to liver CYP1A, heart rate, ejection fraction and higher energy stores in cardiac and muscle tissues. In conclusion, both NAP and PYR alone appear to cause cardiac toxicity through arrhythmia associated with oxidative stress, while AhR inhibition, CYP1A inhibition and free radical scavenging all were associated with improved cardiac function and higher energy stores. These results provide support for a complex interaction between normal cardiac and metabolic control with oxidative stress and AhR signaling.

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LIST OF ABBREVIATIONS:

µg/g	Micrograms per gram
µg	Microgram
µg/L	Micrograms per litre
µm	Micrometre
°C	Degrees centigrade
AHR	Aryl hydrocarbon receptor
AMR	Active metabolic rate
ANOVA	Analysis of variance
ASE	Accelerated solvent extractor
AV	Atrial-ventricular
BaP	Benzo(a)Pyrene
BMR	Basal metabolic rate
BNF	β-naphthoflavone
BPM	Beats per minute
CCME	Canadian Council of Ministers of the Environment
cm	Centimetre
CO	Cardiac output
COT	Cost of transport
CS	Citrate synthase
CYP1A	Cytochrome P4501A monooxygenase
d	Day
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDV	End diastolic volume
EF	Ejection fraction
EROD	Ethoxyresorufin-o-deethylase
ESV	End systolic volume

F-AS	Factorial aerobic scope
FL	Fluoranthene
g	Gram
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GSR	Glutathione-S-reductase
GSSG	GSH oxidation to form oxidized GSH
GST	Glutathione-S-transferase
HOAD	β -hydroxyacyl coenzyme A dehydrogenase
HSP	Heat shock protein
HPLC-FD	High performance liquid chromatography-fluorescence detection
K	Condition factor
Kg	Kilogram
L	Litre
LC50	Lethal concentration required to kill 50% of population
LOD	Limit of detection
log Kow	Octanol-water partition coefficient
LPL	Lipoprotein lipase
LSD	Last significance difference
m	Metre
miRNA	MicroRNA
mg	Milligram
mg/kg	Milligrams per kilogram
mg/L	Milligrams per litre
mL	Millilitre
MO ₂	Oxygen consumption
mm	Millimetre
mRNA	Messenger RNA
m/s	Meters per second
MS-222	Tricaine methanesulfonate

n	Number of samples
ng/g	nanograms per gram
NAP	Naphthalene
NAWQA	National Ambient Water Quality Assessment
nrf	nuclear factor erythroid
NSERC	Natural Sciences and Engineering Research Council of Canada
O ₂	Oxygen
PCA	Principal components analysis
PYR	Pyrene
PAH	Polycyclic aromatic hydrocarbon
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SEM	Standard error of the mean
SMR	Standard metabolic rate
SOD	Superoxide dismutase
SV	Stroke volume
TCA	tricarboxylic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Ucrit	Critical swimming speed
US EPA	United States Environmental Protection Agency

PREFACE

The general format of this thesis is as chapters with manuscripts as separate chapters; thus, some duplication of material is expected. Chapter 1 of this thesis is a general introduction and literature review, while Chapter 4 is a general discussion with overall major conclusions. Chapter 2 and 3 are both manuscripts that will be submitted for publication in scientific journals. The first data chapter, Chapter 2 is an investigation of the acute cardiovascular and metabolic impairment of acute naphthalene and pyrene exposure in adult zebrafish, while Chapter 3 is an investigation of whether acute naphthalene and pyrene toxicity in adult zebrafish depends on the relative potency as aryl-hydrocarbon receptor agonists and cytochrome P450 inducers are indicative of relative toxicity or whether oxidative stress is a better indicator. These two chapters relate to each other because chapter two investigated the concentration-response relationship to cardiorespiratory and metabolic impairments of the naphthalene and pyrene and chapter three was determining their mechanism of toxicity

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

1.1.1 Overview

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with two or more fused aromatic rings that are produced by the combustion of oil, coal, fossil fuels and as by-products through incomplete combustion of organic matter at high temperatures. PAHs are naturally found in the environment, mainly due to forest fires, volcanic activities and in areas of bitumen deposits. However, PAHs found in the ambient environment are more often the result of anthropogenic mobilization of contaminants and processes e.g. petroleum spills and oil sands activities, with the latter being a major source of emission in Canada (Neff et al., 2005). PAHs are ubiquitous in the aquatic environment worldwide and throughout Canada in biota, groundwater, lakes, rivers, sediments and soils (CCME, 2008) which results in toxicity to inhabiting species. Benzo-a-pyrene (BaP) has been heavily studied and is used as a representative PAH. BaP produces acute adverse effects in adult zebrafish (*Danio rerio*) on cardiovascular and respiratory function (Gerger & Weber, 2015). The PAHs of interest for this thesis, naphthalene (NAP) and pyrene (PYR), are both components of oil or bitumen and are released into the air from burning coal, oil or tobacco smoke (Neff et al., 2005). Like many other PAHs, they are listed on the US Environmental Protection Agency's priority pollutant list, making them chemical pollutants regulated with national discharge standards (US EPA, 2014). However, minimal research has been done with NAP and PYR examining acute adult toxicity. For this thesis, zebrafish will be used as a model freshwater species.

1.1.2 Polycyclic aromatic hydrocarbons (PAHs)

PAHs are defined as having two or more aromatic rings. They are non-polar, hydrophobic compounds that generally have high melting and boiling points, low vapour pressures, low water solubility and do not ionize (CCME, 2008). Although they are abundant in the environment, the fate of PAHs is determined by chemical processes such as volatilization, photolysis, hydrolysis, microbial degradation and adsorption to sediments (Wolska et al., 2012). PAHs enter organisms, then some are thought to interact with the aryl hydrocarbon receptor (AhR) and induce pathogenic changes through three different mechanisms (Incardona et al., 2011). PAHs that bind to AhR activate an adaptive response where cytochrome P4501A monooxygenase (CYP1A) enzymes are induced for metabolism of the ligand. However, this metabolic activity often instead leads to bioactivation of the parent compound into reactive, toxic metabolites. Reactive quinone metabolites cause excess production of reactive oxygen species (ROS), with superoxide production each time the quinone cycles to a semi-quinone and back, thereby depleting glutathione (GSH) stores and leading to oxidative stress (Salvo et al., 2016; Stohs et al., 2002). Oxidative stress by itself can cause cytotoxicity and increases cancer risk (Nebert et al., 2004; Rengarajan et al., 2015). Species with a greater ability of inducing CYP1A enzymes have been reported to have an increase in susceptibility to toxicity (Incardona, 2017; Okey, 1990). Carcinogenic PAHs are activated by both CYPs and epoxide hydrolase into “K-region” epoxides, previously reported as the ultimate carcinogenic metabolite. However, diol epoxide metabolites of PAHs with space(s) between the aromatic rings, known as the “bay region”, are mutagenic, highly reactive towards DNA and thus are concluded to be the ultimate carcinogenic metabolites of BaP (Gelboin, 1980; Shimada & Fujii-Kuriyama, 2004). These diol epoxides form adducts by

reacting with DNA and affecting cell replication (Shimada & Fujii-Kuriyama, 2004). The degree of reactivity and mutagenicity is determined by the location of the “bay region”. PAH exposure in animal studies has also determined that activation of the ras oncogene can cause tumor induction (Ding et al., 2006). Most PAH mechanisms of action have been inferred from BaP, but not all PAHs may act in the same manner as BaP. Barron et al. (2004) studied the relative potency of PAHs as AhR agonists in fish relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Generally, they found that two and three ring unsubstituted PAHs, such as NAP, were largely inactive at the AhR in fish. In contrast, the highest potency PAHs had 4-6 aromatic rings with an exposed bay region, similar to PYR. Thus, PYR will be studied in this thesis because it is predicted to act largely via AhR activation, but yet knowledge of its mechanism of toxicity is limited compared to that of BaP.

1.1.2.1 *Naphthalene*

The simplest PAH, NAP ($C_{10}H_8$), is a white, crystal solid consisting of two aromatic rings fused together. It has the highest vapour pressure of all the PAHs (US EPA, 1998). Due to its low molecular weight, it is easily removed by volatilization from aquatic environments; a process considered insignificant for PAHs with three or more aromatic rings (CCME, 2008). The estimated half-life of NAP ranges from 4.2 to 7.3 hours in surface waters (CCME, 2008). Thus, toxicity to the aquatic environment tends to occur immediately after an oil spill when volatile components such as NAP are present in high quantities rather than weathered crude oil (Incardona, 2017). NAP has a low solubility of 33.72 g/L in water at 25°C (Ran et al., 2002), but solubility increases as temperature increases. This results in less than 10% of NAP settling into sediments through the association with organic matter and instead it tends

to undergo biodegradation or photolysis, depending on the water chemistry and concentration (USEPA, 2003).

In addition to being a common component in crude oil and refined products containing PAHs, NAP is also a widely used commercially in mothballs, carbamate insecticides, surface-active agents, synthetic resins and in the production of phthalic anhydride (USEPA, 2003; Vuchetich et al., 1996). The majority of NAP in the environment is from direct release into the air, accounting for 90% of total discharge and only 2.7% is found in the terrestrial environment. Approximately 5% of NAP entering the environment is released into water primarily from coal tar production, distillation processes, oil seepage and spills directly into surface water (USEPA, 2003). Therefore, NAP has the potential of directly affecting inhabiting freshwater species.

Kerr & Melton (1999) analyzed 48 different crude oils from around the world to determine the concentrations of priority PAHs within each oil. Despite the variability in the content of the PAHs from each site, NAP is present in the highest concentration in all 48 samples, ranging anywhere between 1.2-3700 mg/kg oil and averaging 427 mg/kg oil. The U.S. Geological Survey National Ambient Water Quality Assessment (NAWQA) program reviewed and analyzed water samples and found that detection frequencies and concentrations for drinking water wells to be relatively low; at 1 µg/L or lower (USEPA, 2003). These drinking water levels fall into the range of concentrations of NAP found in the aquatic environment, being 0.4-70 µg/L, (CCME, 2008).

Microsomal metabolism is initiated when NAP is converted into 1-naphthol and then 1,2-dihydro-1,2-dihydroxynaphthalene (O'Brien et al., 1985). Through the process of

epoxidation, NAP is also converted into reactive metabolites; naphthalene 1,2-oxide and sulphate, glucuronide and GSH conjugates. Wilson et al. (1996) determined that GSH depletion and direct toxicity to human hepatic microsomes is caused by 1-naphthol, 1,2-naphthoquinone and 1,4-naphthoquinone, and not by the primary metabolite, 1,2-epoxide. Cytotoxicity and genotoxicity of NAP is due to the formation of quinones from 1-naphthol, not from the formation of the primary metabolite, resulting in oxidative stress and ROS formation. NAP exposure has also been reported to cause increased DNA damage in the brain and liver of mice (Bagchi et al., 2000). In the same study, the tumor suppressor gene p53 was shown to play a role in NAP toxicity with the use of p53 deficient mice being more susceptible to NAP than the controls. The toxic effects induced by NAP is thought to be mediated by oxidative stress, the production of ROS, GSH oxidation to form oxidized GSH (GSSG) and DNA damage, particularly related to carcinogenicity and cytotoxicity (Matés, 2000; Vuchetich et al., 1996). The important information to note for this thesis is that NAP is a PAH that is thought to work through AhR-independent mechanisms, but whether these oxidative stress mechanisms are also relevant for acute sub-lethal toxicity in fish is not known.

1.1.2.2 *Pyrene*

PYR ($C_{16}H_{10}$) is larger than NAP, but the smallest of the peri-fused PAHs, and is a colorless solid consisting of 4 fused benzene rings. Besides occurring ubiquitously as a product of incomplete combustion, anthropogenic sources of PYR in the environment include automotive care products, laundry and dishwashing products, personal care products, water treatment products and relatively high quantities in coal tar (Douben, 2003). Major sources of PYR in aquatic environments are from petroleum spillage, atmospheric deposition, wastewaters, and surface-land runoff (Eisler, 1987). PYR originating from crude

oil is less abundant in aquatic environments than NAP, ranging from non-detectable limits to 9.2 mg/kg oil (Kerr & Melton, 1999). Thus, aquatic levels resulting from various inputs were only 0.025 µg/L in aquatic environments, as reported by CCME (2008). Volatilization and microbial degradation are not important degradation pathways for PYR in aquatic systems, while photodegradation is important for PYR (Suess, 1976). PYR has a solubility of 0.134 mg/L (Ran et al., 2002) and a half-life greater than 8 weeks. Thus PYR is considered a persistent substance (CCME, 2008).

Adult goldfish (*Carassius auratus*) exposed to PYR for one week and determined that PYR induces oxidative stress through ROS production in fish liver. After 24 hours of PYR exposure at acutely toxic levels, bioaccumulation in the liver has been reported to occur and maximum levels reached (Yin et al., 2014). The hydroxyl radical ($\bullet\text{OH}$) is generated shortly after PYR exposure, increases significantly with the exposure period, and decreases until the end of measured exposure period (Yin et al., 2014).

Zebrafish larvae exposed to PYR showed different defects at different developmental stages, such as anemia, mild pericardial edema, a minor dorsal curvature, peripheral vascular defects and neuronal cell death (Incardona et al., 2004; 2006). However, cardiac conduction does not appear to be affected (Incardona et al., 2004). The mechanism of toxicity of PYR-induced developmental deformities is proposed to be through changes in the hepatic antioxidant enzyme superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase activity (Incardona et al., 2005; 2004). All three enzymes activities reported to reach maximum levels within 48 hours and then gradually decreased until day 21 (end of the experiment) with continuing aqueous PYR exposure in larval *Carassius auratus* (Yin et al., 2014). Production of GSSG followed the levels of antioxidant responsive genes, while GSH

levels were inversely related, with maximum increase and decrease, respectively at 48 hours after exposure (Yin et al., 2014). This was followed by a gradual return to normal throughout the remaining exposure period. These results indicate that PYR bioaccumulating in the liver resulted in redox cycling and the production of free radicals is as important mechanisms of PYR toxicity in goldfish liver. It is noted that these defects are very similar to those described by the potent ligand for the AhR, TCDD. Therefore, it is likely that PYR also acts through the AhR (Incardona et al., 2005; 2004), but direct evidence for this is lacking. Furthermore, whether acute sub-lethal toxicity in fish is also caused by AhR activation and oxidative stress mechanisms is unknown.

1.1.2.3 Toxic Concentrations for Pyrene & Naphthalene

The estimated acute toxicity of the PAHs of interest to this thesis were identified by Neff et al (2005) in marine and freshwater invertebrates and fish. He reported octanol-water partition coefficient (K_{ow})-corrected acute LC_{50} values of 4780 $\mu\text{g/L}$ and 61 $\mu\text{g/L}$ for NAP and PYR respectively (see Table 1.1). Corrected chronic toxicity LC_{50} values were estimated to be 970 $\mu\text{g NAP /L}$ and 12 $\mu\text{g PYR/L}$ (Table 1.1). While both acute and chronic toxicity concentrations are approximately three orders of magnitude higher than normal ambient environmental concentrations (Table 1), the levels of NAP and PYR approach these toxicity levels in the immediate vicinity of a recent aquatic oil spill (Aas et al., 2000). Moreover, these values are based on lethality, but relevant acute (24-48h) sub-lethal toxicity, such as cardiorespiratory or metabolic impairment could occur at much lower concentrations in fish. NAP levels in most crude oils are very high (Neff et al., 2005), thus despite the short half-life, the potential for acute adult cardiorespiratory or metabolic toxicity is of concern. In contrast, the longer half-life of PYR combined with its lower threshold for lethality (Payan et

al., 2008) suggests it may also have potential to exert acute adult fish toxicity after an oil spill, but its potential for toxicity will continue over a longer time frame.

Table 1.1. Summary of the average environmental concentrations (Government of Canada, Environment Canada, & Health Canada, 1993), estimated acute and chronic toxicity levels (Neff et al., 2005), water concentrations sampled after an oil spill (Aas et al., 2000), environmental half-life (CCME, 1999), and water quality guideline values for the protection of aquatic life (CCME, 2008) of PAHs of interest.

PAH	Environmental concentrations (µg/L)	Acute Toxicity (µg/L)	Chronic Toxicity (µg/L)	Water Concentration (µg/L)	Environmental Half-Life ($t_{1/2}$)	Canadian Water Guideline value (µg/L)
NAP	0.0036	4780	970	0.764	4.2-7.3 hours	1.1
PYR	0.0003	61	12	0.038	>8 weeks	0.025

1.1.2.4 *Cytochrome P4501A Monooxygenase and Aryl Hydrocarbon Receptor*

Induction of CYP450 associated enzymes in the liver have been associated with exposure to contaminants, especially CYP1A in the liver. It has been assumed that all PAH exposure in fish leads to a receptor-mediated induction of CYP1A gene expression (Folkerts et al., 2017), where the CYP1A enzymes have important functions in the biotransformation of PAH into reactive metabolites or in the detoxification process. AhR activation has been reported to increase the transcription and translation of CYP isozymes 1A, CYP1B1 (Nebert et al., 2000) and CYP1C1 which is exclusive to fish species (Wang et al., 2006). High affinity ligands for the AhR include a variety of anthropogenic and naturally occurring pollutants, such as TCDD and co-planar halogenated biphenyls. Teleosts have two AhR genes due to genome duplication, AhR1 and AhR2 (Hahn, 2002). AhR2 transcripts are more abundant, widely distributed, and have been reported as the only functional receptor to TCDD and halogenated biphenyls in fish species (Andreasen, 2002; Hahn, 2002). However, Incardona

et al. (2005) was able to demonstrate that both toxic and non-toxic PAHs induce CYP1A in zebrafish embryo through both AhR1 and AhR2. BaP and β -naphthoflavone (BNF) are moderately strong AhR agonists capable of causing similar cardiac deformities as strong binding ligands like TCDD (Clark et al., 2010; Gerger et al., 2014). Hepatic CYP1A is a common biomarker of AhR agonist exposure in zebrafish, but extrahepatic organs including the kidney, heart and gills also have a high amount of CYP1A activity during AhR agonist exposure (Sarasquete & Segner, 2000). However, there has long been a debate as to whether CYP1A is merely a marker of AhR agonist exposure or whether it is involved directly in mediating toxicity. Recently, it was reported that zebrafish embryos exposed to weak AhR agonists and simultaneously to an inhibitor of CYP1A activity show greater developmental cardiotoxicity (Van Tiem & Di Giulio, 2011). The suggested explanation was that CYP1A activity is necessary to detoxify the weak AhR agonist or that low-level CYP1A activity is needed for normal zebrafish cardiac development (Brown et al, 2014). It is unknown whether weak AhR agonists, when combined with a CYP1A inhibitor also alter cardiorespiratory or metabolic function in adult fish.

1.1.3 Cardiac physiology in fish

Zebrafish (*Danio rerio*) are a model indicator freshwater fish species used for aquatic toxicology. This is due to their well-studied physiology and fully sequenced genome, making molecular and biochemical studies easier than in other fish species (Briggs, 2002). The zebrafish cardiovascular system is fully functional approximately 24h post fertilization (Schwerte & Fritsche, 2003), thus allowing the measurement of heart rate and oxygen consumption at early life stages. The adult zebrafish heart is approximately 1mm in size, is located anterior of the body cavity, ventral to the esophagus and functions to pump

oxygenated blood throughout the body (Menke et al., 2011). The ventricle consists of numerous trabeculae, each having a compact outer layer of muscle and spongy inner layer. When the ventricle contracts, it generates high pressure which leads the blood through the ventriculobulbar valve into the bulbus arteriosus; a thick muscular layer of fibro-elastic tissue and smooth muscle fibers. The afferent branchial arterioles distribute blood from the ventral aorta to the gills (Menke et al., 2011). After circulating oxygenated blood to the peripheral tissues, deoxygenated venous blood will return to the heart and enter the sinus venosus, a thin collagenous connective tissue. The blood then moves passively into the atrium which has a thinner muscle than the ventricle. During ventricular diastole and during atrial contraction, the blood enters passively, then actively through the atrioventricular valve into the ventricle (Menke et al., 2011). Cardiac activity and blood flow throughout the body is determined by the metabolic demand, with cardiac output increasing in response to increasing oxygen demand caused by increased temperature, exercise, detoxification or increased metabolism (Claireaux & Davoodi, 2010; Pinsky, 2016).

1.1.4 Respiration and metabolism in fish

1.1.4.1 *Respiration*

When water moves in a unilateral direction over the gill membranes, respiration is accomplished as water is moved through the mouth, into the buccal cavity, through the pharynx and over the gills (Menke et al., 2011). Adult zebrafish gills are responsible for oxygen uptake, gas exchange, ion and water balance, nitrogenous waste excretion, and acid-base maintenance (Rombough, 2002). The operculum covering the opening in the gills drives water flow with the buccal chamber, located anterior to the gill, and the opercular chamber, posterior to the gills, through alternate expanding and contracting. During swimming, water

flow is achieved through ram ventilation: water is forced through the pharynx, over the gills and out the opercular chamber and gill slits (Menke et al., 2011). Zebrafish gills consists of four bilateral gill arches which contain skeletal muscles and are supported by bony and cartilaginous tissue (Menke et al., 2011).

1.1.4.2 *Metabolism*

Lipoprotein lipase (LPL) is a water-soluble enzyme responsible for hydrolyzing triglycerides in lipoproteins into free fatty acids and 2-monoacylglycerol, which are released into the bloodstream for tissue utilization (Murray et al., 2010). Within tissues, LPL is also needed to mobilize fatty acids for catabolism if fat has been stored in the tissue. In adult mammals, LPL is most abundant in adipose, lactating mammary glands, heart and skeletal muscle tissues, but not in the liver (Mead et al., 2002). In contrast, fish have higher levels of LPL in the liver and muscle (Tocher, 2003). Free fatty acid molecules are catabolized in the process known as β -oxidation (López-Patiño et al., 2014). This process occurs within the mitochondria, producing acetyl-CoA, which then feeds into the citric acid cycle to produce ATP. The rate limiting enzyme, 3-hydroxacyl-CoA-dehydrogenase (HOAD), is responsible for the catalysis of acyl-CoA into acetyl-CoA (López-Patiño et al., 2014) and is commonly used as a biomarker of as β -oxidation capacity (McClelland et al., 2006).

Zebrafish depend on the oxidation of acetate derived mainly from fatty acid metabolism to produce ATP rather than carbohydrate or protein metabolism (Rajotte & Couture, 2002). Aerobic organisms rely on the tricarboxylic acid (TCA) cycle, also known as the citric acid cycle or the Krebs cycle for ATP production (Goertzen et al., 2012; Rajotte & Couture, 2002). The TCA cycle is a series of enzyme-catalyzed chemical reactions to generate

energy. After glycolysis and pyruvate production, the TCA cycle starts with the methyl carbon of acetyl-CoA binding its methyl carbon to the carbonyl carbon of oxaloacetate, producing citrate (Rajotte & Couture, 2002). Citrate synthase (CS) is the rate limiting enzyme that catalyzes this step in aerobic energy production, being common to both fatty acid and glucose aerobic metabolism (Goertzen et al., 2012; Rajotte & Couture, 2002). Therefore, CS activity can be used as an index of aerobic metabolic activity.

1.1.5 Swimming and metabolic rates in fish

Swimming is the horizontal or vertical locomotion without specification for spatial position, orientation and velocity. Measuring oxygen consumption rate (MO_2) and observing swim performance along with cardiac function in fish is important to determine fish fitness after sub-lethal toxicant exposure (Mager & Grosell, 2011). Swimming is essential to many fish behaviours such as predatory avoidance, reproduction and obtaining food. Therefore, negative effects on any of these behaviours would have a much greater effect than just the single organism alone. By measuring the critical swimming speed (U_{crit} ; a measure of aerobic swimming capacity and swimming endurance), along with oxygen consumption rate, aerobic performance and metabolic capacity during exposure to a stressor (Thomas et al., 2013). Standard metabolic rate (SMR) is the minimum metabolic rate in order for life to be sustained, while the active metabolic rate (AMR) is the metabolic rate at the maximum water velocity during a U_{crit} test (Brett, 1972; Gerger & Weber, 2015). Both are both important values to measure alone as indicators of metabolism, but also to calculate metabolic capacity. The ratio of AMR/SMR gives factorial aerobic scope (F-AS), indicative of aerobic capacity (Gerger & Weber, 2015; Killen et al., 2007).

1.2 Acute versus chronic development PAH toxicity

Acute (<24h) toxicity of PAHs to fish has historically been reported to cause reverse narcosis (Di Toro et al., 2000; French-McCay, 2002) due to PAHs partitioning across cell membranes of nervous tissues and disrupting the central nervous system (Barron et al., 2004). However, embryonic exposure to sub-lethal concentrations resemble the symptoms of blue-sac disease, which is usually caused by exposure to planar halogenated aromatic compounds and several PAHs (Incardona et al., 2004). Acute (<24h) cardiotoxicity of three-ring and larger PAHs in fish embryos (such as PYR) has been shown to selectively disrupt cardiac function, while having minimal effect on neuronal function. Moreover, PAHs do not immobilize embryos or render them insensitive to mechanosensory stimulation, all of which are requirements for narcosis (Incardona et al., 2004). Thus, acute functional cardiotoxicity in larval fish is mediated by a mechanism other than narcosis, while the contribution to acute adult toxicity is unknown. Chronic (>96h) toxicity is much more specific and is usually correlated to PAH metabolite residues found within tissues (Aas et al., 2000). Mechanisms of actions are dependent on the chemical and physical properties of the PAH, therefore are predicted to differ between the extremely volatile NAP and the peri-fused PYR (Martin et al., 2013). Based on our limited knowledge of NAP and PYR mechanisms of toxicity, acute adult cardiorespiratory and metabolic toxicity are likely to be mediated by differing mechanisms, but this must be confirmed with the work proposed in this thesis.

1.2.1 How cardiac function is affected by PAHs

Incardona et al. (2004) exposed zebrafish larvae acutely (<24h) with to a variety of PAHs, including NAP and PYR. Larvae exposed to 5 μ M PYR displayed mild pericardial edema, slight bradycardia, and severely reduced peripheral blood circulation in the head. Cell death

in the neural tube caused a less pronounced dorsal curvature, anemia, and peripheral vascular defects (Incardona et al., 2004). However, cardiac conduction was not affected by PYR (Incardona et al., 2004). In the same study, zebrafish larvae exposed to 78 μ M NAP through static exposure had grossly normal anatomical features, however displayed mild bradycardia. Zhang et al., (2012) conducted a similar study with and found that low level PYR levels failed to alter the AhR1a, AhR1b, AhR2 and CYP1A expression. However, NKx2.5, a homeodomain transcription factor that has an essential role in development of the cardiovascular system, was downregulated (Zhang et al., 2012).

When adult zebrafish were acutely (48h) aqueously exposed to BaP, cardiac output and stroke volume significantly decreased (Gerger & Weber, 2015). End diastolic and systolic volumes were also significantly decreased when adult zebrafish were acutely (48h) exposed to BaP, indicative of decreased filling in the ventricle, thereby leading to less blood being pumped by the heart. Ventricular heart rate, but not atrial contractile rate, significantly declined with BaP exposure, causing bradycardia. This is similar to that reported for embryos exposed to BaP (Huang et al., 2012; Incardona et al., 2011) and adult zebrafish exposed to BNF (Gerger et al., 2014). When ventricular contractile rate drops without a change in atrial rate, this is due to a conduction blockade through the atrioventricular node, an effect thought to be mediated through oxidative stress in mammals (Oudit et al., 2006). Current knowledge of cardiovascular effects of NAP and PYR in adult zebrafish are virtually absent, but if these compounds work via oxidative stress, they may mediate atrioventricular blockade and diminish cardiac output in adult fish, similar to that observed for BaP and BNF.

1.2.2 How respiration and metabolism are affected by PAHs

Gerger & Weber (2015) found MO_2 of all zebrafish, regardless of treatment, significantly increased with increasing swim speeds. Zebrafish exposed to BaP had higher MO_2 at a given swimming speed compared to controls (Gerger & Weber, 2015). Zebrafish embryos exposed to weathered crude oil consisting of PAH mixtures at sub-lethal concentrations impaired swimming performance and changed ventricular shape (Hicken et al., 2011). An investigation of the impacts of the *Erika* oil spill off the southern coast of Brittany, France, on sole (*Solea solea*) reported histomorphological alterations of the gill epithelium in 85% of fuel-exposed sole. At the highest dose, the secondary lamellae respiratory epithelium had thinned or completely disappearing in some fish, but no alteration of the primary lamellae was observed (Claireaux et al., 2004).

TCDD has been demonstrated to affect CS mRNA expression in mice and humans (Gohlke et al., 2009). Moreover, TCDD is reported to increase triglyceride levels in tissue and reduce the ability to swim at high speeds in zebrafish (Marit & Weber, 2012). Glycogen, the storage form of glucose in animals, has been reported to have changed after NAP exposure in fish. Dangé (1986) found that tilapia exposed to sub-lethal concentrations of NAP caused a decrease in liver and muscle glycogen levels. The similar impact was observed when rainbow trout were also exposed to sub-lethal concentrations of NAP, resulting in a dose dependent decrease in a 6-hour period due to mobilization of glycogen (Tintos et al., 2006). Decreased glycogen after NAP exposure was attributed to enhanced use of glucose in the liver, either from glycogen stores or from the blood stream (Tintos et al., 2006). Thus, although some research has been done on the acute effects of PAHs on aerobic glycolysis in adult fish, relatively little is known about PAH-mediated effects on fatty acid mobilization

and fatty acid oxidation. Characterizing whether PAHs with different modes of action have differential acute effects on glycolysis and fatty acid metabolism in adult fish is a goal of this thesis.

Adult zebrafish metabolism was significantly altered after acute (48h) exposure to BaP, with elevated SMR, no change in AMR and reduced factorial aerobic scope (F-AS) (Gerger & Weber, 2015). This is consistent with the impacts of the *Erika* oil spill on sole; no change in basal metabolic rate (BMR), but significant reduction in SMR (Claireaux et al., 2004). In another study, AMR decreased 27% after exposure to resulting in a reduction on aerobic metabolic capacity, indicative of changes in the ability of fish to face environmental challenges (Davoodi & Claireaux, 2007). Aside these reports of respiratory and metabolic effects in adult fish acutely (24-48h) exposed to PAHs, they are limited to crude oil as a whole or BaP. PAHs other than BaP are a concern if they also alter adult fish metabolism after acute (24-48h) exposure, but is largely unknown and will be investigated in this thesis.

1.2.3 How swimming is affected by PAHs

Swimming performance is indirectly negatively affected by cardiac toxicity caused by crude oil, as reported by Hicken et al. (2011), who found an 18% decline in the aerobic performance (U_{crit}) in adult zebrafish. With increasing concentrations of PAHs exposure, swimming activity has been reported to decrease with increasing number of lethargic juvenile gilthead seabream (*Sparus aurata*) fish (Gonçalves et al., 2008). The fish behavioral responses to three PAHs, including PYR, caused 60-75% of fish to show no swimming activity at the highest dose (50µg/L) (Gonçalves et al., 2008). In contrast, adult zebrafish exposed to acutely (48h) to BaP had significantly increased COT (cost of transport), yet U_{crit} was not

significantly altered (Gerger & Weber, 2015). A decline in swimming performance has ecological relevance at the population level and thus must be further investigated.

1.3 Unanswered questions/problems to be addressed with my research

- i. Does NAP and PYR cause cardiorespiratory and metabolic impairment after acute (48h) exposure in adult fish?
- ii. After 48-hour exposure, will the parent compound (or metabolite) be found within tissues and correlate to biological effect?
- iii. Will NAP and PYR affect energy stores of triglycerides and glycogen and/or levels of key metabolic enzymes?
- iv. Are NAP and PYR AhR agonists? Is CYP1A induction indicative of toxicity or is oxidative stress a better indicator?

1.3.1 Rationale for experiments

PAHs are pervasive contaminants whose toxicity to aquatic species is due to more than just BaP. While BaP is the representative PAH whose chemistry and mechanism of action are well known, it may differ from other important petrogenic PAHs, such as NAP and PYR. Both NAP and PYR have some ability to act as AhR agonists, however with much different binding affinities (Barron et al., 2004). It is assumed, yet not definitively proven, that NAP affinity for the AhR is significantly less than PYR. PAHs in weathered crude oils are developmentally cardiotoxic and show a strong correlation with AhR activation (Zhang et al., 2012). In contrast, the mechanism of action of NAP and PYR, specifically on adult zebrafish cardiorespiratory and metabolic function is unclear. Additional investigation is needed into whether relative potency AhR agonist and CYP1A induction are indicative of relative toxicity

or whether oxidative stress is a better indicator. This can be studied by inhibiting these three endpoints and looking at the overall effects on NAP and PYR exposure to zebrafish. Based on the literature, proposed mechanisms of action for NAP and PYR that will be investigated in this thesis are shown in Figure 1.1.

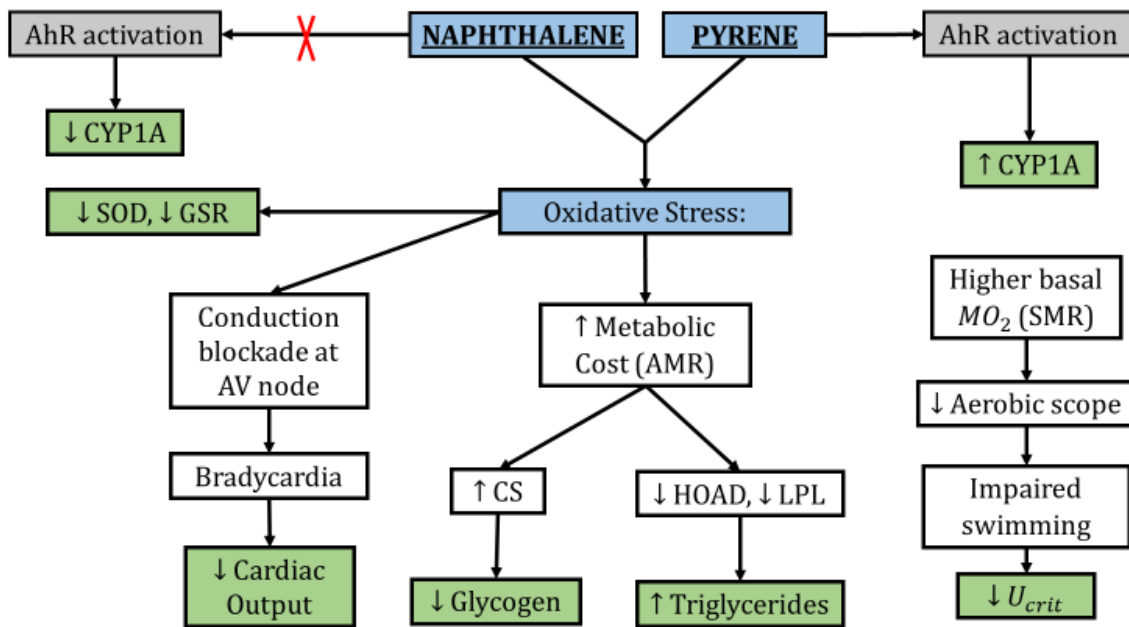


Figure 1.1: Schematic representation of hypothesized mechanism of action during acute (48h) exposure of NAP and PYR to adult zebrafish.

1.4 Hypotheses

- i. Acute (48h) exposure to NAP and PYR will also cause cardiorespiratory and metabolic impairment in adult fish.
- ii. After 48-hour exposure, the parent compound metabolites will be found within tissues.
- iii. NAP and PYR will deplete energy stores of glycogen, but increase triglycerides with differential effects on LPL, HOAD and CS.
- iv. PYR, but not NAP, is a weak AhR agonist and will cause an increase in CYP1A gene expression.
- v. CYP1A expression increases stimulated via AhR will be independent of cardiovascular (atrioventricular conduction blockade, decreased cardiac output) and metabolic toxicity.
- vi. NAP and PYR will cause cardiorespiratory and metabolic toxicity through oxidative stress.

1.5 Research Design

1.5.1 Experiment 1 & 2: Basic characterization of acute NAP and PYR cardiorespiratory and metabolic toxicity in adult zebrafish

The ubiquitous nature of PAHs found in the ambient environment is a cause for concern for freshwater aquatic species. Important petrogenic PAHs, such as NAP, found in high concentrations in crude oil, and PYR, which has a low threshold of toxicity, have had minimal research in comparison to the representative BaP. Therefore, the purpose of my research is to investigate the cardiorespiratory and metabolic impairment of NAP and PYR

to adult zebrafish, to resemble toxicity immediately after an oil spill. To characterize the concentration-response relationship of acute toxicity of NAP and PYR to adult zebrafish, high-resolution cardiovascular ultrasound and swim tunnel analysis examined the cardiorespiratory toxicity of NAP and PYR. Quantifying the changes in energy stores content and determining the changes in mRNA levels of important metabolizing enzymes were used to characterize the tissue specific (heart, liver and skeletal muscle) changes in metabolism. Finally, tissue specific changes in mRNA levels of CYP1A and antioxidant enzymes were used to determine the toxicity of NAP and PYR to adult zebrafish.

Experiments were performed in clean, dechlorinated, aerated municipal water with dissolved PAHs using static renewal conditions. Adult zebrafish (0.3-0.8g) were randomly selected from holding tanks, placed in static aerated glass beakers (4 fish/1 L beaker) containing vehicle control (0.05% DMSO), 0.25, 2.5, 25 $\mu\text{g/L}$ of PYR dissolved in $27\pm 1^\circ\text{C}$ water and PYR renewal at 24 hours for a total of 48-hour exposure. NAP test will be conducted exactly the same, using 3.7, 370, 3700 $\mu\text{g/L}$. The test concentrations were chosen on the basis of aquatic environment relevant concentrations (CCME, 1999) and the highest reported concentrations found in crude oil (Pampanin & Sydnes, 2013). Post 48-hour exposure, half of the fish in each treatment groups ($n=16/\text{treatment}$) were subjected to the swim tunnel and the other half subjected to cardiac ultrasound ($n = 16/\text{treatment}$). Fish were dissected where the heart, liver and skeletal muscle were saved. Fish that were for triglyceride and glycogen analysis were only subjected to the cardiac ultrasound. The remaining dissected fish will be used for mRNA levels of CYP1A, CS, LPL, HOAD, GSR and SOD. Parent compound analysis of NAP and PYR were performed on a sub-set of remaining

fish not needed for other analyses from both swim testing and ultrasound using the whole carcass.

1.5.2 Experiment 3: Pharmacological characterization of mechanisms of toxicity of acute NAP and PYR cardiorespiratory and metabolic toxicity in adult zebrafish

Once the cardiorespiratory and metabolic toxicity of NAP and PYR was characterized, the mechanism of toxicity was determined using a threshold concentration for each PAH determined in the previous experiment. With the use of pharmacological inhibitors, adult zebrafish were then co-exposed with the threshold concentration of each PAH of interest. The most sensitive end-points from experiment 1 (swim tunnel analysis not measured), were repeated.

From experiment 1 and 2, the threshold concentration of NAP and PYR exposure was chosen for our co-exposure. Adult zebrafish were co-exposed to each PAH (370 µg NAP/L AND 2.5 µg PYR/L) and 3 antagonistic treatments for 48 hours:

- 3334 µg/L CH-223191 (Ko & Shin, 2012)
- 500 µg/L fluoranthene (FL) (Brown et al., 2014)
- 172 µg/L Tempol (4-hydroxy-TEMPOL) (Epperly et al., 2012)

FL is a known CYP1A inhibitor (Wassenberg & Di Giulio, 2004), while Tempol is a known antioxidant scavenger for free radicals (Chatterjee et al., 2000). Inhibiting CYP1A activity, or detoxifying ROS which blocks the need for CYP1A, can determine if biotransformation of NAP or PYR into reactive metabolites causing oxidative stress is responsible for the toxic effects or if CYP1A aids in the detoxification process. To determine if this process is independent of AhR binding, a potent AhR antagonist that does not stimulate AhR-dependent transcription

CH-223191 will be used (S. Kim et al., 2006). Comparing these three antagonist treatments will determine the mechanism of action NAP and PYR causing the expected cardiorespiratory and metabolic effects. Co-exposure to FL and CH-223191 are expected to have similar results.

Naphthalene (NAP), Pyrene (PYR), dimethyl sulfoxide (DMSO), ethyl 3 aminobenzoate methanesulfonate (MS-222) fluoranthene (FL), CH-223191 and tempol were all purchased from Sigma-Aldrich (Oakville, ON, Canada). The anesthesia, Aquacalm, will be purchased from Syndel Laboratories (Vancouver, BC, Canada).

Summary of experiments

- Experiment 1 – NAP exposure
 - 48-hours exposure
 - n = 32 fish
 - Treatments: 0 (0.05% DMSO), 37, 370, 3700 µg/L

- Experiment 2 – PYR exposure
 - 48-hours exposure
 - n = 32 fish
 - Treatments: 0 (0.05% DMSO), 0.25, 2.5, 25 µg/L

- Experiment 3 – NAP and PYR mechanism of action investigation
 - 48-hours exposure
 - n = 32 fish
 - Treatments:
 - Control: 0.05% DMSO
 - 370 µg/L NAP or 2.5 µg/L PYR
 - 500 µg/L FL + + 370 µg/L NAP or 2.5 µg/L PYR
 - 3334 µg/L CH-223191 + 370 µg/L NAP or 2.5 µg/L PYR
 - 172 µg/L Tempol + 370 µg/L NAP or 2.5 µg/L PYR

CHAPTER 2: THE CARDIORESPIRATORY AND METABOLIC TOXICITY OF ACUTE NAPHTHALENE VERSUS PYRENE EXPOSURE IN ADULT ZEBRAFISH (*DANIO RERIO*).

2 Preface

The research in this chapter was designed to address the acute cardiovascular and metabolic impairment of acute naphthalene and pyrene exposure in adult zebrafish. The author contributions to Chapter 2 of this thesis were as follows:

Chanel Yeung (University of Saskatchewan) performed 100% of all animal experiments, collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript. Lynn Weber (University of Saskatchewan) supervised, helped with study design, provided scientific input and guidance, and edited the manuscript. Chapter 2 is being prepared for submission to *Comparative Biochemistry & Physiology, Part C: Toxicology & Pharmacology*.

2.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds consisting of two or more aromatic rings that are found naturally in the environment from forest fires, volcanic activity and in areas of bitumen deposits. However, anthropogenic sources of PAHs also occur from incomplete combustion of organic matter at high temperatures, fossil fuel extraction, coal mining, oil spills and oil sands wastes (Neff et al., 2005). Abundant sources and continued input leads to ubiquitous PAH contamination in the aquatic environment resulting in toxicity to aquatic species (Jaward, 2012; CCME, 1999). Toxicity occurs for some

PAHs when they interact with the aryl hydrocarbon receptor (AhR), a ligand activated transcription factor known to mediate toxicological effects of many environmental pollutants (Gerger & Weber, 2015; Schmidt & Bradfield, 1996). The well characterized response to AhR activation is increased transcription/translation of phase one cytochrome P450 1A monooxygenase (CYP1A), which in turn causes production of reactive oxygen species and stimulation of the antioxidant system. Alternatively, some PAHs or their metabolites may cause oxidative stress in an AhR-independent manner (Guigal et al., 2000; Raha et al., 1995). The well-studied and abundant aquatic PAH contaminant, benzo-a-pyrene (BaP), is a known AhR agonist that biotransforms via CYP1A metabolism into reactive metabolites that are mutagenic, cytotoxic and produce oxidative stress (U.S. EPA., 2017). Important antioxidant enzymes, superoxide dismutase (SOD) (Kim & Lee, 1997; Miller & Ramos, 2001) and glutathione reductase (GSR; Miller & Ramos, 2001; Wells et al., 1997) have been shown to have protective against BaP embryotoxicity.

Acute effects of exposure to complex PAH mixtures (e.g. crude oil) on metabolism have been studied in adult fish. Most studies report an increase in basal or standard metabolic rate (SMR) and no change or decrease in active metabolic rate (AMR) after acute PAH mixture exposure, leading to a fairly consistent finding of decreased aerobic scope (AS) (Davoodi & Claireaux, 2007; Gerger & Weber, 2015; Nelson et al., 2017). However, zebrafish chronically exposed to pyrolytic PAHs via ingestion found no significant changes in SMR, AMR or F-AS at both juvenile and adult life stages (Lucas et al., 2016). Juvenile chinook salmon fed a PAH-contaminated diet had a reduction in total body lipid and plasma triglycerides (Meador, 2006). It has been reported that the potent AhR agonist, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), increases triglyceride levels in zebrafish (Marit & Weber,

2012). TCDD also alters the rate limiting enzyme in the citric acid cycle, citrate synthase (CS) mRNA expression in humans and mice (Gohlke et al., 2009). Sub-lethal concentrations of NAP caused a decrease in tilapia liver and muscle glycogen levels (Dangé, 1986) and rainbow trout liver (Tintos et al., 2008). Although some research has been done on the acute effects of PAHs on aerobic glycolysis in adult fish, relatively little is known about PAH-mediated effects on fatty acid mobilization and fatty acid oxidation. Characterizing whether PAHs with different modes of action have differential acute effects on glycolysis and fatty acid metabolism in adult fish is a goal of this thesis.

Adequate energy reserves and metabolism directly affect normal growth, survival and reproduction in fish, but also have a strong influence on swimming and cardiovascular function (Davoodi & Claireaux, 2007; Tocher, 2003). Increased metabolic demand is well known to drive increases in cardiac output in fish (Farrell, 1991; Kopp et al., 2014; Nelson et al., 2017). Despite the fact that PAHs should therefore increase cardiac output, instead developmental exposure to petroleum contaminants and specifically BaP are well known to cause lethal cardiac deformities and cardiac dysfunction in fish embryos or larvae (Esbaugh et al., 2016; Hicken et al., 2011; Incardona et al., 2004, 2006, 2010). More recently, acute exposure to petroleum or BaP has been shown to exert cardiorespiratory and metabolic effects in adult fish (Guy Claireaux & Davoodi, 2010; Gerger & Weber, 2015). Specifically, acute BaP exposure in adult zebrafish has been reported to cause arrhythmia (atrioventricular conduction blockade) and increased metabolic demand (oxygen consumption) with decreased aerobic scope (Gerger & Weber, 2015). This is similar to the reported effects of acute exposure to PAH mixtures derived from crude oil or individual tricyclic PAHS. In these studies, PAH mixture exposure in various aquatic species

consistently produced arrhythmias, decreased heart rate and impaired contractility (Incardona et al., 2009; Incardona & Scholz, 2017; Sørhus et al., 2016). While BaP has been studied as a representative of all PAHs, other petrogenic PAHs such as naphthalene (NAP) and pyrene (PYR) are often more abundant in petrogenic contamination and are often present at high enough concentrations to be of environmental concern (Barron & Holder, 2003; Neff et al., 2005). Previous studies have reported that low level PYR is cardiotoxic to zebrafish embryos, changing heart morphology (elongated ventricle, string-like morphology and edema) similar to that described for the potent AhR agonist TCDD (Incardona et al., 2004; Zhang et al., 2012). Developmental PYR exposure caused altered cardiac function (elevated heart rate, but decreased end diastolic, end systolic, and stroke volumes) in larval fish, without a change in CYP1A mRNA expression (Zhang et al., 2012). Conversely, developmental exposure of zebrafish larvae to the less potent PAH, NAP, induced mild bradycardia and edema, without the profound teratogenic effects on the heart (Incardona et al., 2004). This difference in developmental cardiotoxicity between BaP, NAP and PYR could be related to differences in affinity for the AhR (Barron et al., 2004), but could also be related to differences in mechanisms of toxicity. Moreover, whether these same differences in toxicity would be evident in adult fish acutely exposed to NAP or PYR compared to known BaP cardiorespiratory toxicity is unclear.

Therefore, the overall objective of the present study was to investigate the acute (48h) cardiorespiratory and metabolic toxicity of aqueous exposure to NAP and PYR in adult zebrafish. NAP and PYR body burdens were measured using high performance liquid chromatography with fluorescence detection (HPLC-FD). After 48hr exposure, fish underwent high frequency cardiac ultrasound to measure cardiac function, or swim tunnel

respirometry to determine changes in swimming endurance, metabolic rate and aerobic capacity. Quantitative reverse transcription PCR (qRT-PCR) was then used to quantify mRNA levels of important metabolic enzymes (citrate synthase, CS; 3-hydroxacyl-CoA dehydrogenase, HOAD), markers of AhR activation (CYP1A), and genes known to be responsive to oxidative stress (GSR and SOD) in heart, liver and skeletal muscle. Finally, changes in triglyceride and glycogen levels were measured in the same tissues as indicators of energy storage.

2.2 Materials and Methods

2.2.1 Chemicals

Naphthalene (NAP), pyrene (PYR), dimethyl sulfoxide (DMSO), and ethyl 3-aminobenzoate methanesulfonate (MS-222), dichloromethane, acetone, acetonitrile, triglyceride and glycogen kits, were all purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2.2 Test species

Adult zebrafish were obtained from university colonies and acclimated in 30-L holding tanks with particulate, charcoal and ammonia biofilters for a minimum of two weeks prior to experiments. Zebrafish were kept on a 14-hour light and 10-hour dark photoperiod. Clean, dechlorinated, aerated municipal water was maintained at 27 ± 1 °C with 80% water change performed weekly during basic maintenance. Zebrafish were fed twice a day with Nutrafin basic flake food (Hagen Inc., Montreal, QC, Canada), with the exception of the last 24h of exposure or during swim performance or cardiac function testing (Gerger et al., 2014; Gerger & Weber, 2015). Experiments were conducted according to procedures approved by

the University of Saskatchewan Animal Care and Use committee according to the Canadian Council on Animal Care guidelines.

2.2.3 Aqueous exposure

Adult zebrafish (0.3-0.8g) were randomly selected from holding tanks, placed in aerated 2-L glass beakers (4 fish/1 L exposure solution) containing vehicle control (0.05% DMSO), PYR (0.25, 2.5, or 25 µg/L) or NAP (37, 370, 3700 µg/L) in 27±1°C water for 48-hour exposure (static with renewal at 24-hr). Test concentrations were chosen on the basis of water quality guidelines for the protection of aquatic life (CCME, 1999) and the highest reported concentrations found in crude oil (Pampanin & Sydnes, 2013). After 48-hour exposure, half of the fish in each treatment groups (n=16/treatment) were subjected to swim tunnel and the other half were used for cardiac ultrasound (n = 16/treatment). After ultrasound, heart, liver and skeletal muscle from a portion of the zebrafish were stored at -80°C for triglyceride and glycogen analyses. The heart, liver and skeletal muscle from a portion of the zebrafish after swim tunnel testing were stored at -80°C in RNAlater until use for mRNA levels of CYP1A, CS, HOAD, GSR and SOD. All remaining zebrafish carcasses minus the gastrointestinal tract and head after either ultrasound or swim tunnel testing were used for PYR and NAP parent compound analysis and frozen at -80°C in ethanol-washed foil until extraction for HPLC analyses.

2.2.4 Total PAH body burden analysis

Whole fish carcasses were freeze dried using a Virtis Genesis 25ES freeze drier, and stored at -20 °C until extraction. Extractions were carried out using a Dionex ASE 3—Accelerated Solvent extractor (ASE). Samples were weighed and placed into methanol-

rinsed 1ml stainless-steel cells, fitted with a cellulose filter at both ends prior being sealed and situated in the carousel of ASE 200 system. The conditions were as follows: oven temperature of 125 °C with a 6 min heat-up time with a pressure of 1500 psi and two static cycles with a static time of 5 min. Samples were purged using pressurized nitrogen for 1 min with a flush volume of 10%. The solvent mixture used consisted of 1:1 dichloromethane:acetone.

Following ASE extraction, samples were evaporated to near dryness under a stream of nitrogen and then re-suspended in 1 mL acetonitrile. ASE-extracted samples or exposure water samples were then glass syringe-filtered through Whatman GMF 0.45 µM filters into labeled 2 mL amber vials. An Agilent 1260 infinity HPLC-FD was then used to analyze NAP and PYR parent compound concentrations according to the Agilent method (Volk & Gratzfeld-Huesgen, 2011) with some modifications. An aliquot of 10 µL was injected into an Agilent PAH pursuit column maintained at 25 °C. The run time was set for 25 min with a solvent flow rate of 1.5 mL/min starting at a gradient of 60:40 acetonitrile:water and gradually increasing to 95:5 acetonitrile:water by 20 min. NAP eluted at ~3.5 min with a constant excitation wavelength of 260nm and an emission wavelength of 350 nm. PYR eluted at ~7.5 min with a constant excitation wavelength of 260 nm and an emission wavelength of 410 nm. Total NAP and PYR masses were calculated for extracted carcass samples by comparing to peak height of PAH standards at different concentrations. Recovery of NAP and PYR from blank fish samples spiked with a known amount of standard was $111 \pm 12\%$ and $84 \pm 2.9\%$ (n=5-6 independent spike/recovery samples for each), respectively.

2.2.5 *In Vivo* high frequency cardiovascular ultrasound

After 48hr exposure, physiological changes to cardiac function were measured using a VEVO 3100 high frequency ultrasound machine (VisualSonics, Markham, ON Canada) equipped with B-mode imaging, pulsed-wave Doppler and colour flow Doppler according to methods adapted from rodents (VisualSonics, 2008) and previously described in zebrafish (Gerger et al., 2014; Gerger & Weber, 2015). Zebrafish were anesthetized with Aquacalm (20 mg/L; Oakville, ON, Canada) in clean aerated, dechlorinated municipal water in a grooved holding dish, ventral side up, prior to ultrasonography and anesthesia is maintained throughout. Using a recirculating water bath with aerated anesthetic water, temperature was maintained at 27 ± 0.5 °C (the same as housing temperature) to prevent temperature-induced effects on cardiac function.

The short and long-axis views of the ventricle were obtained using an MX 700 scanhead (30-70 MHz; resolution to 30 μ m) in B-mode. Simpson's rule of disks calculated ventricular volume (Mercier et al., 1982; VisualSonics, 2008). The ventricle volume is the sum of the cylindrical disks plus an apical bullet-shaped disk. Because zebrafish do not have a hollow ventricular chamber like mammals do, the cross-sectional areas using traces of the outer edges of the ventricle were measured ($A_1 - A_3$) at three different levels of the ventricle in the short axis view. The height of each ventricle disk was then calculated by measuring the ventricular length (L) in the long axis view and dividing by the number of short axis views ($h=L/3$). Volume was calculated using an average of at least three different measurements in an individual fish during end-systole and during end-diastole for each measurement.

$$V = (A_1 + A_2)h + \left[\left(\frac{A_3 h}{2} \right) + \left(\frac{\pi}{6h^3} \right) \right]$$

(Mercier et al., 1982)

Stroke volume (V_s) for each fish was calculated by subtracting end-diastolic volume from end-systolic volume. Ventricular and atrial contractile rates were calculated by counting individual chamber contractions in 5 second B-mode ultrasound video loops, then converting to beats per minute (BPM). Ventricular heart rate (f_H) multiplied by V_s was used to calculate cardiac output (Q).

$$Q = V_s * f_H$$

Atrioventricular (AV) ratio was used as a measure of the efficiency of electrical excitation from the atrium to the ventricle via the atrioventricular (AV) node by dividing the atrial contractile rate by ventricular contractile rate for each individual fish. This value should equal 1.0 if cardiac electrical conduction is ideal.

$$AV \text{ ratio} = \frac{\text{Atrial } f_H}{\text{Ventricular } f_H}$$

2.2.6 Swimming performance and oxygen consumption

After 48 hour exposure, swim testing was conducted using a 170 mL swim tunnel respirometer (Loligo Systems, Tjele, Denmark). Fish were swim-tested in clean, dechlorinated, aerated municipal water, kept at 28° C by a 20-L recirculating water bath (VWR International, Mississauga, ON, Canada). The swim tunnel was submerged in the 20-L buffer tank and the water velocities calibrated using constant temperature anemometry according to the Loligo System instructions (Gerger et al., 2014; Gerger & Weber, 2015). Oxygen consumption rate was calculated using AutoResp™ 1 software (Loligo Systems, Denmark) during each speed increment of the swimming performance trial for each fish.

Prior to testing, fish were placed into the swim tunnel for 120 minutes acclimation at 0.02 m/s and then a step-wise increase in the water velocity (0.077m/s (U_{ii})) every 20 minutes (T_{ii}) until the fish fatigued (refused to swim). U_{crit} was calculated using the following equation (Brett, 1972):

$$U_{crit} = U_i + [U_{ii} \left(\frac{T_i}{T_{ii}} \right)]$$

U_i = highest swimming velocity maintained during entire time interval

T_i = time elapsed at fatigue velocity

To account for the variation in fish size, U_{crit} values were converted from m/s to body lengths/s.

Within each 20-minute swim velocity increment, oxygen consumption was measured twice, with a 1-minute flush and a 4-minute re-acclimation time between each measurement. After this, dissolved oxygen in the chamber water was measured for 5 minutes or until oxygen saturation fell to 90%. Oxygen consumption was calculated for each swim velocity. AMR for each fish was determined at the maximum swimming velocity prior to the fish fatiguing during the U_{crit} test. SMR was calculated by plotting oxygen consumption (m/s) against swimming speed using a non-linear fit and extrapolating the oxygen consumption (MO_2) at zero water velocity (Gerger et al., 2014; Thomas et al., 2013). Factorial aerobic scope (F-AS) was calculated as AMR/SMR (Gerger & Weber, 2015). Cost of transport (J/g/m) was calculated by multiplying MO_2 (mg O_2 /g/s) by an oxycaloric value of 14.1 J/mg O_2 and then dividing by the corresponding swimming speed (m/s) (Gerger & Weber, 2015; Thomas et al., 2013).

2.2.7 Determination of triglyceride and glycogen tissue stores

Frozen hearts (2 hearts pooled to create one sample), liver (2 livers pooled to create one sample) and skeletal muscle (measured from individual fish) samples were weighed, homogenized in 0.2M sodium citrate, immediately heated for 5 min at 100°C, then stored at -80°C until assessment for triglyceride and glycogen levels. Briefly, triglycerides were determined using a kit based on a lipase and glycerol kinase colorimetric assay, which has been previously validated for measuring triglycerides in whole fish homogenates in our laboratory (Weber et al., 2003) and glycerol used as a standard. Glycogen was analyzed using an amylase and glucose oxidase-based colorimetric assay and purified Type IX bovine liver glycogen as a standard, as previously described in our group (Weber et al., 2008).

2.2.8 Gene expression analysis – Real time qRT-PCR

Quantitative qRT-PCR in zebrafish heart (2 heart pooled/sample), liver (2 liver pooled/sample) and skeletal muscle (one sample/fish) was examined for three classes of genes in zebrafish tissue using methods previously described in Bugiak & Weber (2009). As an indication of AhR activation, CYP1A mRNA transcript abundance was measured (Sarasquete & Segner, 2000). To determine the effects on metabolism, mRNA levels of rate limiting enzymes for oxidative metabolism, CS, or fatty acid oxidation, HOAD, were analyzed (Rajotte & Couture, 2002). Antioxidant responsive genes were also quantified using GSR and SOD mRNA transcript abundance (Cossu et al., 1997). Total RNA was extracted from frozen tissues with TRIzol (Life Technologies Burlington, ON, Canada) according to manufacturer specifications. A NanoDrop apparatus and software (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the purity and quantity of mRNA. Samples were reverse transcribed

into cDNA from total RNA with superscript II reverse transcriptase (iScript, Invitrogen, (Quanta Biosciences, Maryland, USA) and oligo (dT) primers using the cDNA synthesis kits (BioRad Mississauga, ON, Canada). Chromo4 Multi-Color Real-Time PCR detection system (Biorad, Mississauga, ON, Canada) was used for qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a housekeeping gene and its expression determined for each run of a gene. Each sample was run in at least 3 different dilution for each primer pair along with the housekeeping gene to generate efficiency curves. The method of analysis used to represent the relative expression levels was calculated using $\Delta\Delta CT$, where the amount of the gene of interest was normalized to the amount of G3PDH in that sample as well as to the mean value for the control group and data expressed as fold change from control (Bugiak & Weber, 2009).

Table 2. 1. Primer sequences for cDNA targets, GenBank accession numbers, target genes and their efficiencies that were used for real time qRT-PCR. (Liu et al., 2013; McClelland et al., 2006; Mccurley & Callard, 2008; Tiedke et al., 2013; Uren-Webster et al., 2010).

Gene	Primer	Sequence (5'-3')	Accession number	Efficiency (%)
CYP1A	Forward	GCATTACGATACGTTTCGATAAGGAC	NM131879	114.0
	Reverse	GCTCCGAATAGGTCATTGACGAT		
G3PDH	Forward	AGCACTGTTCATGCCATCAC	NM213094	99.5
	Reverse	TACTTTGCCTACAGCCTTGG		
CS	Forward	AGCGCTGCTATGAATGGTCT	BC045362	93.3
	Reverse	CTGAGGAAGACAGACCCTCG		
HOAD	Forward	CCACAGGACATTCAGTGGTG	ZGI TC279717	93.7
	Reverse	GTCAGTGCCATGAACGACAG		
GSR	Forward	ACAGTCAGTGAGGATGATGTGCCAG	NM001020554	97.0
	Reverse	TAGACCCAAGAGTGGAAGAATACCAGC		
SOD	Forward	TATGCAGCTTCATCACAGCAAGCA	NM199976	100.0
	Reverse	GGTTGTCACATCACCTTGGCC		

CYP1A = cytochrome P450 1A; G3PDH = Glyceraldehyde-3-phosphate dehydrogenase; CS = citrate synthase; HOAD = 3-hydroxacyl-CoA dehydrogenase; GSR = glutathione reductase; SOD = superoxide dismutase

2.2.9 Statistical analysis

One-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc tests were used to determine significant difference among treatment groups for majority of the endpoints measured. For oxygen consumption (MO_2) and cost of transport (COT), data was analyzed using a 2-way repeated measures (with swim speed and treatment as factors) followed by Fisher's LSD post hoc test. All statistical analyses were carried out using SPSS Statistics v19.0 (IBM Corporation, Armonk, NY) and all results are presented as mean \pm standard error of the mean (SEM). A $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Zebrafish NAP and PYR body burdens

Body burdens for adult zebrafish following 48h aqueous exposure to NAP or PYR are shown in Fig 2.1. Exposure of adult zebrafish to nominal concentrations of 0, 37, 370, 3700 $\mu\text{g/L}$ NAP for 48 hours resulted in body burdens that ranged from 1.36 ng/g in control to 1.44 ng/g in NAP treatment groups (Fig 2.1 left panel). However, there were no significant differences in parent NAP body burdens between exposure groups relative to solvent control. Similarly, exposure of adult zebrafish to nominal concentrations of 0, 0.25, 2.5 and 25 $\mu\text{g/L}$ PYR for 48 hours resulted in body burdens that ranged from 8.89 ng/g in control to 6.84 ng/g in exposed fish (Fig 2.1 right panel) with no significant difference among groups.

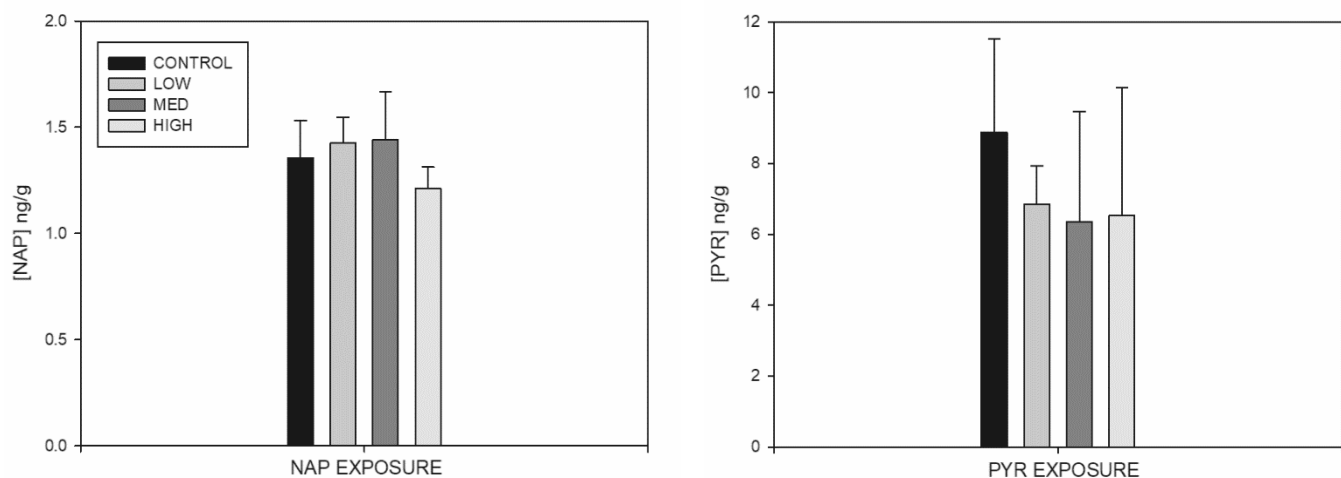


Figure 2. 1: Parent compound body burden (ng/g, dry mass) after 48-hour aqueous exposure to naphthalene (left; low = 37, med = 370, high = 3700 $\mu\text{g/L}$), pyrene (right; low = 0.25, med = 2.5, high = 25 $\mu\text{g/L}$) or solvent control in adult zebrafish. Parent compound was measured using high pressure liquid chromatography with fluorescence detection. Data are mean \pm SEM of $n = 5-6$ fish/group. No significant differences were detected for body burdens in separate one-way ANOVA analyses for naphthalene (NAP) and pyrene (PYR).

2.3.2 Mortalities and morphometrics

Following 48-hour aqueous exposure to either NAP or PYR, there were no statistically significant differences in body mass, body length or condition factor among exposure groups (Table 2.2). Only one mortality, in the 370 μg NAP/L exposure group, occurred during the duration of the entire experiment, resulting in no statistically significant differences among groups in mortalities.

Table 2. 2. Mortalities and morphometrics of adult zebrafish aqueously exposed to solvent control, naphthalene or pyrene for 48 h.

Nominal PAH Concentration	Mortalities (%)	Body Weight (g)	Body Length (cm)	Condition Factor (K)
Naphthalene				
Control	0	0.37 ± 0.02	3.04 ± 0.06	1.33 ± 0.07
37 µg/L	0	0.36 ± 0.02	2.98 ± 0.03	1.38 ± 0.05
370 µg/L	3.1 ± 3.6	0.37 ± 0.02	2.95 ± 0.06	1.46 ± 0.10
3700 µg/L	0	0.36 ± 0.01	2.98 ± 0.06	1.43 ± 0.09
Pyrene				
Control	0	0.37 ± 0.02	3.04 ± 0.06	1.33 ± 0.07
0.25 µg/L	0	0.34 ± 0.02	2.95 ± 0.04	1.33 ± 0.06
2.5 µg/L	0	0.37 ± 0.02	3.00 ± 0.06	1.37 ± 0.07
25 µg/L	0	0.39 ± 0.02	3.01 ± 0.06	1.51 ± 0.14

Data are mean ± SEM with n = 30-32/group for morphometrics; n=4 replicates/group for mortalities. Condition factor (K) = (weight (100/length³)). No significant treatment effects were detected in separate one-way ANOVA analyses.

2.3.3 Critical swimming speed and metabolic rates

Oxygen consumption, indicated by MO_2 , increased in all control, NAP- and PYR-exposed zebrafish with increasing swim speed (Figure 2.2A, 2.2B). Despite the fact that both control groups tended to have the lowest MO_2 , no significant effect of PAH treatment was detected on MO_2 . However, some of the treatment effect at higher swim speed is likely to have been masked because the number of fish declines as the swim tunnel test progressed to higher speeds due to fatigue. Only the lowest three swim speeds can be reliably compared since these have the full sample size of fish still swimming in each group. Overall, COT of all groups decreased as swim speed increased, regardless to exposure concentration to either NAP or PYR (Figure 2.2C, 2.2.D). Similar to MO_2 , there were no significant effects of treatment on COT. Finally, neither NAP nor PYR significantly altered U_{crit} (Figure 2.3).

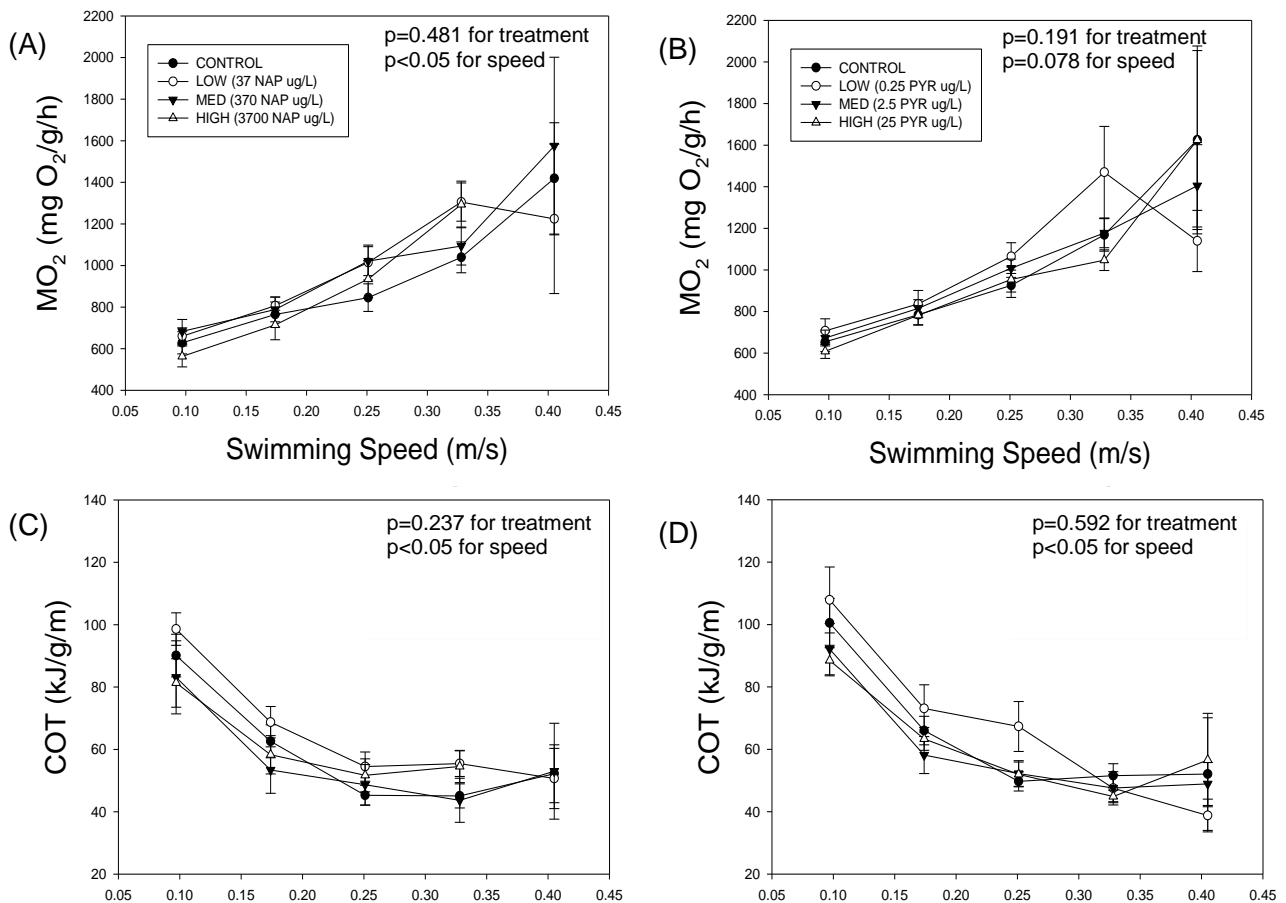


Figure 2. 2: Oxygen consumption rates (MO_2) (A, B) and cost of transport (COT) (C, D) as a function of swimming speed in adult zebrafish following aqueous exposure to naphthalene (left), pyrene (right) or solvent control for 48 hours. Data are mean \pm SEM of n = 14-16 fish/group. *p < 0.05 in Fisher's LSD after two-way ANOVA with swim speed and treatment as a factor. No significant differences were detected on (MO_2) and (COT) in separate two-way ANOVA analyses (speed and treatment as factors) for naphthalene (NAP) and pyrene (PYR). Exact p-values for both factors are shown in each graph. No significant interactions were found between swimming speed and treatment.

MO_2 of all treatment groups significantly increased with increasing swim speed, including the control group (Figure 2.2). However, metabolic capacities in adult zebrafish after 48 hour of aqueous NAP or PYR exposure showed minimal change compared to control. Standard metabolic rate (SMR) remained statistically unchanged across all exposure concentrations for both NAP and PYR (Figure 2.4A). In contrast, active metabolic rate (AMR)

was significantly greater in zebrafish exposed to 0.25 μg PYR/L compared to corresponding controls (Figure 2.4B), while zebrafish in all other PYR exposures and all NAP exposures were not significantly different from control. Despite this significant change in the 0.25 μg PYR/L AMR, F-AS of all NAP and PYR-treated groups did not differ significantly from control (Figure 2.4C). COT of all treatment groups tended to decrease with increasing swim speed, however this was not significantly different from the control group.

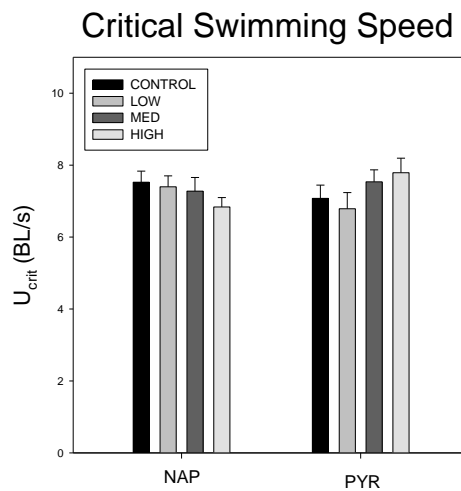


Figure 2. 3: Critical swimming speed (U_{crit}) of adult zebrafish following aqueous exposure to naphthalene (left; NAP; low = 37, med = 370, high = 3700 $\mu\text{g}/\text{L}$), pyrene (right; PYR; low = 0.25, med = 2.5, high = 25 $\mu\text{g}/\text{L}$) or solvent control for 48 hours. Data are mean \pm SEM of $n = 14-16$ fish/group. No significant differences among groups were observed using Fisher's LSD after one-way ANOVA.

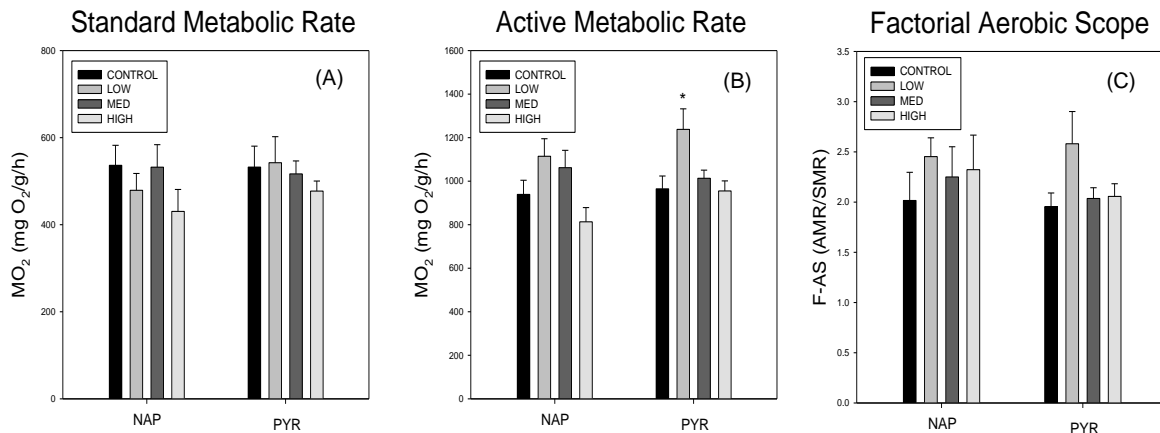


Figure 2. 4: Standard metabolic rate (SMR) (A), active metabolic rate (AMR) (B) and factorial aerobic scope (F-AS) (C) of adult zebrafish following aqueous exposure to naphthalene (left; NAP; low = 37, med = 370, high = 3700 $\mu\text{g/L}$), pyrene (right; PYR; low = 0.25, med = 2.5, high = 25 $\mu\text{g/L}$) or solvent control for 48 hours. Data are mean \pm SEM of n = 14-16 fish/group. * $p < 0.05$ in Fisher's LSD after one-way ANOVA compared to the corresponding control group.

2.3.4 Cardiac function

B-mode echocardiograms in long and short axes of the zebrafish heart were used to measure ventricular end diastolic volume (EDV), end systolic volume (ESV), stroke volume (V_s) and ejection fraction in zebrafish after 48-hour exposure to NAP, PYR or solvent control (Figure 2.5A-D). In the zebrafish exposed to 370 μg NAP/L, but not the higher or lower NAP exposures, EDV was significantly elevated compared to corresponding control with no change in ESV, leading to an increase in V_s (Figure 2.5 A-C). Despite the EDV and V_s change in the 370 μg NAP/L group, there was no significant change in ejection fraction (Figure 2.5D). In contrast, zebrafish exposed to 37 μg NAP/L showed significantly higher ejection fraction despite no significant changes in EDV, ESV or V_s (Figure 2.5 A-D). Zebrafish exposed for 48 hours to the highest dose of PYR (25 $\mu\text{g/L}$) had significantly reduced ESV compared to corresponding control without changes in EDV or SV (Figure 2.5A-C), leading to a greater ejection fraction (Figure 2.5D).

Effects of 48-hour exposure to NAP and PYR in adult zebrafish on heart rate and efficiency of cardiac conduction were also assessed from echocardiographic loops. However, 48-hour NAP exposure at all concentrations in adult zebrafish did not significantly affect cardiac output, atrial contractile rate, ventricular contractile rate or the ratio of atrial to ventricular rates (Figure 2.6). In contrast, 48-hour aqueous PYR exposure at 2.5 and 25 $\mu\text{g/L}$ significantly decreased both atrial and ventricular contractile rates without a change in the

ratio of atrial to ventricular rates (Figure 2.6 A, B, C). Despite decreased heart rate in the PYR groups, no significant change in cardiac output was observed in these groups compared to corresponding control (Figure 2.6D).

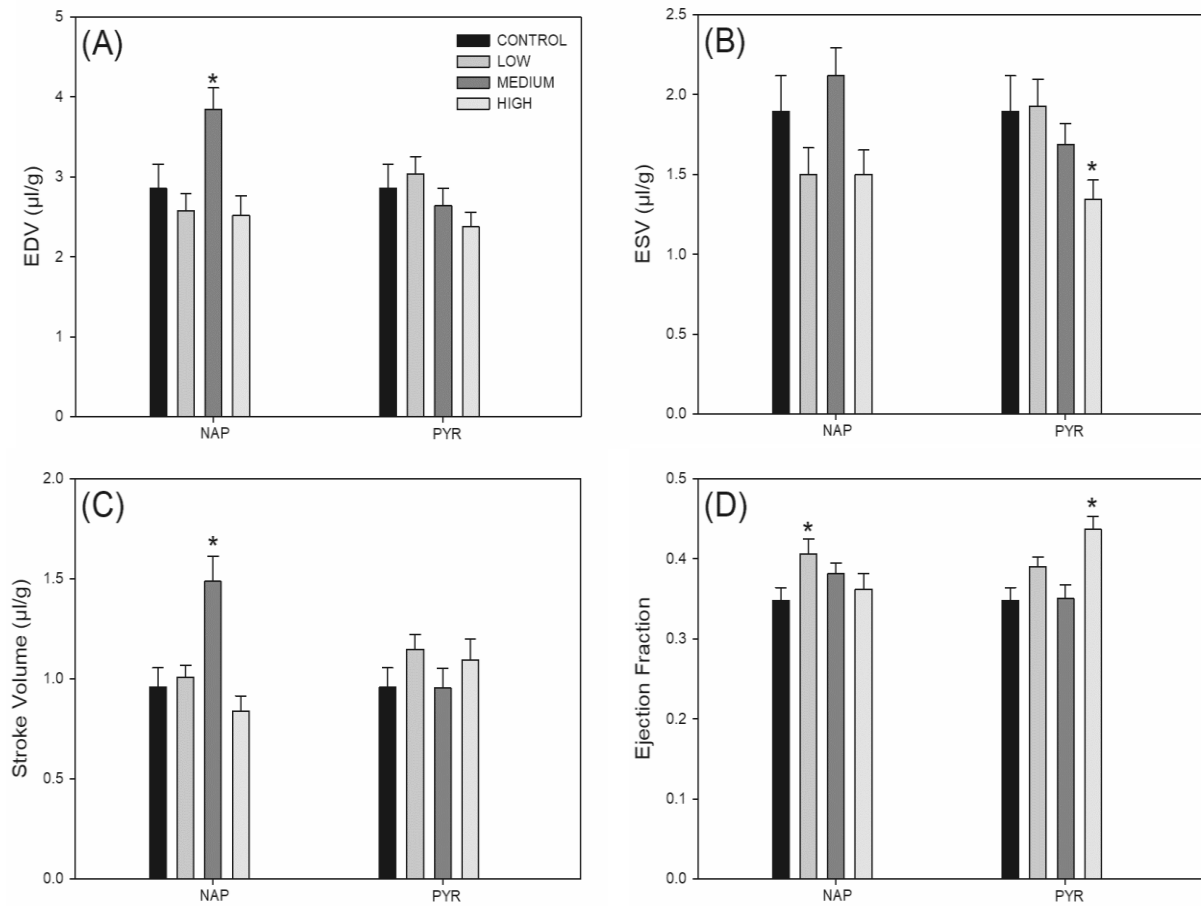


Figure 2. 5: Echocardiography was used to assess cardiac function with end diastolic volume (EDV; A), end systolic volume (ESV; B), stroke volume (C) and ejection fraction (D) in adult zebrafish shown following aqueous exposure naphthalene (left; low = 37, med = 370, high = 3700 $\mu\text{g/L}$), pyrene (right; low = 0.25, med = 2.5, high = 25 $\mu\text{g/L}$) or solvent control for 48 hours. Data are mean \pm SEM of $n = 14-16$ fish/group. * $p < 0.05$ in Fisher's LSD after one-way ANOVA compared to the corresponding control group.

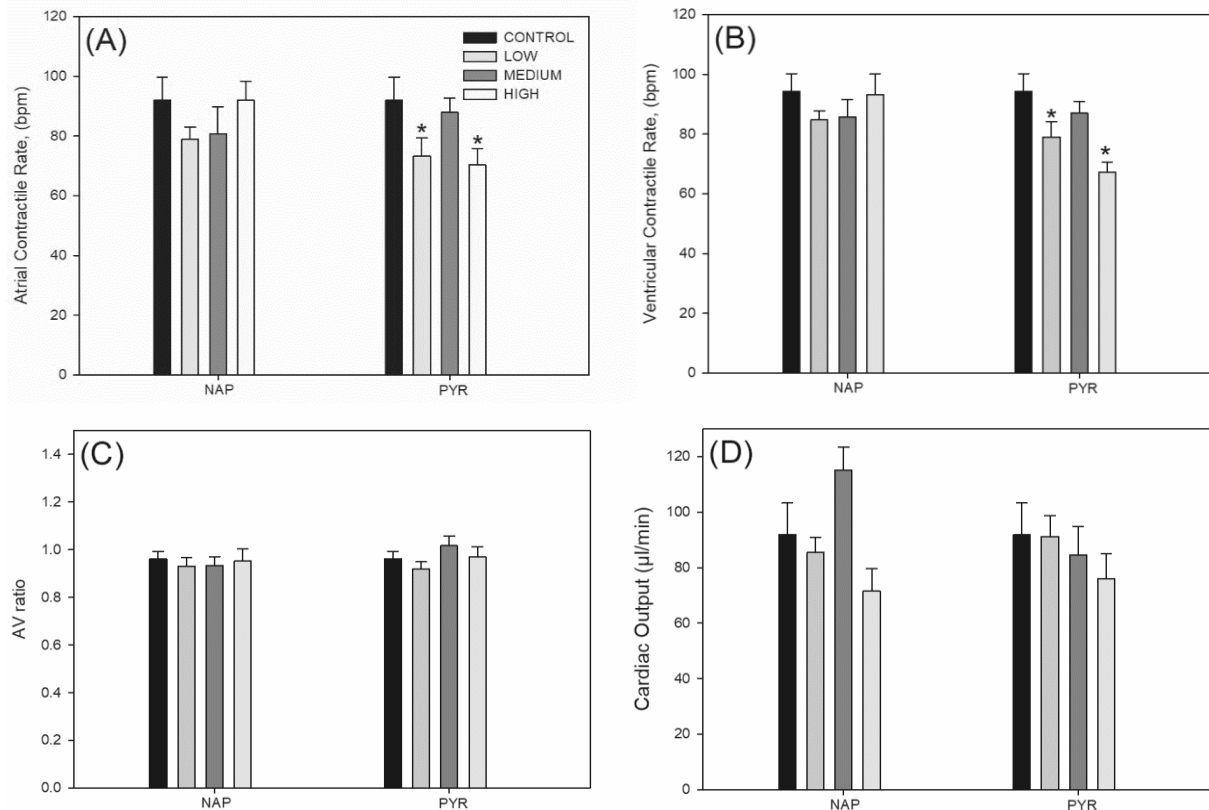


Figure 2. 6: Atrial contractile rate, ventricular contractile rate, ratio of atrial to ventricular rates (AV ratio), and cardiac output of adult zebrafish following 48 hour aqueous exposure to naphthalene (left; low = 37, med = 370, high = 3700 µg/L), pyrene (right; low = 0.25, med = 2.5, high = 25 µg/L) or solvent control. Data are mean ± SEM of n = 14-16 fish/group. *p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding control group.

2.3.5 Energy storage and key metabolizing enzyme gene expression

The mRNA transcript abundance of two rate limiting aerobic energy metabolizing enzymes, citrate synthase (CS) and β -hydroxyacyl coenzyme A dehydrogenase (HOAD) were measured as indicators of metabolic dysfunction, then compared to tissue glycogen and triglyceride content (Figure 2.7). Glycogen content showed a tendency for dose-dependent increase in heart, liver and skeletal muscle from NAP-exposed zebrafish. Only at the highest dose was there a significant difference compared to control in the heart (Figure 2.7A). However, mRNA level of CS was significantly increased in all three tissues from zebrafish

exposed to 370 µg/L NAP as well as in heart from zebrafish exposed to 37 µg/L NAP compared to control (Figure 2.7B). Triglyceride content was elevated in NAP-exposed zebrafish heart and skeletal muscle (Figure 2.7D). The mRNA transcript abundance of HOAD increased in the heart and skeletal muscle from NAP-exposure (Figure 2.7D).

For zebrafish exposed to PYR, all tissues showed a tendency to have increased glycogen content similar what was observed after NAP exposure, but only liver from zebrafish exposed to 2.5 µg PYR/L was significantly increased compared to control (Figure 2.7A). Also similar to NAP, mRNA level of CS was significantly increased in heart, liver and skeletal muscle from zebrafish exposed to 0.25 µg PYR/L compared to the control, CS expression in heart from 25 µg PYR/L-exposed zebrafish also showed significant increases in CS (Figure 2.7B). Triglyceride levels were unchanged in heart tissue after PYR exposure, but were significantly higher in liver and skeletal muscle from zebrafish exposed to 25 µg PYR/L compared to control or in skeletal muscle from the 2.5 µg PYR/L group (Figure 2.7C). Changes in HOAD transcript abundance were less consistent after PYR exposure across tissues. HOAD mRNA level was significantly higher in heart from zebrafish exposed to 2.5 µg PYR/L and in skeletal muscle from zebrafish exposed to 25 µg PYR/L compared to corresponding control (Figure 2.7D). In contrast, all three concentrations of PYR caused significant decreases in HOAD mRNA levels in liver compared to control (Figure 2.7D).

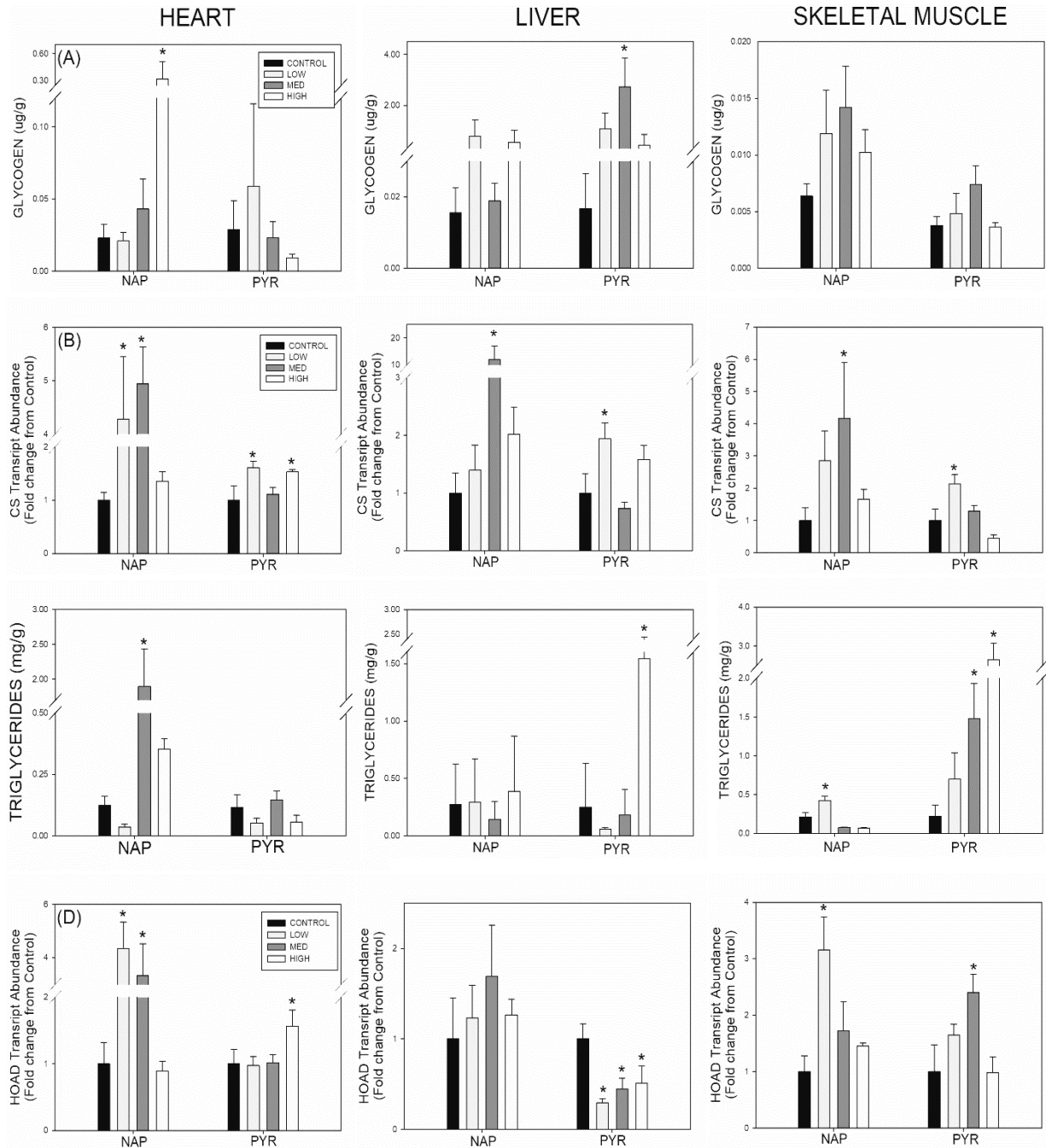


Figure 2. 7: Glycogen (A) and triglyceride (C) tissue content as well as mRNA levels (fold change relative to control) of citrate synthase (CS; B) and β -hydroxyacyl coenzyme A dehydrogenase (HOAD; D) in heart, liver skeletal muscle after aqueous exposure to naphthalene (left; low = 37, med = 370, high = 3700 $\mu\text{g/L}$), pyrene (right; low = 0.25, med = 2.5, high = 25 $\mu\text{g/L}$) or solvent control for 48 hours. Data are mean \pm SEM. For energy storage, heart and liver samples are pooled ($n=2$ hearts or livers/pooled sample) for a total of $n=3-5$ /treatment group, $n=10$ fish/treatment for mRNA levels. * $p < 0.05$ in Fisher's LSD after one-way ANOVA compared to the corresponding control group.

2.3.6 Gene expression of CYP1A and antioxidant enzymes

Heart and skeletal from adult zebrafish aqueously exposed to 37 µg NAP/L had significantly upregulated CYP1A gene expression as well as in skeletal muscle from zebrafish exposed to 370 µg NAP/L compared to corresponding controls (Figure 2.8A). However, in liver, CYP1A expression was unexpectedly significantly decreased in zebrafish exposed to all concentrations of NAP, but achieved significance for the 370 µg NAP/L (Figure 2.8A). A similar pattern of change in response to NAP was observed for GSR and SOD expression. Specifically, heart and skeletal muscle from zebrafish exposed to 370 µg NAP/L showed significant increases in both GSR and SOD mRNA levels compared to corresponding controls, while NAP was without any significant effect on GSR or SOD expression in liver (Figure 2.8 B, C).

After 48-hour exposure in adult zebrafish to all concentrations of PYR tested, both cardiac and liver CYP1A mRNA transcript abundance was significantly decreased, while no change was observed in skeletal muscle CYP1A (Figure 2.8A). Changes in antioxidant enzymes followed a different pattern than CYP1A after PYR exposure. All three tissues (heart, liver and skeletal muscle) from zebrafish exposed to 0.25 µg PYR/L group compared to corresponding control had significantly increased GSR expression (Figure 2.8B). A similar increase in cardiac and hepatic SOD mRNA transcript levels was observed in the same 0.25 µg PYR/L group, as well as heart from the 25 µg PYR/L-exposed zebrafish compared to corresponding controls (Figure 2.8C). However, no significant changes were observed for SOD expression at any PYR concentration in skeletal muscle (Figure 2.8C).

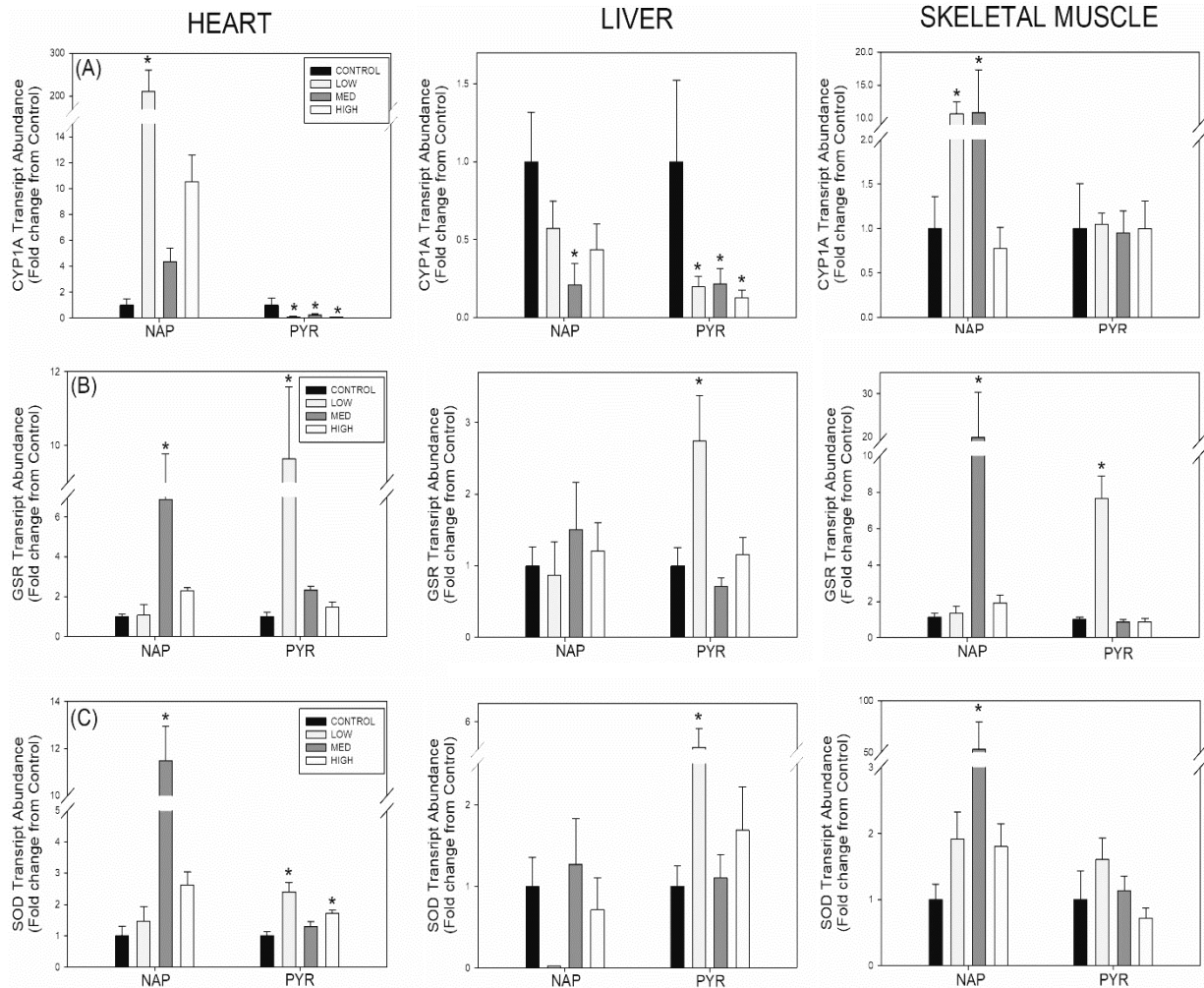


Figure 2. 8: mRNA levels of cytochrome P450 1A (CYP1A; A), glutathione-S-reductase (GSR; B) and superoxide dismutase (SOD; C) in adult zebrafish heart, liver and skeletal muscle following aqueous exposure to naphthalene (left; NAP; low = 37, med = 370, high = 3700 $\mu\text{g/L}$), pyrene (right; PYR; low = 0.25, med = 2.5, high = 25 $\mu\text{g/L}$) or solvent control for 48 hours. Data are mean \pm SEM of n = 10 fish/group. *p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding control group.

2.4 Discussion

2.4.1 Summary of key findings

In this study, we evaluated the cardiorespiratory and metabolic effects of acute aqueous exposure to NAP and PYR in adult zebrafish as well as examining markers of two

key pathways for toxicity, CYP1A and antioxidant enzyme induction. The most prominent findings of the current study were that NAP and PYR demonstrated notable differences in cardiovascular and metabolic effects on adult zebrafish from each other and from BaP. Both PAHs of interest had little to no effects on metabolism, as indicated by SMR, AMR and aerobic scope. More importantly, neither NAP nor PYR had any effect on swimming endurance, as indicated by U_{crit} . Cardiac effects of acute NAP exposure were to increase filling of the heart during diastole and increase stroke volume, while PYR instead slowed cardiac pacemakers to lower heart rate. Overall, both NAP and PYR tended to increase glycogen and triglyceride levels in heart, liver and skeletal muscle, along with increased mRNA levels of CS, a rate-limiting enzyme. Fatty acid catabolism was also upregulated in cardiac and skeletal muscle tissue, as indicated by increased HOAD mRNA levels, in zebrafish exposed to both NAP and PYR. However, PYR caused HOAD transcript abundance in the liver to decrease instead. Potential mechanisms of toxicity for NAP and PYR could be through AhR activation or through increased oxidative stress. To this end, CYP1A mRNA levels was increased in cardiac and skeletal muscle, but was instead decreased in liver compared to control after NAP exposure. In contrast, PYR showed no ability to increase mRNA levels in any of the tissues examined and in fact caused a significant decrease in CYP1A levels in liver and heart instead. Finally, both NAP and PYR tended to increase mRNA transcript abundance of SOD and GSR in the three tissues examined, suggesting the antioxidant response was activated by both PAHs.

2.4.2 NAP and PYR body burdens

The levels of NAP and PYR detected in zebrafish in our current study are substantially lower than levels detect in fish found in contaminated environments and more in line with

reference or control fish (Kalf et al., 1997; McDonald et al., 1995; Rainio et al., 1986). This study failed to detect any change in NAP or PYR parent compound body burden after 48-hour exposure in adult zebrafish. It should be noted that the current study did not examine metabolites and rapid metabolism of the parent compound is the likely explanation for this failure to observe an increase in PAH body burden. In support of this, previous studies in various aquatic species have reported that NAP is biotransformed into two monohydroxylated naphthalene metabolites (Pulster et al., 2017) or reactive metabolites; naphthalene 1,2-oxide and sulphate, glucuronide and GSH conjugates (Krahn et al., 1980). In fact, NAP demonstrated a higher biotransformation and preferential metabolism relative to phenanthrene (Pulster et al., 2017). Based on previous studies in other aquatic species, PYR was also likely rapidly metabolized into pyrene-glucuronide and pyrene-sulfate (Ikenaka et al., 2013), or 1-hydroxypyrene (Oliveira et al., 2012; Vuontisjärvi et al., 2004). Therefore, body burdens after PAH exposure would ideally be determined by the quantification of metabolites (Aas et al., 2000; Budzinski et al., 2004; Oliveira et al., 2012), but technical difficulty in setting up HPLC techniques to quantify the numerous metabolites precluded their measurement in the current study. Since both NAP and PYR exerted observable biological effects in the current study despite no change in NAP or PYR body burdens, both PAHs must have been bioaccumulated in the adult zebrafish over the 48 hours of exposure, but were metabolized by time of sampling for HPLC analysis for parent compounds.

2.4.3 Swimming performance & metabolic rates

Swimming performance is critical to the success of both an individual fish and the entire fish population (Claireaux et al., 2006; Oliveira et al., 2012). While the current study found no effect of NAP exposure on zebrafish swim performance or metabolic rates, other

studies reported NAP increased oxygen consumption and metabolic rate in juvenile Florida Pompanos (*Trachinotus carolinus*; dos Santos et al., 2006). Conversely, the current study found PYR increased zebrafish active metabolic rate with no change in swim endurance, while another previous study reported *Pomatoschistus microps* acutely exposed to PYR (96h) could not resist water flow and covered a shorter swim distance (Oliveira et al., 2012). On the other hand, the more toxic PAH, BaP, was found to increase metabolic rate in adult zebrafish after acute exposure, but with no change in swimming endurance (Gerger & Weber, 2015). The differences in findings among studies could be due to zebrafish being a relatively hardy species that are resilient swimmers compared to other fish species. Taken together, it appears most PAHs including NAP, PYR and BaP, either do not affect or tend to increase metabolic rate, which would cause PAH-exposed fish to use more energy to perform same amount of exercise as unexposed fish. Previous studies examining exposure to a variety of toxicants have also reported elevated standard metabolic rate (Gerger et al., 2014; Thomas & Janz, 2011), with many of these studies similarly failing to find a change in swim performance. This lack of change in U_{crit} in the current study may be further related to the fact that despite some functional changes in the heart, cardiac output remained unchanged after either NAP or PYR exposure. These findings after acute exposure to PAHs in adult fish disagree with other studies where developmental exposures to crude oil or TCDD found persistently higher oxygen consumption, reduced aerobic capacity and lower U_{crit} in surviving adult zebrafish (Hicken et al., 2011; Marit & Weber, 2012). This highlights the high sensitivity of embryonic exposure versus acute adult exposure. Developmental exposure to PAHs and TCDD likely had sublethal teratogenic effects that persisted in adult surviving fish,

making the effects on swim performance greater than that observed after acute exposure in adults.

2.4.4 Cardiac function

Due to the necessity to use ultrahigh resolution ultrasound or highly invasive instruments to measure cardiac function in adult fish, to our knowledge no previous study has reported effects of acute NAP or PYR exposure in adult fish. In the current study, zebrafish exposed to 370 μg NAP/L had significantly elevated EDV, consistent with higher ventricular filling. Starling's Law states that stroke volume increases in response to increasing preload which is the pressure in the venous system that drives filling of the heart during diastole (Farrell, 1991). Although increased ventricular elasticity can also increase EDV, this seems less likely of an explanation due to the acute (48 h) nature of the NAP exposure in the current study. Instead, extensive edema after NAP exposure was noted during dissection in the current study, especially with highest exposure and this is consistent with known edematous effects of NAP (USEPA, 2003). While the current study did not examine this directly, abdominal edema is known to increase venous pressure and preload (Monnet et al., 2016). Despite this increased stroke volume, cardiac output was not significantly changed after NAP exposure in adult zebrafish.

The effects of acute PYR exposure on cardiac function differed markedly from NAP. In the current study, PYR significantly decreased the atrial and ventricular contractile rate without altering the atrial ventricular ratio, suggesting the primary effect was decreased stimulation of the pacemaker cells. Acute exposure to BaP in adult zebrafish also resulted in bradycardia, but was due to AV conduction blockade (decreased AV ratio with no change in

atrial rate), suggesting BaP preferentially affects causes arrhythmias by slowing conduction through the atrioventricular node (Gerger & Weber, 2015), while PYR affects either the pacemaker cells directly to decrease their excitability or increases vagal firing to the sinoatrial node. In addition, BaP further impaired cardiac function by reducing ventricular filling, stroke volume and decreasing cardiac output (Gerger & Weber, 2015), while NAP and PYR both ultimately had no significant effect on cardiac output in the current study. Previous studies reported that zebrafish embryos exposed to PYR or crude oil had abnormal contractions, resulting in increased heart rate, decreased EDV, decreased ESV and decreased stroke volume, reduce cardiac output and reduced swim performance (Hicken et al., 2011; Incardona & Scholz, 2016; Zhang et al., 2012). The observed decrease in ESV after 25 µg PYR/L exposure agrees with these previous studies, but the heart rate changes are opposite to what we observed in adult fish and cardiac output was ultimately unaffected. Again, teratogenic cardiac effects after embryonic exposure were reported (Hicken et al., 2011; Incardona & Scholz, 2016), leading to greater cardiac effects than that observed in the current study after adult fish exposures. Taken together, it appears that NAP and PYR have some cardiac effects after acute exposure in adult fish, but the heart could still compensate to preserve cardiac function. Moreover, the NAP or PYR-induced effects after exposure during adulthood appear to differ significantly from the more cardiotoxic BaP at any life stage in fish.

2.4.5 Energy stores and metabolizing enzymes

Liver and skeletal muscle are both important organs for storage and utilization of glycogen and triglycerides, whereas the heart is a tissue with high metabolic demand (Rajotte & Couture, 2002). Zebrafish prefer to use free fatty acids as their main source of

energy, but will switch to glycolytic energy when needed, especially during the anaerobic metabolism observed in fish fatigued at the end of a swimming challenge (Dangé, 1986; Salmerón, 2018; Thomas & Janz, 2011; Tocher, 2003). If PAHs tend to increase metabolic demand, as PYR did in the current study, then one would predict increased utilization of triglyceride and glycogen energy stores. However, the current study instead found that acute exposure to either NAP or PYR either did not change or caused increased glycogen and triglyceride content in heart, liver and skeletal muscle. Moreover, the observed higher CS mRNA levels in the current study after NAP and PYR exposure indicates higher oxidative capacity in zebrafish that should have again caused more energy stores to be depleted. Previous studies reported that adult tilapia (*Oreochromis mossambicus*) aqueously exposed for up to 10 weeks or female rainbow trout injected intraperitoneal with NAP or BaP had reduced muscle and liver glycogen reserves along with increased serum lactate, indicating NAP caused a switch to glycolytic anaerobic metabolism (Dange & Masurekar, 1982; Tintos et al., 2006; 2008). Differences in exposure duration, exposure route and/or species may explain the difference in observations among studies.

Reported effects of PAHs on tissue triglyceride levels may be more consistent. The current study found significant increases in triglyceride content in the heart and skeletal muscle after NAP, as well as in the liver and skeletal muscle after PYR exposure. This agrees with previous studies examining developmental TCDD exposure or 21-day NAP exposure in adult zebrafish, both of which reported increases in whole body triglycerides and fatty infiltration of the liver, respectively (Chen et al., 2018; Marit & Weber, 2012). In the current study, HOAD mRNA transcript abundance was paradoxically increased after both NAP and PYR exposure in heart and skeletal muscle, but not liver where hepatic HOAD was instead

decreased after PYR exposure. The heart and muscle results agree with a transcriptome analysis of hepatic mRNA in adult zebrafish or whole-body mRNA in larval zebrafish after PYR exposure where upregulation of genes associated with both fatty acid synthesis and branched chain fatty acid β -oxidation were reported (Chen et al., 2018; Goodale et al., 2013). However, changes in mRNA levels do not always translate into changes in enzyme activity. This may explain why other studies reported that hepatic HOAD enzyme activity is unaffected by intraperitoneal injection of NAP or BaP in rainbow trout (Tintos et al., 2006, 2008). Thus, we can speculate that tissues from zebrafish exposed to NAP and PYR respond with fatty acid accumulation, but may be unable to utilize or access the triglyceride stores available. Future studies should examine whether the observed changes in mRNA abundance of both glycolytic and lipolytic enzymes translate into similar changes in enzyme activity after both NAP and PYR exposure. Moreover, studies should also examine genes associated with fatty acid and glycogen synthesis since both catabolism and synthesis would influence tissue glycogen and triglyceride levels.

2.4.6 AhR activation and antioxidant response

The relative potency of PYR at the AhR is substantially weaker than the strongest PAH agonist, BaP, and both are orders of magnitude weaker agonists than TCDD (Barron et al., 2004). On the other hand, NAP reportedly failed to bind to human AhR at the TCDD binding site (Sandoz et al., 1998). Upregulation of CYP1A is a well-known marker of AhR activation and the liver is generally considered the most important organ in this detoxification response (Denison & Nagy, 2003; Marit & Weber, 2012; Prasch et al., 2003). Previous mammalian studies reported that PYR does induce small increases in hepatic ethoxyresorufin-o-deethylase (EROD) activity, but does this through increased CYP1A2, not

CYP1A1, mRNA transcript abundance (Lee et al., 2007; Shimada & Fujii-Kuriyama, 2004). The regulation of CYP1A2 expression by PYR may be through an AhR-independent mechanism involving the hepatic constitutive androstane receptor (Lee et al., 2007). In contrast, BaP caused a much greater induction in EROD activity as well as increased CYP1A1 gene expression in an AhR-dependent manner (Shimada & Fujii-Kuriyama, 2004), while NAP failed to change mammalian EROD activity or CYP1A1/2 expression (Sandoz et al., 1998).

The AhR is known to be different in fish compared to mammals, with most toxicity thought to be mediated by AhR2 in fish (Prasch et al., 2003), while AhR1 was thought to be unresponsive to TCDD-mediated toxicity (Prasch et al., 2003; Ulin et al., 2018). Based on this, it would be expected that differences in CYP1A responsiveness to PYR versus NAP should also differ between mammals and fish. In Nile tilapia (*Oreochromus niloticus*), hepatic EROD was reported to be strongly induced by four-ringed PAHs such as PYR, while two-ringed PAHs such as naphthalene instead decreased EROD activity (Pathiratne & Hemachandra, 2010). Also, in zebrafish at all life stages, BaP has been previously reported to cause significant induction of CYP1A mRNA (Bugiak & Weber, 2009; Fang et al., 2015; Gerger & Weber, 2015). Other studies reported fish toxicity from PYR was linked to AhR activation and CYP1A induction (Hendon et al., 2008; Incardona et al., 2005; 2004). Therefore, it was surprising in the current study that NAP, but not PYR, appeared to activate the AhR (increased CYP1A mRNA levels) in cardiac and skeletal muscle. In fact, CYP1A expression decreased in heart and liver from PYR-exposed zebrafish in the current study. PYR has been reported to bind AhR, but only while AhR was in an antagonist, not agonist conformation through selective activation of AhR-nongenomic calcium signaling (Brinchmann et al., 2018), and failed to upregulate CYP1A gene expression in zebrafish embryos (Zhang et al., 2012).

Thus, this new structure-functional evidence that PYR may prefer to antagonize AhR is consistent with the observations of decreased CYP1A in heart and liver after PYR exposure in the current study. NAP may be similarly antagonizing the AhR in the liver. A class of non-coding RNAs, microRNAs (miRNAs), reported to be important cytoplasmic regulators of gene expression (Catalanotto et al., 2016), could be degrading CYP1A mRNA prior to translation with NAP exposure. NAP could also be affecting the window of time at which peak mRNA transcript abundance could have been missed due to the time at which tissues were sampled. However, Atlantic salmon hepatic CYP1A mRNA levels increased eightfold six hours after intraperitoneal injection of BNF and reached maximum levels 48hr after injection (14-fold) (Grøsvik et al., 1997), evidence CYP1A mRNA levels reach maximum at 48-hours.

However, we do not currently have an explanation for why NAP appeared to stimulate AhR since CYP1A mRNA transcript abundance increased in heart and skeletal muscle from NAP-exposed zebrafish in the current study. What may be related is a recent study in zebrafish which demonstrated a complex cooperative action between AhR1b and the signaling of the antioxidant response system through nuclear factor erythroid-2 (nrf) response elements (Ulin et al., 2018).

On that note, SOD and GSR mRNA transcript abundance was investigated in the current study as indicators of activation of the antioxidant response to PYR or NAP. However, they are not direct indicators of oxidative stress since they do not measure levels of reactive oxygen species. Overall, there was an increase in expression of SOD, GSR or both in all tissues after both NAP and PYR exposure, agreeing with previous studies reporting similar elevations in SOD and GSR using NAP-rich diluted bitumen and oil sand process-affected

water (He et al., 2012; Madison et al., 2015). In contrast, another previous study reported that neither PYR nor NAP had any effect on activity of another antioxidant enzyme, glutathione-S-transferase (GST; Pathiratne & Hemachandra, 2010). A recent meta-analysis of lab and field studies of PAH-exposed fish concluded that upregulation of antioxidant enzymes such as GST, SOD, glutathione peroxidase and GSR was common after most PAH exposures (Santana et al., 2018). Although variable responses in mRNA transcript abundance among different antioxidant enzymes were noted after PAH exposure in fish, the meta-analysis suggested EROD and GST were possible the most reliable biomarkers. However, this same meta-analysis suggested GSR also look promising as a biomarker, but inadequate studies examining GSR precluded a definitive conclusion (Santana et al., 2018). Taken together with the current study, both SOD and GSR may also good indicators of PAH-mediated toxicity.

2.4.7 Hormetic effect of NAP exposure

Hormesis is characterized by stimulation or a beneficial effect at low doses and a toxic or inhibitory effect at high doses (Mattson, 2008). Interestingly, it appears that NAP exposure to adult zebrafish is characterized by a biphasic dose response effect. 370 µg/L NAP significantly increased EDV and SV in the heart, CS transcript abundance in all three tissues and CYP1A transcript abundance in the heart and skeletal muscle. However, these changes were not seen at the low and highest dose of NAP, indicating a hormetic response to NAP exposure. Another possibility could be the full body edema witnessed upon dissection with the fish in the highest dose group. Instead of toxicity occurring at a molecular level, the entire body is overwhelmed and experiencing a full body toxicity, therefore resulting in a lack of increase in the endpoints listed in the highest exposure group.

2.5 Conclusion

In conclusion, due to the rapid transformation into more hydrophilic metabolites for excretion, aqueous exposure to NAP and PYR resulted in only trace amounts of the parent compound detected after 48 h aqueous exposures (renewal at 24 h) in adult zebrafish. Cardiac effects of NAP versus PYR differed from each other and from that previously reported for BaP. Overall, both NAP and PYR cardiac effects were compensated and did not change cardiac output or produce arrhythmia, suggesting these PAHs are less cardiotoxic to acutely exposed adult zebrafish compared to BaP. Overall, both NAP and PYR tended to increase glycogen and triglyceride levels in heart, liver and skeletal muscle, along with increased mRNA levels of CS and HOAD, rate-limiting enzymes in the citric acid cycle and fatty acid catabolism, respectively, in most of these same tissues. Coupled with no changes or minor increases in metabolic rates and aerobic scope, with no change in swimming endurance, this suggests again that NAP and PYR exposure at 48 h in adult zebrafish is starting to potentially affect the fish's ability to access its energy stores, but has not yet affected swimming. This again differs from BaP which exerted greater metabolic toxicity and impaired swimming after only 48 h exposure (Gerger & Weber, 2015). Surprisingly, the mechanisms of action may involve AhR activation for NAP, but not PYR, although this may involve a complex interaction with a common upregulated antioxidant response after exposure to both PAHs. In conclusion, acute NAP and PYR exerted cardiorespiratory toxicity, metabolic alterations and mechanisms activated had some similarities with BaP, but BaP is clearly a stronger AhR agonist and much more toxic than either NAP or PYR.

CHAPTER 3: PHARMACOLOGICAL INVESTIGATION OF ACUTE NAPHTHALENE AND PYRENE TOXICITY IN ADULT ZEBRAFISH (*DANIO RERIO*).

3 Preface

The research in this chapter was designed to investigate whether acute naphthalene and pyrene in adult zebrafish depends on the relative potency as aryl-hydrocarbon receptor agonists and cytochrome P450 inducers are indicative of relative toxicity or whether oxidative stress is a better indicator. The author contributions to Chapter 3 of this thesis were as follows:

Chanel Yeung (University of Saskatchewan) performed 100% of all animal experiments, collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript. Lynn Weber (University of Saskatchewan) supervised, helped with study design, provided scientific input and guidance, and edited the manuscript. Chapter 2 is being prepared for submission for publication in *Aquatic Toxicology*.

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds consisting of two or more aromatic rings and, are among the most ubiquitous contaminants present in the aquatic environment today (Jaward et al., 2012). PAHs are generated through the incomplete combustion of organic matter at high temperatures. Natural sources include forest fires and volcanic activity. However, the environmental concern is from the anthropogenic sources, including oil spills, automotive exhaust and industrial activity (Abdel-Shafy & Mansour,

2016; Neff et al., 2005). Previous studies have focused on the representative PAH, benzo-a-pyrene (BaP), which induces toxicity through binding to the aryl hydrocarbon receptor (AhR; Miller & Ramos, 2001). The well characterized response to AhR activation is increased transcription/translation of phase one cytochrome P4501A monooxygenase (CYP1A), which in turn causes production of reactive oxygen species and stimulation of the antioxidant system (Nebert et al., 2000; Thirman et al., 1994). The reactive BaP metabolites are mutagenic and cytotoxic, in addition to their ability to produce oxidative stress (U.S. EPA., 2017). When a ligand binds to the AhR, there is an upregulation of phase I and II metabolic enzymes, ranging from CYPs, aldehyde dehydrogenase 3, NADPH quinone oxidoreductase, glutathione S-transferase and UDP-glucuronosyltransferases (Brown, Clark, Garner, & Di Giulio, 2016; Denison & Nagy, 2003; Nebert et al., 2000). Anthropogenic ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls, and several PAHs have been heavily studied and are known to bind to the AhR and induce AhR dependent gene expression (Brown et al., 2016; Kafafi et al., 1993; Waller & Mckinney, 1995). However, some PAHs or their metabolites may cause oxidative stress in an AhR-independent manner (Guigal et al., 2000; Raha et al., 1995).

Previous studies have determined the relative potency of PAHs as AhR agonists in fish compared to TCDD. Generally, they found that two and three ring unsubstituted PAHs, such as NAP, were largely inactive at the AhR in fish. In contrast, the highest potency PAHs had 4-6 aromatic rings with an exposed bay region, similar to PYR (Barron et al., 2004; Sandoz et al., 1998). Thus, PYR will be studied in this thesis because although it is predicted to act largely via AhR activation, knowledge of its mechanism of toxicity is limited compared to that of BaP. Despite NAP being reported to be inactive in fish (Barron et al., 2004), in a study

analyzing 48 different crude oils from around the world, NAP was present in the highest concentration in all 48 samples (Kerr & Melton, 1999). Important detoxification antioxidant enzymes, superoxide dismutase (SOD) and glutathione reductase (GSR) (Kim & Lee, 1997; Miller & Ramos, 2001) have been shown to be protective against oxidative stress and BaP toxicity, therefore will be measured in this study.

Zebrafish prefer using free fatty acid metabolism rather than carbohydrate or protein metabolism (Dangé, 1986; Salmerón, 2018; Thomas & Janz, 2011; Tocher, 2003). The rate limiting enzyme in β -oxidation is 3-hydroxyacyl-CoA dehydrogenase (HOAD), is responsible for the catalysis of acyl-CoA into acetyl-CoA (López-Patiño et al., 2014), and is a biomarker of β -oxidation capacity. HOAD mRNA levels will be compared to triglyceride levels and lipoprotein lipase (LPL) mRNA levels. LPL is responsible for hydrolyzing triglycerides into free fatty acids, allowing them to be imported from plasma into cells or from cellular lipid stores for use in aerobic metabolism (Rajotte & Couture, 2002; Salmerón, 2018). Aerobic metabolism of both glucose liberated from glycogen and fatty acids occurs via the citric acid cycle to generate ATP, with citrate synthase (CS) as the rate limiting enzyme (Krebs, 1970).

Developmental exposure to PAHs in various aquatic species consistently demonstrated an effect on heart rate, arrhythmias and impaired contractility (Collier et al., 2013; Incardona et al., 2009; Incardona & Scholz, 2017; Sørhus et al., 2016). Acute BaP exposure in adult zebrafish has also been reported to have negative effects on cardiovascular function, namely arrhythmia (atrioventricular conduction blockade), bradycardia and reduced cardiac output reported (Gerger & Weber, 2015). PAH mixtures derived from crude oil or individual tricyclic PAHs have been reported to have similar toxicities (Incardona et

al., 2015). Low level PYR is cardiotoxic to zebrafish embryos, changing heart morphology similar to that described for the potent AhR agonist TCDD (Incardona et al., 2004; Zhang et al., 2012). Developmental PYR exposure caused altered cardiac function (elevated heart rate, but decreasing end diastolic, end systolic, and stroke volumes) in larval fish, without a change in CYP1A mRNA expression (Zhang et al., 2012). Conversely, developmental exposure of zebrafish larvae to NAP, induced mild bradycardia and edema, without the profound teratogenic effects on the heart (Incardona et al., 2004). This difference in developmental cardiotoxicity between BaP, NAP and PYR could be related to differences in affinity for the AhR (Barron et al., 2004), but could also be related to differences in other potentially AhR-independent mechanisms of toxicity such as oxidative stress. Moreover, whether these same differences in toxicity would be evident in adult fish acutely exposed to NAP or PYR compared to known developmental BaP cardiorespiratory toxicity is unclear.

The purpose of this study is to investigate whether relative potency as AhR agonists and CYP1A inducers are indicative of relative toxicity or whether oxidative stress is a better indicator. This can be studied by inhibiting these three endpoints pharmacologically and looking at the overall effects on NAP and PYR exposure to zebrafish. From previous studies done in our lab (Chapter 2 of this thesis), the concentrations chosen for this co-exposure were sub-maximal, but produced consistent changes in gene expression, without major cardiac function effects (370 µg NAP/L; 2.5 µg PYR/L). Adult zebrafish were exposed to each PAH, alone or in combination with three antagonistic treatments for 48 hours. To determine if cardiometabolic process were dependent of AhR stimulation, a potent AhR antagonist that does not stimulate AhR dependent transcription, CH-223191 (3334 µg/L), was used (Kim et al., 2006; Ko & Shin, 2012). Fluoranthene (FL; 500 µg/L) is a PAH, but is also a known CYP1A

inhibitor (Brown et al., 2014), while Tempol (4-hydroxy-TEMPOL; 172 µg/L) is a known antioxidant scavenger of free radicals (Chatterjee et al., 2000; Epperly et al., 2012). After 48-hour exposure, fish underwent high frequency cardiac ultrasound technology to measure cardiac function. Quantitative reverse transcription PCR (qRT-PCR) was then used to quantify mRNA transcript abundance of important metabolic enzymes (CS, HOAD, LPL), a biomarker of AhR activation (CYP1A), and indicators of activation of the antioxidant response cytochrome (glutathione-S-reductase, GSR and superoxide dismutase, SOD) in excised heart, liver and skeletal muscle. Finally, changes in triglyceride and glycogen levels were measured in the same tissues as indicators of energy storage.

3.2 Materials and Methods

3.2.1 Test compounds

Naphthalene (NAP), Pyrene (PYR), dimethyl sulfoxide (DMSO), ethyl 3 aminobenzoate methanesulfonate (MS-222), fluoranthene (FL), CH-223191 and tempol were all purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.2.2 Test species

Adult zebrafish were obtained from university colonies and acclimated in 30-L holding tanks with particulate, charcoal and ammonia biofilters for a minimum of two weeks prior to experiments. Zebrafish were kept on a 14-hour light and 10-hour dark photoperiod. Clean, dechlorinated, aerated municipal water was maintained at 27 ± 1 °C with 80% water change performed weekly during basic maintenance. Zebrafish were fed twice a day with Nutrafin basic flake food (Hagen Inc., Montreal, QC, Canada), with the exception of the last 24h of exposure or during swim performance or cardiac function testing (Gerger et al., 2014;

Gerger & Weber, 2015). Experiments were conducted according to procedures approved by the University of Saskatchewan Animal Care and Use committee according to the Canadian Council on Animal Care guidelines.

3.2.3 Aqueous exposure

Experiments were performed in clean, dechlorinated, aerated municipal water with PAHs dissolved in DMSO (0.05% concentration in exposure water) using static renewal conditions. Adult zebrafish (0.3-0.8g) were randomly selected from holding tanks, placed in static aerated glass beakers (4 fish/1 L beaker) containing vehicle control (0.05% DMSO), PYR (2.5 µg/L) or NAP (370 µg/L) alone, or co-exposed with either 500 µg/L fluoranthene (FL), 3333.9 µg/L CH-223191 or 172.2 µg/L Tempol dissolved in 28°C water for 48 hour (renewal at 24 hours). Test concentrations were chosen on the basis of the results of our previous study. Pharmacological antagonist concentrations were based on previous studies (Brown et al., 2014; Epperly et al., 2012; Ko & Shin, 2012). After 48-hour exposure, fish were used for cardiac ultrasound (n = 16/treatment). After ultrasound, heart, liver and skeletal muscle from a portion of the zebrafish were stored at -80°C for triglyceride and glycogen analyses or in RNAlater until use for mRNA levels of CYP1A, CS, HOAD, GSR, LPL and SOD.

3.2.4 *In vivo* high frequency cardiovascular ultrasound

After 48 hour exposure, physiological changes to cardiac function were measured using a VEVO 3100 high frequency ultrasound machine (VisualSonics, Markham, ON Canada) equipped with B-mode imaging, pulsed-wave Doppler, colour flow Doppler and tissue Doppler according to methods adapted from rodents (VisualSonics, 2008) and previously described in zebrafish (Gerger et al., 2014; Gerger & Weber, 2015). Zebrafish were

anesthetized with Aquacalm (20 mg/L; Oakville, ON, Canada) in clean aerated, dechlorinated municipal water in a grooved holding dish, ventral side up, prior to ultrasonography and anesthesia is maintained throughout. Using a recirculating water bath with aerated anesthetic water, temperature was maintained at 27 ± 0.5 °C (the same as housing temperature) to prevent temperature-induced effects on cardiac function.

The short and long-axis views of the ventricle were obtained using an MX 700 scanhead (30-70 MHz; resolution to 30 μm) in B-mode. Simpson's rule of disks calculated ventricular volume (Mercier et al., 1982; Visualsonics, 2008). The ventricle volume is the sum of the cylindrical disks, forming an apical disk shape. Because zebrafish do not have a hollow ventricular chamber like mammals do, the cross-sectional areas using traces of the outer edges of the ventricle were measured ($A_1 - A_3$) at three different levels of the ventricle in the short axis view. The height of each ventricle disk was then calculated by measuring the ventricular length (L) in the long axis view and dividing by the number of short axis views ($h=L/3$). Volume was calculated using an average of at least three different measurements in an individual fish during end-systole and during end-diastole for each measurement.

$$V = (A_1 + A_2)h + \left[\left(\frac{A_3 h}{2} \right) + \left(\frac{\pi}{6h^3} \right) \right]$$

(Mercier et al., 1982)

Stroke volume (V_s) for each fish was calculated by subtracting end-diastolic volume from end-systolic volume. Ventricular and atrial contractile rates were calculated by counting individual chamber contractions in 5 second B-mode ultrasound video loops, then

converting to beats per minute (BPM). Ventricular heart rate (f_H) multiplied by V_s was used to calculate cardiac output (Q).

$$Q = V_s * f_H$$

Atrioventricular (AV) ratio was used as a measure of the efficiency of electrical excitation from the atrium to the ventricle via the atrioventricular (AV) node by dividing the atrial heart rate by ventricular heart rate for each individual fish. This value should equal 1.0 if cardiac electrical conduction is normal.

$$AV \text{ ratio} = \frac{\text{Atrial } f_H}{\text{Ventricular } f_H}$$

3.2.5 Determination of triglycerides and glycogen storage

Frozen heart (2 hearts pooled to create one sample), liver (2 livers pooled to create one sample) and skeletal muscle (measured from individual fish) samples were weighed, homogenized in 0.2M sodium citrate, immediately heated for 5 min at 100°C, then stored at -80°C until assessment for triglyceride and glycogen levels. Briefly, triglycerides were determined using a kit (Sigma-Aldrich, Oakville, ON, Canada) based on a lipase and glycerol kinase colorimetric assay, which has been previously validated for measuring triglycerides in whole fish homogenates in our laboratory (Thomas & Janz, 2011; Weber et al., 2008) and glycerol used as a standard. Glycogen was analyzed using an amylase and glucose oxidase-based colorimetric assay and purified Type IX bovine liver glycogen as a standard, as previously described in our group (Weber et al., 2008).

3.2.6 Gene expression analysis – Real time fluorescence qRT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) for gene expression analyses in zebrafish heart (2 heart pooled/sample), liver (2 liver pooled/sample) and skeletal muscle (one sample/fish) was examined for three classes of genes in zebrafish tissue using methods previously described in Bugiak & Weber, (2009). As an indication of AhR activation, CYP1A mRNA levels was measured (Sarasquete & Segner, 2000). To determine the effects on metabolism, mRNA levels of rate limiting enzymes for oxidative metabolism, CS, or fatty acid oxidation, HOAD, were analyzed (Rajotte and Couture, 2002). Activated anti-oxidant responsive genes were also quantified using GSR and SOD mRNA levels (Cossu et al., 1997). Total RNA was extracted from frozen tissues with TRIzol (Life Technologies Burlington, ON, Canada) according to manufacturer specifications. A NanoDrop apparatus and software (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the purity and quantity of mRNA. Samples were reverse transcribed into cDNA from total RNA with superscript II reverse transcriptase (iScript, Invitrogen, (Quanta Biosciences, Maryland, USA) and oligo (dT) primers using the cDNA synthesis kits (BioRad Mississauga, ON, Canada). Chromo4 Multi-Color Real-Time PCR detection system (Biorad, Mississauga, ON, Canada) was used for qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a housekeeping gene and its transcript abundance determined for each run of a gene. Primer sequences for cDNA targets, GenBank accession numbers, target genes and their efficiencies that were used for real time qRT-PCR (Liu et al., 2013; McClelland et al., 2006; Mccurley & Callard, 2008; Tiedke et al., 2013; Uren-Webster et al., 2010). Each sample was run in at least 3 different dilution for each primer pair along with the housekeeping gene to generate efficiency curves. The method of analysis used to represent the relative mRNA

levels was calculated using $\Delta\Delta CT$, where the amount of the gene of interest was normalized to the amount of G3PDH in that sample as well as to the mean value for the control group and data expressed as fold change from control (Bugiak & Weber, 2009).

Table 3. 1. Primer sequences for cDNA targets, genbank accession numbers, target genes and their efficiencies that were used for real time qrt-pcr. (Liu et al., 2013; McClelland et al., 2006; McCurley & Callard, 2008; Tiedke et al., 2013; Uren-webster et al., 2010).

Gene	Primer	Sequence (5'-3')	Accession number	Efficiency (%)
CYP1A	Forward	GCATTACGATACGTTTCGATAAGGAC	NM131879	114.0
	Reverse	GCTCCGAATAGGTCATTGACGAT		
G3PDH	Forward	AGCACTGTTTCATGCCATCAC	NM213094	99.5
	Reverse	TACTTTGCCTACAGCCTTGG		
CS	Forward	AGCGCTGCTATGAATGGTCT	BC045362	93.3
	Reverse	CTGAGGAAGACAGACCCTCG		
LPL	Forward	CGCAGGAGCAGCAAGATG	NM131127	107
	Reverse	GTTCAAAGTAGGCATAATGTAGGG		
HOAD	Forward	CCACAGGACATTTCAGTGGTG	ZGI TC279717	93.7
	Reverse	GTCAGTGCCATGAACGACAG		
GSR	Forward	ACAGTCAGTGAGGATGATGTGCCAG	NM001020554	97.0
	Reverse	TAGACCCAAGAGTGGAAGAATACCAGC		
SOD	Forward	TATGCAGCTTCATCACAGCAAGCA	NM199976	100.0
	Reverse	GGTTGTCACATCACCTTGGCC		

CYP1A = cytochrome P450 1A; G3PDH = Glyceraldehyde-3-phosphate dehydrogenase; CS = citrate synthase; HOAD = 3-hydroxacyl-CoA dehydrogenase; GSR = glutathione reductase; SOD = superoxide dismutase

3.2.7 Statistical analysis

Samples sizes (n) indicate either the number of fish/treatment or the number of pooled samples analyzed/treatment. Outliers were identified as those values more than two

standard deviations from the mean and were removed, as appropriate. One-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test was used to determine significant difference among treatment groups for all endpoints measured. Gene expression data are expressed as $\Delta\Delta CT$ values, using expression of the housekeeping gene (G3PDH) as an internal control, and then normalized to control group expression to calculate fold-change in expression with treatment for the genes of interest. CYP1A mRNA transcript abundance was log-transformed to achieve normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). All statistical analyses were carried out using SPSS Statistics v19.0 (IBM Corporation, Armonk, NY) and all results are presented as mean \pm standard error of the mean (SEM). Principal components analysis was performed initially using mean values for each of the 10 treatment groups in this study for each endpoint in each tissue measured (36 end-points total). Factors were reduced to two components and end-points included in the analysis reduced until $\geq 70\%$ of the variance in the data was explained.

3.3 Results

3.3.1 Mortalities and morphometrics

Following aqueous exposure to NAP, PYR and co-exposure with various pharmacological antagonists, subsequent cardiovascular ultrasound, there was no statistically significant differences in body mass, body length or condition factor among exposure groups (Table 3.2). No mortalities were observed in any treatment group.

Table 3. 2. Mortalities and morphometrics of adult zebrafish aqueously exposed to solvent control, naphthalene (NAP, 370 µg/L) or pyrene (PYR, 2.5 µg/L), alone or in the presence of AhR antagonist (CH223191, 3334 µg/L), CYP1A antagonist (FL = fluoranthene, 500 µg/L), or free radical scavenger (tempol, 172 µg/L) for 48 h.

Nominal PAH Concentration	Mortalities	Body Weight (g)	Body Length (cm)	Condition Factor (K)
NAP				
Solvent control	0	0.30 ± 0.02	3.02 ± 0.04	1.09 ± 0.03
NAP alone	0	0.27 ± 0.02	2.94 ± 0.05	1.06 ± 0.03
NAP + CH223191	0	0.26 ± 0.02	2.84 ± 0.05	1.13 ± 0.03
NAP + FL	0	0.25 ± 0.02	2.79 ± 0.05	1.11 ± 0.03
NAP + TEMPOL	0	0.22 ± 0.02	2.83 ± 0.07	0.96 ± 0.03
PYR				
Solvent control	0	0.31 ± 0.01	3.01 ± 0.03	1.12 ± 0.03
PYR alone	0	0.32 ± 0.02	3.04 ± 0.06	1.10 ± 0.20
PYR + CH223191	0	0.23 ± 0.02	2.82 ± 0.05	1.03 ± 0.08
PYR + FL	0	0.25 ± 0.02	2.81 ± 0.07	1.10 ± 0.03
PYR + TEMPOL	0	0.31 ± 0.02	3.02 ± 0.07	1.10 ± 0.03

Data are mean ± SEM with n = 20/group for morphometrics; n=4 replicate beakers/group for mortalities. Condition factor (K) = (weight (100/length³)). No significant differences among treatment groups were detected for any of the data shown using 1-way ANOVA.

3.3.2 Cardiovascular ultrasound analysis

While neither NAP nor PYR alone significantly altered cardiovascular function at the moderate exposure levels examined (Figure 3.1), co-exposure of each pharmacological antagonist with NAP significantly reduced ventricular filling and lowered end-systolic volume to significantly increase ejection fraction compared to untreated control (Figure 3.1). Similarly, NAP and PYR by themselves also had no effect on heart rate or cardiac output (Figure 3.2), but yet adding CYP1A inhibition to both PAHs increased heart rate, while adding an antioxidant suppressed it, leading to a significant decrease in cardiac output in only the PYR + tempol group (Figure 3.2). This suggests the antagonists by themselves could have affected cardiac function. However, since we did not do singular exposure of each of the

pharmacological antagonists, the combination of the antagonist plus PAH could have increased the effects of each other.

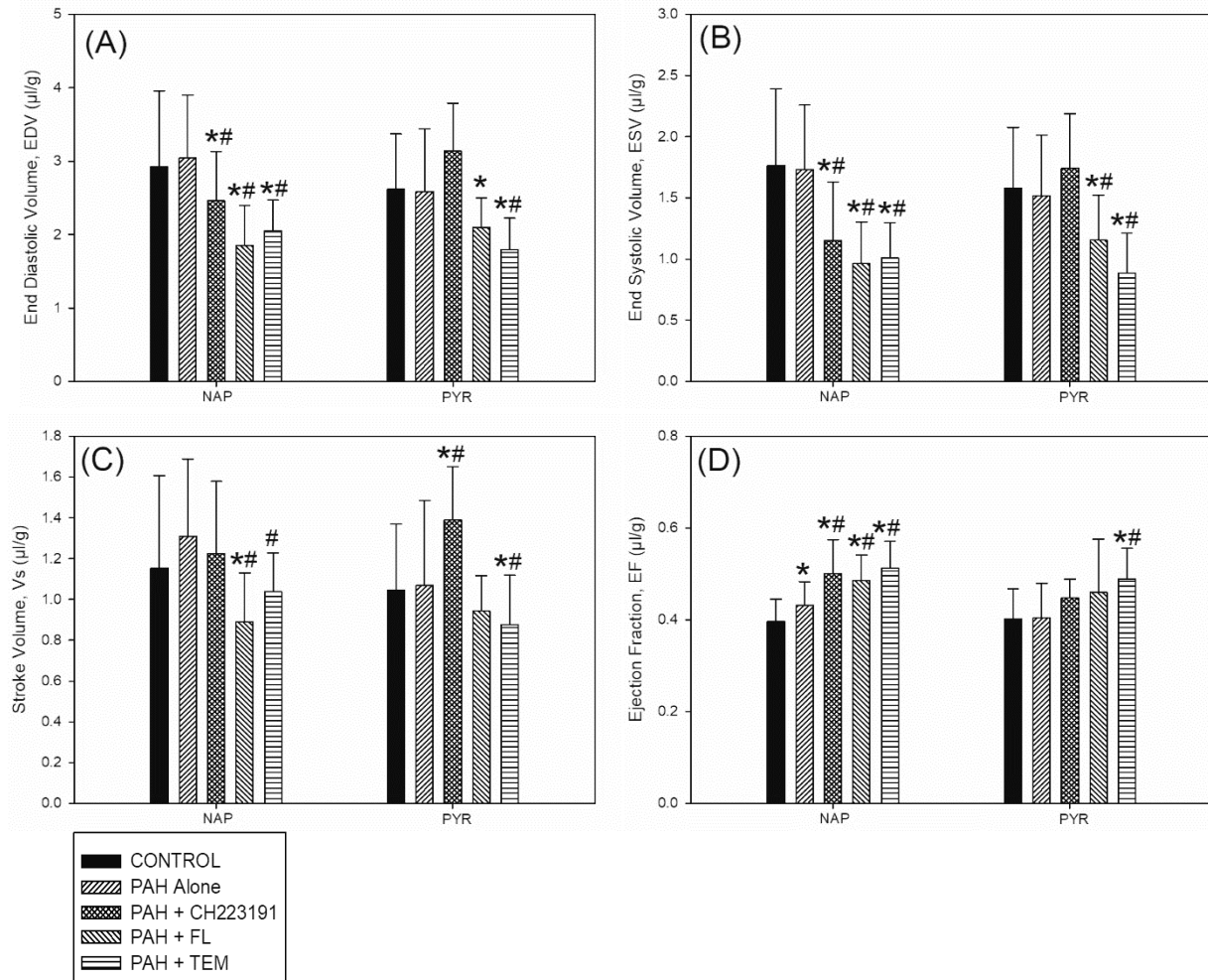


Figure 3. 1: Echocardiography was used to assess cardiac function with end diastolic volume (EDV; A), end systolic volume (ESV; B), stroke volume (C) and ejection fraction (D) in adult zebrafish shown following aqueous exposure to solvent control, naphthalene (left; 370µg/L) or pyrene (right; 2.5 µg/L) alone, or with CH-223191(33334 µg/L), fluoranthene (500 µg/L) or tempol (172 µg/L) for 48 hours. Data are mean ± SEM of n = 14-16 fish/group. * = p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding solvent control group, # = p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding PAH alone group.

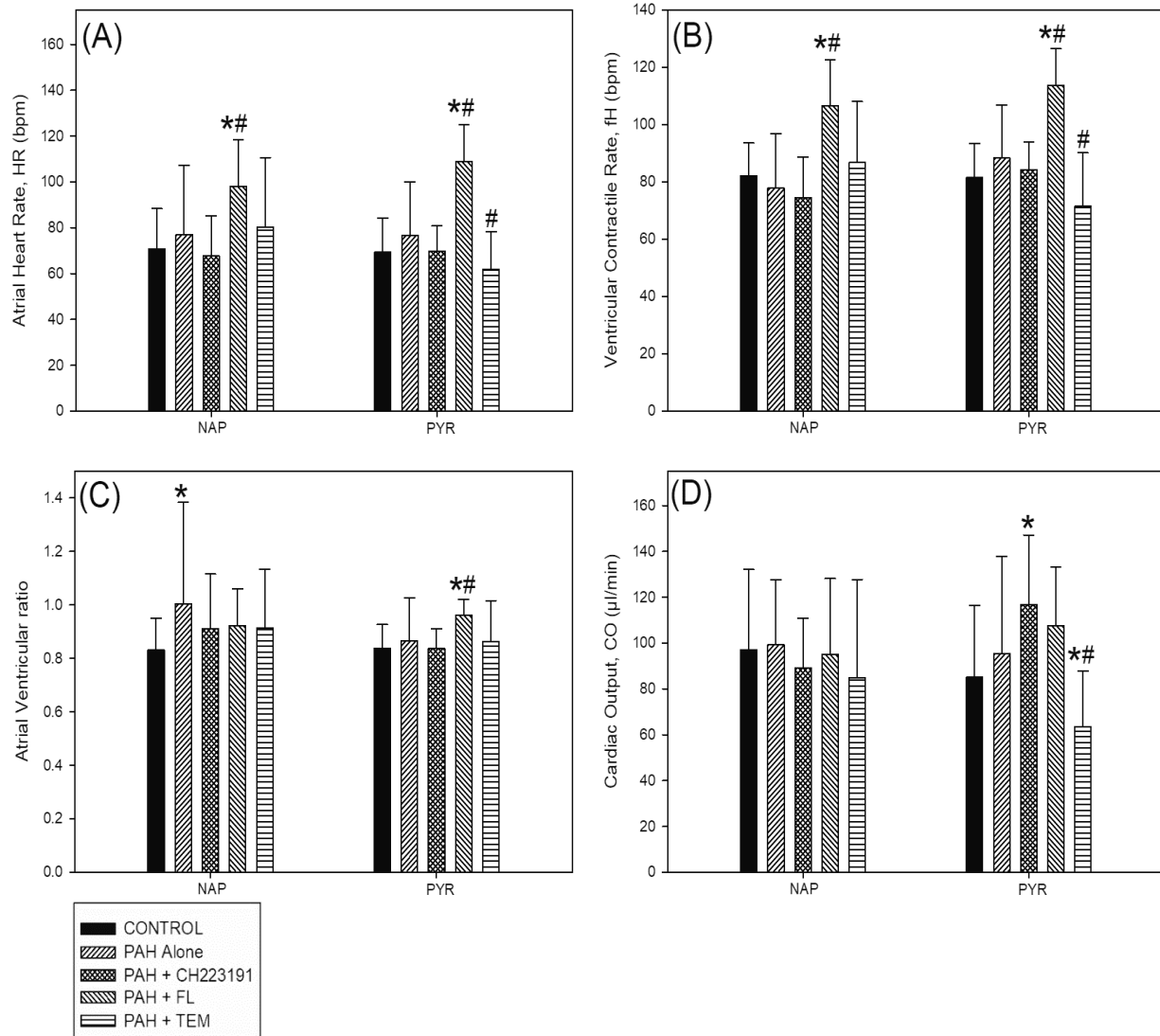


Figure 3. 2: Atrial contractile rate, ventricular contractile rate, ratio of atrial to ventricular rates (AV ratio), and cardiac output in adult zebrafish shown following aqueous exposure to solvent control, naphthalene (left; 370µg/L) or pyrene (right; 2.5 µg/L) alone, or with CH-223191(3333.9 µg/L), fluoranthene (500 µg/L) or tempol (172.2 µg/L) for 48 hours. Data are mean ± SEM of n = 14-16 fish/group. * = p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding solvent control group, # = p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding PAH alone group.

3.3.3 Energy storage and metabolizing enzymes

To examine effects on metabolism, glycogen, triglycerides and qRT-PCR of key metabolic genes were measured in heart, liver and skeletal muscle. While NAP and PYR by themselves again had little effect on tissue glycogen or triglyceride levels (Figure 3.3), adding

AhR or CYP1A inhibitors with these PAHs tended to increase glycogen in most tissues and all three inhibitors tended to increase tissue triglycerides. In contrast, NAP exposure by itself increased mRNA levels of both citrate synthase (CS) and 3-hydroxacyl-CoA dehydrogenase (HOAD) in all tissues, with all three inhibitors tending to reverse this NAP-induced increase in most tissues (Figure 3.3). On the other hand, PYR by itself either had no effect or decreased CS and HOAD mRNA levels except for HOAD in skeletal muscle where PYR instead increased transcript abundance (Figure 3.3).

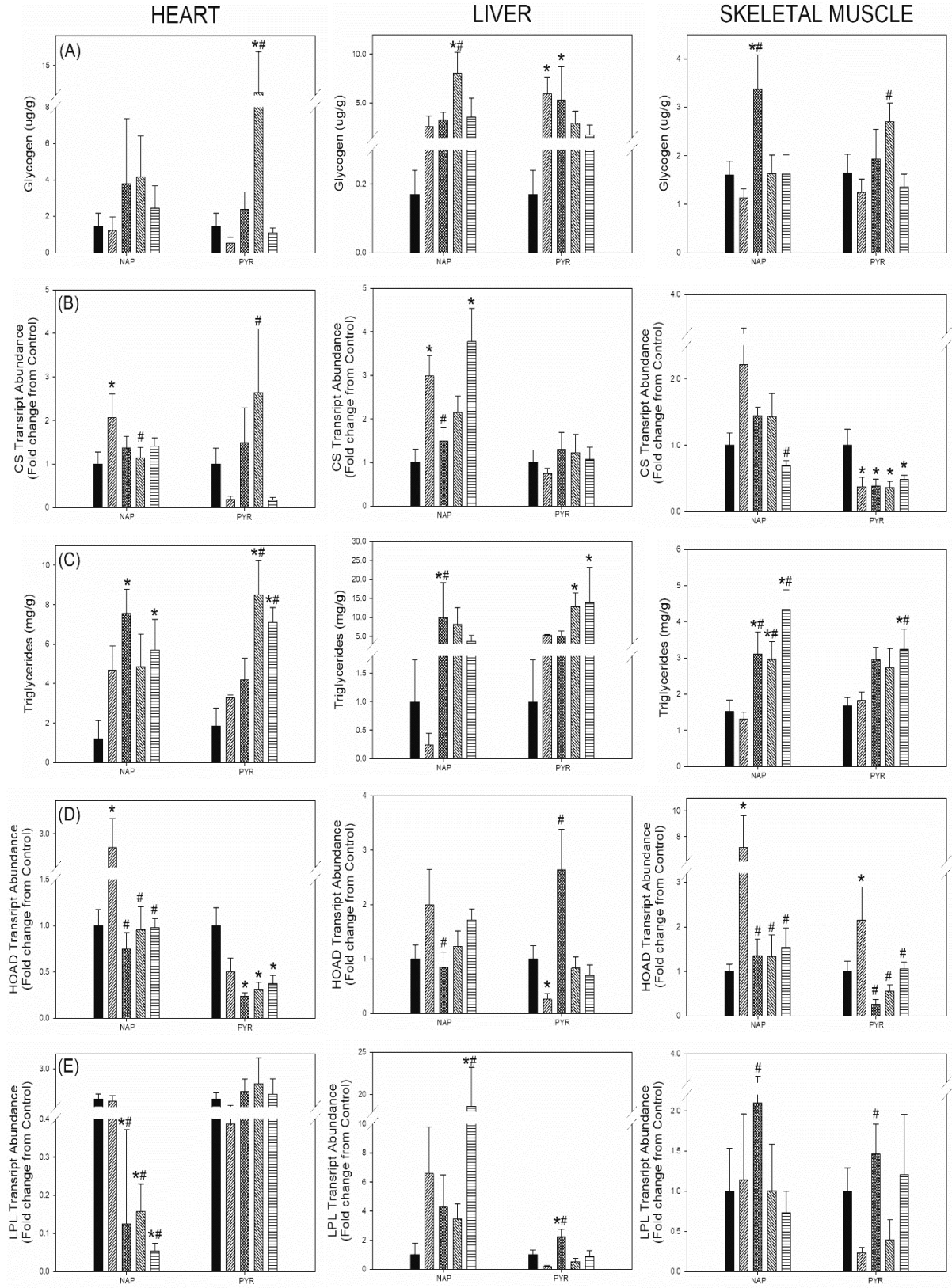


Figure 3. 3: Glycogen (A) and triglyceride (C) tissue content as well as mRNA levels quantified using qRT-PCR (expressed as fold change relative to control) of citrate synthase (CS; B) β -hydroxyacyl coenzyme A dehydrogenase (HOAD; D), and lipoprotein lipase (LPL; E) in heart, liver skeletal muscle after aqueous exposure to solvent control, naphthalene (left; 370 μ g/L) or pyrene (right; 2.5 μ g/L) alone, or with CH-223191(3334 μ g/L), fluoranthene (500 μ g/L) or tempol (172 μ g/L) for 48 hours. Data are mean \pm SEM of n = 4-5 pooled samples of heart or liver/group, n = 14-16 skeletal muscle samples/group. * = p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding solvent control group, # = p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding PAH alone group.

3.3.4 Gene Expression of CYP1A and antioxidant enzymes

In heart, CYP1A inhibition was able to reverse the PYR-mediated decrease in CS mRNA levels, while no inhibitors were able to reverse this PYR-mediated decrease in CS in skeletal muscle (Figure 3.4). The opposite was true for PYR-mediated changes in HOAD mRNA level, where all three inhibitors were without effect in the heart, but yet all three inhibitors were able to reverse the PYR-mediated increase in skeletal muscle HOAD transcript abundance and AhR inhibition was able to reverse the PYR-mediate decrease in liver HOAD. LPL mRNA levels were not significantly altered by either NAP or PYR exposure alone, but adding an AhR antagonist to PYR exposure in liver and skeletal muscle caused significant increases in lipoprotein lipase (LPL) transcript abundance compared to PYR alone (Figure 3.4). In contrast, all three antagonists when combined with NAP in heart caused significant decreases in LPL mRNA levels. Similar to what was observed in previous studies (Chapter 2 of this thesis), NAP alone caused increased CYP1A transcript abundance in several tissues, while PYR alone instead decreased CYP1A mRNA levels. However, the three inhibitors reversed the NAP-mediate CYP1A increases only in skeletal muscle, while CYP1A inhibition instead potentiated CYP1A transcript abundance with NAP in the heart. Moreover, all inhibitors were without effect on PYR-mediated decreases in CYP1A in the

heart, but instead reversed the PYR-mediated decrease in CYP1A in liver. Finally, NAP alone increased GSR and/or SOD in heart and skeletal muscle, with no clear pattern of effect of the three inhibitors.

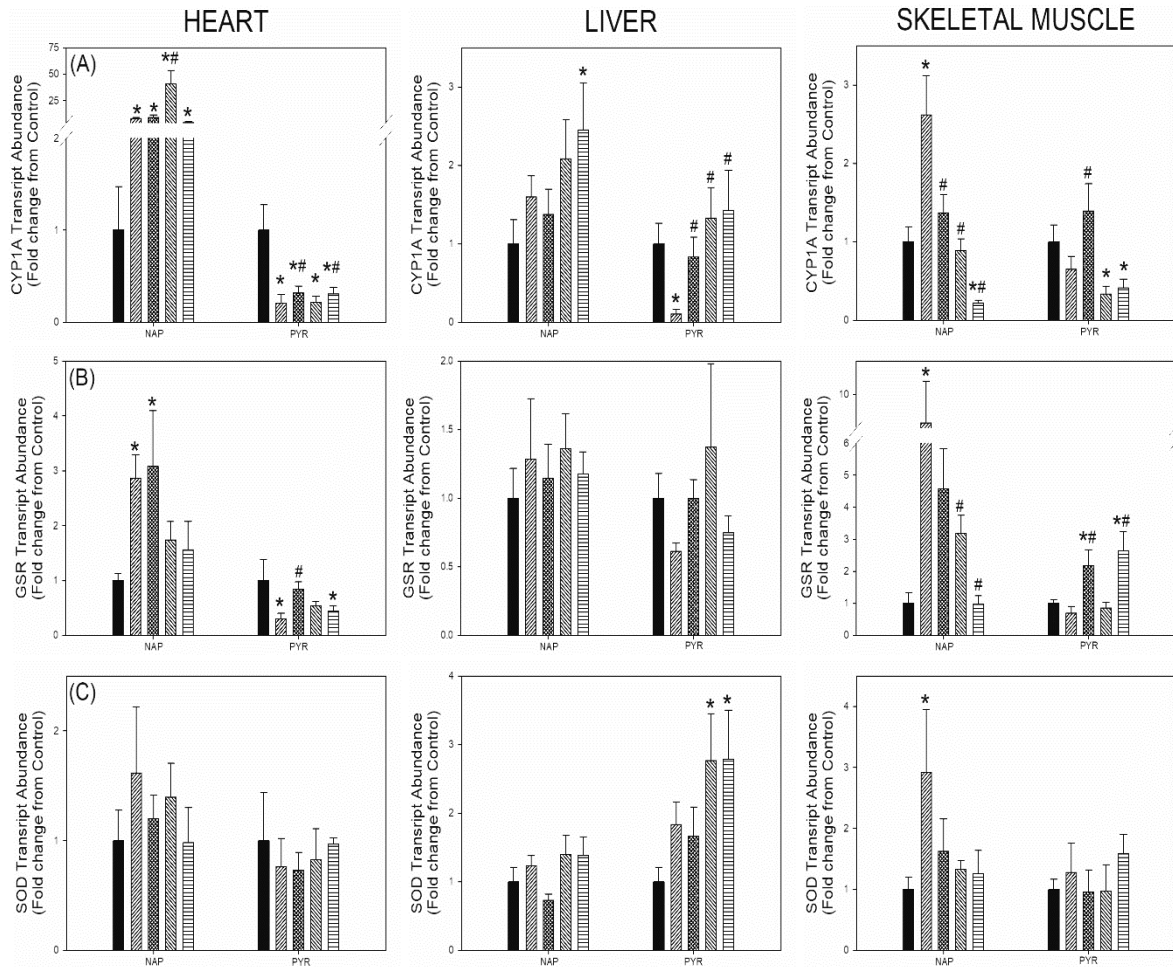


Figure 3. 4: mRNA levels of cytochrome P450 1A (CYP1A; A), glutathione-S-reductase (GSR; B) and superoxide dismutase (SOD; C) in adult zebrafish heart, liver and skeletal muscle following aqueous exposure to solvent control, naphthalene (left; 370 μ g/L) or pyrene (right; 2.5 μ g/L) alone, or with CH-223191(3333.9 μ g/L), fluoranthene (500 μ g/L) or tempol (172.2 μ g/L) for 48 hours. Data are mean \pm SEM of n = 4-5 pooled samples of heart or liver/group, n = 14-16 skeletal muscle samples/group. * = p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding solvent control group, # = p < 0.05 in Fisher's LSD after one-way ANOVA compared to the corresponding PAH alone group.

3.3.5 Factor Analyses

A limitation of the study design analyzed using ANOVA analyses after acute NAP and PYR exposure in adult zebrafish, alone and with inhibitors, is low power to detect differences among treatments due to the large number of treatment groups. This likely led to a high Type II error rate and false negatives. In order to explore the data generated in this study in a different way, principal components analysis was conducted. The first step in the type of factor analysis is regression of each variable against all others. Initial analyses using all endpoints measured in this study were narrowed from 36 endpoints to include only those endpoints (17 endpoints in the final analysis) that were highly correlated to the top two factors to explain 71.69% of the variance (see Table 3.2 for the component loadings). Key regression relationships stemming from the first component show that as muscle CYP1A mRNA levels increase, so does HOAD, GSR and SOD in both skeletal muscle and cardiac tissue (Figure 3.5). All of these variables were also positively related to cardiac AV ratio (Figure 3.5). In contrast, component 2 was more strongly related to cardiac ejection fraction and heart rate (both atrial and ventricular), with these variables showing strong positive correlations to cardiac and muscle energy stores (triglycerides and glycogen), as well as liver CYP1A levels (Figure 3.6). Finally, the sum of total scores for each of the top 17 variables were used to calculate scores for each component and the two scores plotted against each other for each treatment group (Figure 3.7). This plot revealed that all PYR groups clustered to the left, with the NAP plus inhibitor and control groups clustered near these points (Figure 3.7). Of note, the PYR alone group clustered most closely to the control groups, while the NAP alone group was quite distinct from any of the other treatment groups, located to the far right of the graph (Figure 3.7).

Table 3. 3. Principal components analysis of cardiac, metabolic, antioxidant and AhR-related endpoints measured in heart, liver and skeletal muscle after 48 h exposure in adult zebrafish to vehicle control, naphthalene (NAP) or pyrene (PYR), alone or in combination with an AhR antagonist (CH223191), CYP1A inhibitor (fluoranthene) or an antioxidant (Tempol). The top 17 variables explaining the greatest variance were included in the final analysis and are listed in descending order along with corresponding component loadings. Component loadings represent the strength of correlation of the endpoint to each component. Furthermore, relationships of the variables to each other can be inferred through their respective relationships to each component.

	Component 1	Component 2
Total variance explained	44.90%	26.48%
Variable	Component loadings	
Muscle GSR	0.950	0.014
Muscle SOD	0.930	-0.041
Muscle CS	0.930	-0.090
Cardiac HOAD	0.923	-0.176
Cardiac SOD	0.917	0.095
Muscle HOAD	0.860	-0.203
Cardiac GSR	0.830	0.149
Muscle CYP1A	0.816	-0.368
AV ratio	0.677	0.591
Liver CS	0.587	0.514
Ejection fraction	0.117	0.797
Cardiac TG	0.048	0.788
Muscle TG	-0.267	0.744
Liver CYP1A	0.414	0.710
Cardiac glycogen	-0.234	0.706
Atrial rate	-0.073	0.690
Ventricular rate	-0.301	0.592

AhR = aryl hydrocarbon receptor; GSR = glutathione-S-reductase; SOD = superoxide dismutase; CS = citrate synthase; HOAD = 3-hydroxacyl-CoA dehydrogenase; CYP1A = cytochrome P450 1A; AV ratio = ratio of atrial to ventricular contractile rates; CS = citrate synthase; TG = triglycerides.

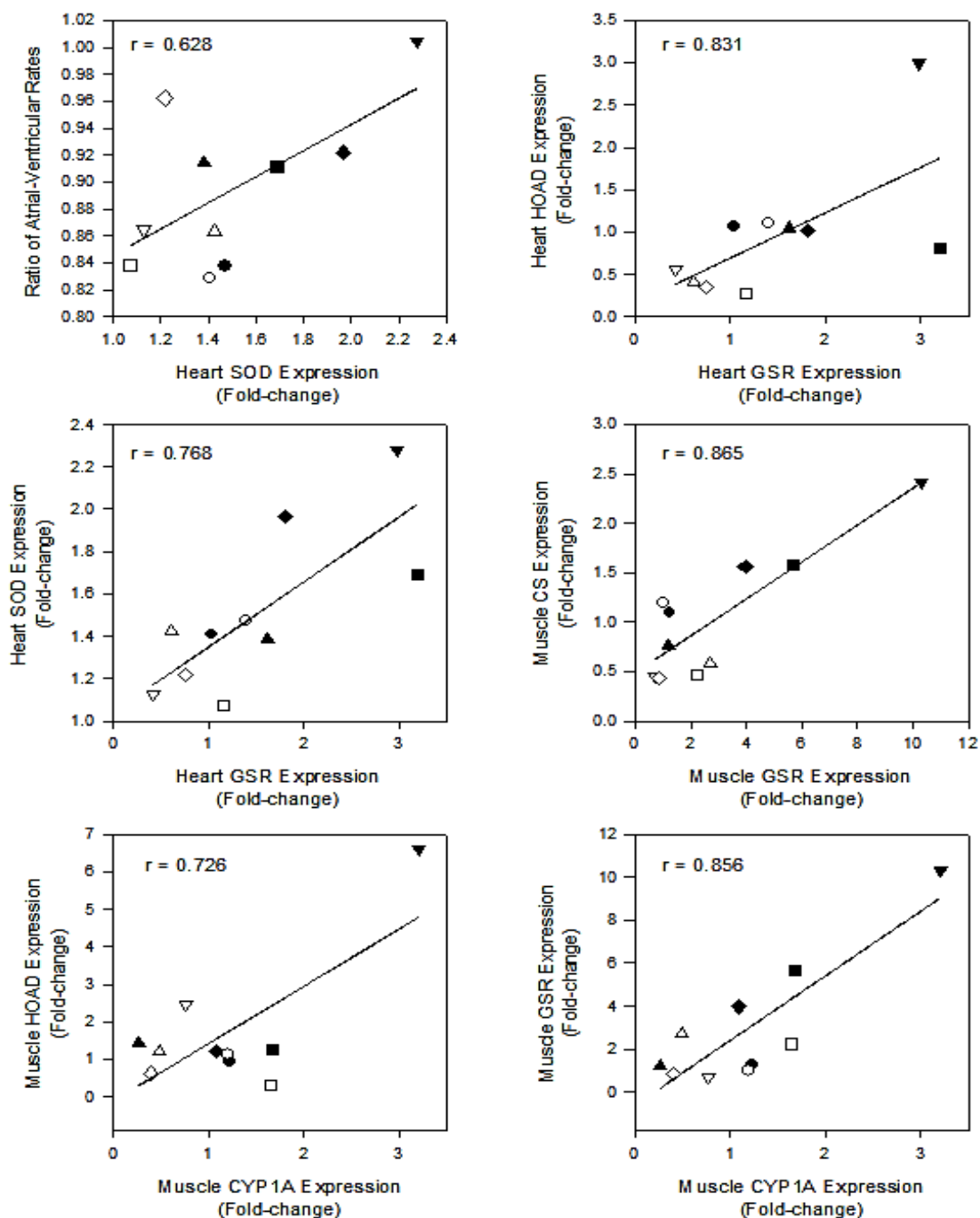


Figure 3. 5: Linear regressions ($p < 0.05$ for all relationships shown) for variables that were strongly related to component 1 of the Principal Components Analysis conducted in this study. Cardiac, metabolic, antioxidant and AhR-related endpoints were measured in heart, liver and skeletal muscle after 48 h exposure in adult zebrafish to vehicle control (circles), naphthalene (NAP, black symbols) or pyrene (PYR, white symbols), alone (inverted triangle) or in combination with an AhR antagonist (CH223191, squares), CYP1A inhibitor (fluoranthene, diamonds) or an antioxidant (Tempol, triangle). Mean values for each treatment are shown as individual points.

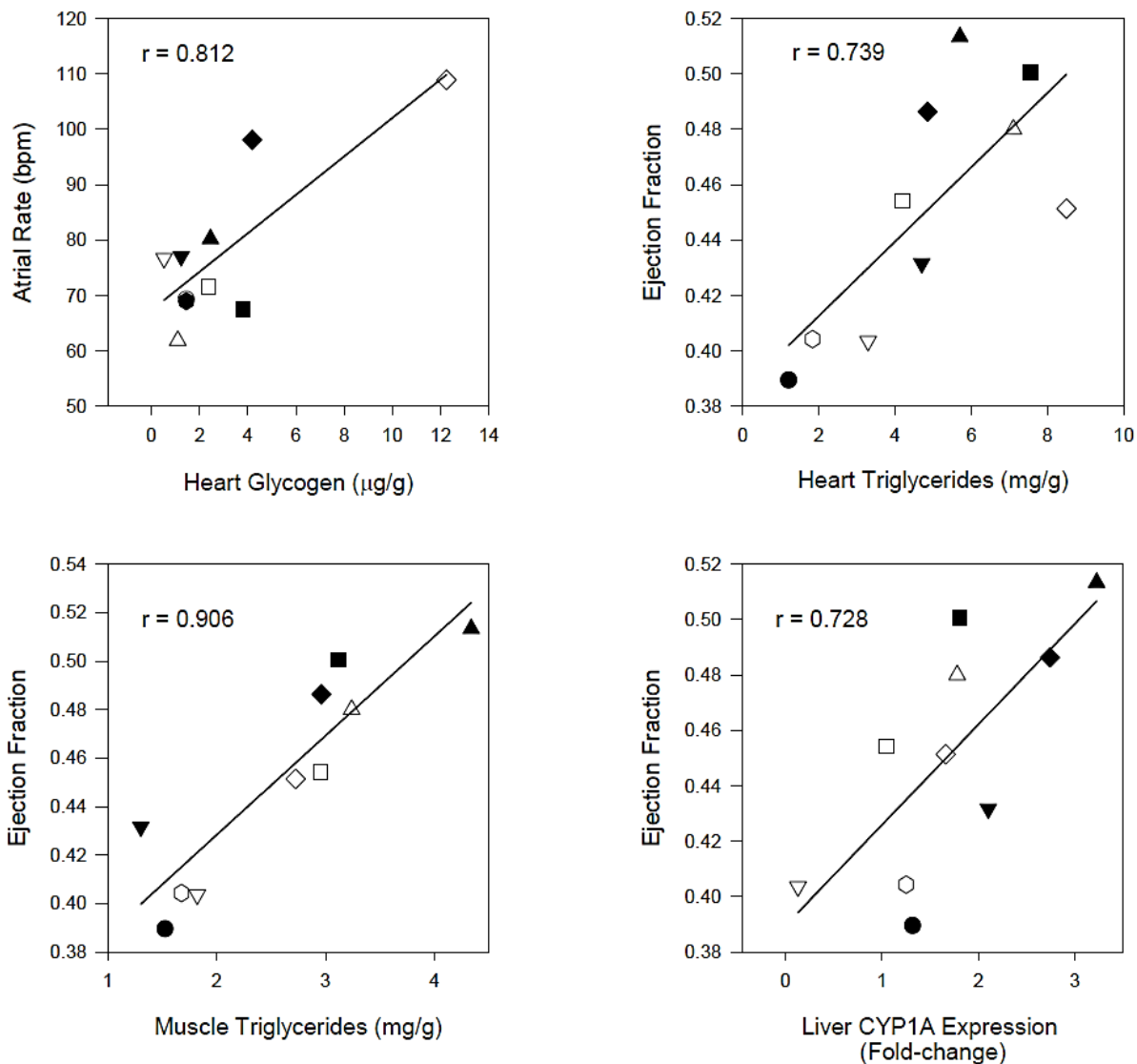


Figure 3. 6: Linear regressions ($p < 0.05$ for all relationships shown) for variables that were strongly related to component 2 of the Principal Components Analysis conducted in this study. Cardiac, metabolic, antioxidant and AhR-related endpoints were measured in heart, liver and skeletal muscle after 48 h exposure in adult zebrafish to vehicle control (circles), naphthalene (NAP, black symbols) or pyrene (PYR, white symbols), alone (inverted triangle) or in combination with an AhR antagonist (CH223191, squares), CYP1A inhibitor (floranthene, diamonds) or an antioxidant (Tempol, triangle). Mean values for each treatment are shown as individual points.

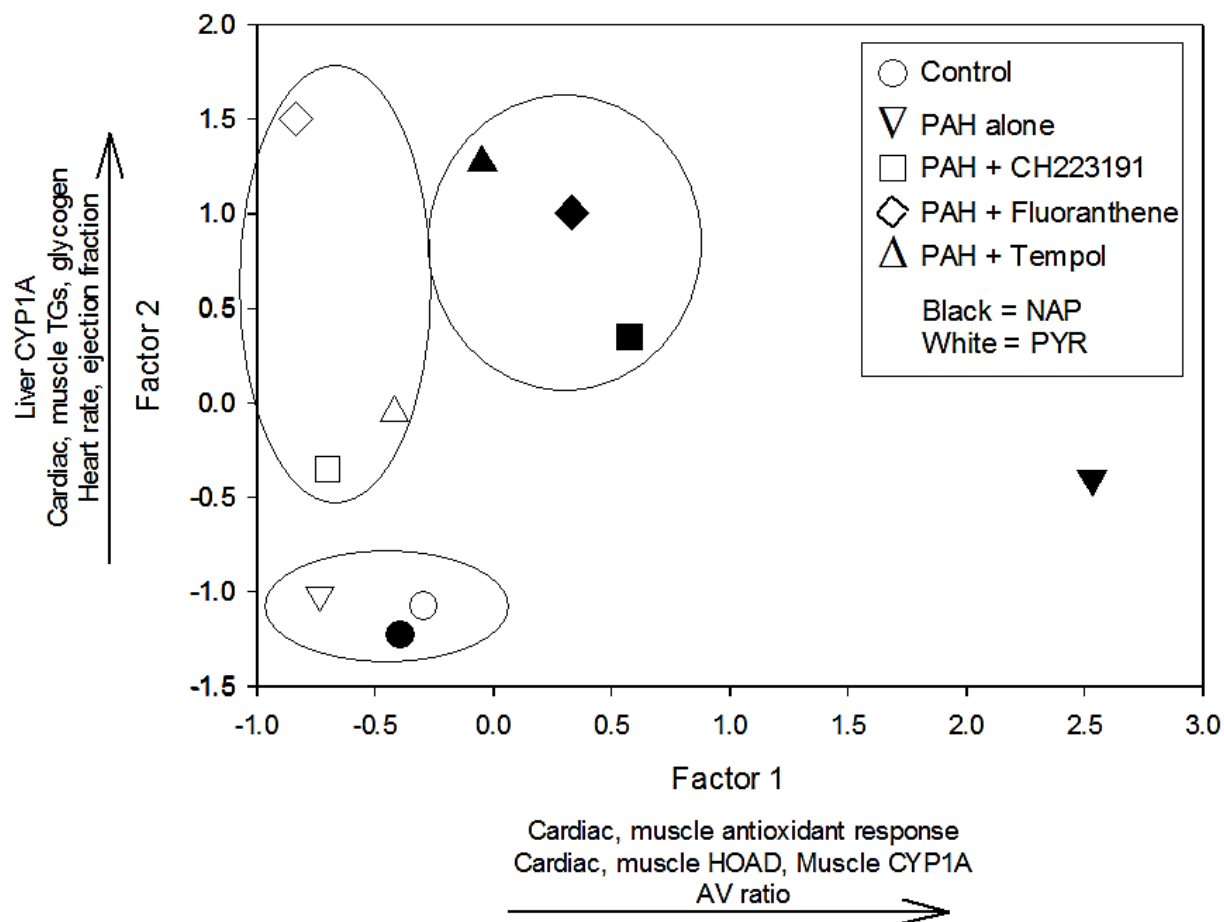


Figure 3. 7: A plot of factor scores for components 1 versus 2 for each of the treatment groups in this study from the Principal Components Analysis. Cardiac, metabolic, antioxidant and AhR-related endpoints were measured in heart, liver and skeletal muscle after 48 h exposure in adult zebrafish to vehicle control, naphthalene (NAP) or pyrene (PYR), alone or in combination with an AhR antagonist (CH223191), CYP1A inhibitor (fluoranthene) or an antioxidant (Tempol).

3.4 Discussion

3.4.1 Summary of key findings

In this thesis chapter, I evaluated the cardiovascular and metabolic effects of acute aqueous exposure NAP and PYR to adult zebrafish as well as examining markers of two key pathways for toxicity, CYP1A and antioxidant enzyme induction. This was the first study, to our knowledge, to examine the effects of acute NAP and PYR co-exposure with three pharmacological antagonists, on important cardiovascular endpoints and metabolism. In PCA, the first component was the one that best distinguished NAP and PYR alone groups from controls. This component was positively correlated with GSR, SOD and HOAD in cardiac and skeletal muscle as well as muscle CYP1A and higher ratio of atrial-ventricular rates, but NAP was a clear outlier in strongly increasing all of these endpoints in component 1. The second component distinguished among groups treated with the three inhibitors, being positively correlated to liver CYP1A, heart rate, ejection fraction and higher energy stores in cardiac and muscle tissues. Taken together, both NAP and PYR alone appear to activate a defense mechanism through arrhythmia associated with oxidative stress, while AhR inhibition, CYP1A inhibition and free radical scavenging all were associated with improved cardiac function and higher energy stores.

3.4.2 Cardiovascular function

The lack of effect of NAP and PYR at the exposure concentrations used agrees well with findings from the previous study (Chapter 2 of this thesis). What was surprising was the large magnitude of effect to stimulate heart rate and contractility (ejection fraction) when an AhR antagonist, CYP1A inhibitor and/or free radical scavenger were included in

exposures. Zebrafish embryos co-exposed to fluoranthene in combination with known weak AhR agonists phenanthrene, a three ringed PAH, increased pericardial edema (Brown et al., 2014), yet singular exposure had no significant difference from the solvent control. Zebrafish with CYP1A knockdown also had higher incidences of cardiac abnormalities (Billiard et al., 2006). A greater than additive cardiac effect was exhibited in zebrafish embryos when binary exposed to fluoranthene and α -naphthoflavone (Billiard et al., 2002, 2006; Van Tiem & Di Giulio, 2011) through the AhR mediated pathway. Adding a CYP1A inhibitor could significantly slow down AhR agonists from being metabolized. This would result in the parent compound having an extended half-life, therefore increasing toxicity (Billiard et al., 2006; Matson et al., 2008; Wassenberg & Di Giulio, 2004). These findings along with finding in this study suggest that CYP1A metabolism has protective properties from cardiac defense mechanism associated with exposure to a weak AhR ligand.

Conversely, PYR co-exposure with tempol had the most significant effects on zebrafish cardiovascular function, relative to the solvent and PAH control in the current study. Tempol, a known scavenger for superoxide anions (Chatterjee et al., 2000; Laight et al., 1997) and “SOD mimetic” (Chatterjee et al., 2000; Krishna et al., 1996) has been successfully used to reduce oxidative stress-mediated renal injury and dysfunction (Chatterjee et al., 2000), as well as reduce myocardial damage caused by acute pancreatitis in rats (Marciniak et al., 2016). Despite the evidence that tempol has protective effects in rats, when co-exposed with NAP and PYR in zebrafish, cardiovascular function instead appeared to be stimulated, based on increased heart rate and ejection fraction. In cardiac tissue, endogenous ROS is now known to contribute to normal cell function (i.e. cell differentiation, migration, adhesion, senescence, growth and apoptosis (Chen et al., 2012).

Endogenous, normal ROS is likely produced by constitutive activity of NADPH oxidase 4 (NOX4), a highly expressed enzyme in cardiovascular tissues. However, oxidative stress will arise when generation exceeds endogenous antioxidant capacity (Sack et al., 2017). The endogenous source of ROS production is insensitive to Hsp90 inhibitors and is regulated in response to a wide range of stimuli, including various cardiovascular diseases (Chen et al., 2012). Instead of having a protective affect against oxidative stress induced by the PAHs, tempol could be compromising the function of NOX4 in the cardiovascular system by scavenging for the endogenous ROS.

Co-exposure with AhR antagonist, CH223191, had the least significant effects on cardiovascular function compared to the other antagonists. This does not correlate with the findings of Incardona et al. (2004), who reported that PYR toxicity to zebrafish embryos was similar enough to TCDD and that it must act through the AhR pathway. The difference in findings is likely due to the early life stage and teratogenic effects of PYR versus adult in the current study. Based on observed changes with the AhR antagonist in the current study, it strongly suggests that AhR has a role in normal heart function. This is supported by that fact that AhR mRNA is highly expressed in the heart (Dolwick et al., 1993). Others have reported an endogenous signal of the AhR pathway that plays a crucial role in cardiovascular development and function (Zhang, 2011). AhR knockout mice demonstrate cardiac hypertrophy, leading to significant reduction in systolic and diastolic aortic pressure, cardiac output and stroke volume (Vasquez et al., 2003; Zhang, 2011), further supporting a physiological role for AhR in the cardiovascular system, in the absence of toxicants or other exogenous ligands.

3.4.3 Energy stores

In fish, their primary source of energy is free fatty acids (Salmerón, 2018). The strong positive relationship between increased antioxidant enzyme expression (GSR and SOD) with HOAD mRNA levels suggests that genes associated with the antioxidant response may also be needed for lipid mobilization in fish. Yang et al. (2006) found a positive correlation between total antioxidant capacity and LPL activity in rats fed a high fat diet. While LPL was largely unaffected by any treatment and did not appear as an important factor in PCA in the current study, HOAD transcript abundance in cardiac and muscle was highly affected, particularly by NAP, not PYR. However, PYR was previously reported to increase blood glucose and triglycerides in the common carp, suggesting PYR can act similar to NAP at a suitable dose or in different fish species (Shirdel et al., 2016). This relationship between lipid and glycogen metabolism with ROS needs to be further explored to see if NOX or other enzymes controlling ROS directly regulate energy metabolism in fish or are merely correlated.

HOAD is a key mitochondrial enzyme involved in β -oxidation of fatty acids (Rajotte & Couture, 2002). In our present study, there is a high correlation between NAP co-exposure and pharmacological antagonists increasing HOAD mRNA levels, muscular CYP1A mRNA levels and the antioxidant response in the heart and muscle. Further examination of the PCA analysis revealed that adding the three inhibitors with NAP, the three treatment groups clustered together closer towards control treatments relative to NAP alone treatment (Figure 3.7). If HOAD mRNA translated into HOAD protein, we would have also expected proportional decrease in triglyceride content. However, triglyceride content either remained unchanged or increased and was not considered in component one. Therefore, it appears

that lipid metabolism (i.e. HOAD) is likely influenced by ROS and the antioxidant response. Previous studies have shown that negative regulators of NOX expression, the peroxisome proliferator-activated receptor (PPAR) family, play a critical role in the expression of genes involved in energy homeostasis, including triglyceride metabolism, fatty acid handling and storage (Plutzky, 2011). PPARs are also directly involved with inflammatory and vascular response in endothelial and vascular smooth muscle cells, processes that also involve ROS production (Manea et al., 2015). Thus, it is possible that the pharmacological antagonists along with NAP exposure could be disrupting fatty acid metabolism by targeting PPARs and should be explored in future experiments.

3.4.4 Gene expression of CYP1A, key metabolizing and antioxidant enzymes

Activation of the AhR activation is often assumed to be indicated by the upregulation of CYP1A since its magnitude increase tends to be largest after AhR agonist exposure among liver enzymes during the detoxification response (Denison & Nagy, 2003; Marit & Weber, 2012; Prasch et al., 2003). The known AhR antagonist, BaP causes a large induction in EROD activity, a biomarker for chemical exposure (Whyte et al., 2000) as well as increased CYP1A1 gene expression in an AhR-dependent manner (Shimada & Fujii-Kuriyama, 2004). BaP has also been previously reported to cause significant induction of CYP1A mRNA in zebrafish at all life stages (Bugiak & Weber, 2009; Fang et al., 2015; Gerger & Weber, 2015). However, NAP has been reported to have no effect on EROD activity or CYP1A1/2 expression in mammals (Sandoz et al., 1998). AhR-regulated enzymes also did not contribute significantly to NAP bioactivation in rodents, but the possibility could not be completely ruled out (Genter et al., 2006). Similar to what was found in our previous study (Chapter 2 of this thesis), NAP alone increased CYP1A mRNA levels in heart and skeletal muscle, but not liver. Thus, NAP

may be antagonizing the AhR in the liver, but not the higher metabolic tissues in the current study. Another explanation could be micro mRNA (miRNA), a class of non-coding RNA. miRNAs have been reported as important cytoplasmic regulator of gene expression (Catalanotto et al., 2016) and could potentially degrade CYP1A mRNA prior to translation. Since tissues were sampled after 48h exposure, the peak window of time mRNA transcript abundance could have been missed due to the time at which tissues were sampled at. Ulin et al., (2018) reported an interaction between various regulatory pathways, i.e. AhR1b antioxidant response system and nuclear factor erythroid-2 (nrf) response elements. Zebrafish embryos exposed to TCDD and AhR antagonists induced nrf transcripts, but these were significantly decreased upon knockdown of AHR1b (Ulin et al., 2018). NRF2 plays a significant role in regulating oxidative stress and is activated when the AhR is bound to xenobiotic response element (Ooi et al., 2018). Therefore, it appears nrf2 could be affected by NAP exposure, leading to increased CYP1A activation and activation of the antioxidant response (increased SOD and GSR mRNA levels). With or without the inhibitors, NAP exposure activates the antioxidant response, as indicated through the upregulation of SOD and GSR mRNA transcript abundance. The strong association of increased cardiac and muscular mRNA levels of GSR and SOD with skeletal muscle CYP1A agrees with previous studies reporting similar elevations in SOD and GSR using NAP-rich diluted bitumen and oil sand process-affected water (He et al., 2012; Madison et al., 2015). Santana et al. (2018) concluded that upregulation of antioxidant enzymes such as GST, SOD, glutathione peroxidase and GSR was common after most PAH exposures. However, this study did suggest that GSR also look promising as a biomarker, but inadequate studies examining GSR precluded a definitive conclusion (Santana et al., 2018). In our current study, both GSR and

SOD are highly associated with one another and have a strong relationship with each other. Therefore, we can conclude that both are good indicators of the antioxidant response.

PYR failed to induce CYP1A transcript abundance in the heart, liver and skeletal muscles, agreeing with our previous study (Chapter 2 of this thesis). However, when PYR was added with CH223191, CYP1A transcript abundance was immediately restored to control levels, an unexpected result for an inhibitor of the AhR pathway. Although PYR has been reported previously to activate AhR, a previous study in human microvascular endothelial and embryonic kidney cells reported a similar lack of change in CYP1A gene expression after PYR exposure, but a similar increase in CYP1A after pre-treatment with AhR antagonists (Brinchmann et al. 2018). Thus, interaction of AhR with weak PAH agonists in the presence of AhR or CYP1A inhibitors/other weak AhR agonists is more complex than previously thought and requires further experimentation to delineate the response pathway. PYR defence mechanism is highly associated with an upregulation in hepatic CYP1A mRNA levels (Figure 3.7), indicating that there is likely tissue specific activation, as reported in previous studies (Hendon et al., 2008; Incardona et al., 2005; 2004). However, it was surprising in the current study that PYR did not seem to activate the AhR (increased CYP1A mRNA levels) in cardiac and skeletal muscle. Previous studies have reported PYR to bind AhR, but only while AhR was in an antagonist, not agonist conformation through selective activation of AhR-nongenomic calcium signaling (Brinchmann et al., 2018). Similar to findings in our study, zebrafish embryos exposed to PYR failed to upregulate CYP1A gene expression (Zhang et al., 2012). Mammalian studies have shown PYR to increase EROD activity in the liver through increased CYP1A2, not CYP1A1, mRNA expression (Lee et al., 2007; Shimada & Fujii-Kuriyama, 2004). Therefore, tissue specific responses and CYP1A

isoform specific changes need to be clarified. Antioxidant parameters were investigated in this present study because oxidative stress might arise during the process of pyrene biotransformation (Honkanen et al., 2008; Oliveira et al., 2012). Goldfish aqueously exposed to PYR for 21d determined the mechanism of PYR toxicity originated from activation of redox cycling due to ROS production and oxidative stress (Yin et al., 2014). PYR co-exposed with CH-223191 and fluoranthene restored changes in GSR levels to those observed with PYR alone in heart and skeletal muscle. In contrast, both fluoranthene or tempol resulted in elevated GSR levels compared to solvent control, thereby increasing PYR defense mechanism. Thus, it appears that high metabolic demand tissues (i.e. heart and skeletal muscle) are more sensitive to changes in AhR-mediated pathways (Incardona et al., 2006). In contrast, the major detoxifying organ, liver, is more sensitive to ROS (Lee et al., 2007; Yin et al., 2014).

3.5 Conclusion

PAHs are pervasive contaminants whose toxicity to aquatic species is due to more than just BaP. While BaP is the representative PAH whose chemistry and mechanism of action are well known, we now know it differs from other important petrogenic PAHs, such as NAP and PYR. Previous studies from our lab suggest that NAP may indeed be an AhR agonist, resulting in induction of CYP1A mRNA levels. The most interesting finding in this study was that pharmacological antagonists of AhR, CYP1A and a free radical scavenging had effects of their own on cardiovascular function. This could indicate the need for AhR-mediated pathways and free radicals for normal cardiovascular function. In conclusion, both NAP and PYR defense mechanism does not resemble BaP and all three PAHs cause toxicity

through different mechanisms of actions. Separate from reversing either NAP or PYR effects, the pharmacological antagonists were not effective in determining mechanism of action. Instead, a complex interaction between AhR, CYP1A, oxidative stress was revealed that appears to regulate baseline metabolism and cardiovascular function. Clarification with further experimentation is required.

CHAPTER 4: GENERAL DISCUSSION

4.1 Major conclusions of research

The purpose of this thesis was to determine the cardiorespiratory and metabolic toxicity of NAP and PYR to adult zebrafish. From our findings, we determined that both NAP and PYR cardiac effects were compensated and did not change cardiac output or produce arrhythmia, suggesting these PAHs are less cardiotoxic to acutely exposed adult zebrafish compared at BaP. Exposures to NAP and PYR had no significant change on swimming endurance and aerobic scope. The lowest PYR concentration increased AMR, but did not alter aerobic scope or swimming endurance, which was consistent with NAP exposure. NAP and PYR exposure also increased glycogen and triglyceride levels in heart, liver and skeletal muscle, along with increased mRNA levels of CS and HOAD, rate-limiting enzymes in the citric acid cycle and fatty acid catabolism, respectively, in most of these same tissues. This suggests that acute exposures (48h) to NAP and PYR exposure in adult zebrafish inhibit the fish's ability to access energy stores, without affecting swimming, differing from BaP acute toxicity (Gerger & Weber, 2015). Surprisingly, NAP and not PYR increased CYP1A mRNA transcript abundance in the heart and skeletal muscle, indicating AhR activation. A similarity between NAP and PYR defence mechanism in fish is the upregulation of SOD and GSR, important antioxidant enzymes, upregulated in response to NAP and PYR. Both PAHs of interest cause an imbalance in pro-oxidant and endogenous ROS, resulting in cytotoxicity and increases cancer risk from oxidative stress (Nebert et al., 2004; Rengarajan et al., 2015). Although GSR expression has been reported to be a poor biomarker for the antioxidant

response (Santana et al., 2018), from our findings its clear that oxidative stress plays a role in NAP and PYR defence mechanism.

Due to its chemistry, NAP is assumed to have a weaker affinity to the AhR than PYR (Barron et al., 2004). The results of our second chapter determined NAP may indeed be an AhR agonist, resulting in induction of CYP1 mRNA levels. With the addition of pharmacological antagonists, blocking various points of the AhR dependent pathway, we attempted to determine NAP and PYR mechanism of action, however discovered that toxicity of these PAHs is more complex than a linear pathway. The pharmacological antagonists could be causing an effect of their own on cardiovascular function. This is evidence that normal cardiovascular function may involve the AhR-mediated pathway and free radicals (Zhang, 2011). NAP and PYR binary exposures with pharmacological inhibitors reversed HOAD levels in high metabolically demanding tissues. Separate from reversing either NAP or PYR effects, the pharmacological antagonists were not effective in determining mechanism of action. Principal components analysis (PCA) was performed to determine response patterns and relationships among the end-points (17 endpoints in the final analysis). PCA revealed highly correlated to the top two factors to explain 71.69% of the variance. The first component was the one that best distinguished NAP and PYR alone groups from controls showed that as muscle CYP1A mRNA transcript abundance increases, so does HOAD, GSR and SOD in both skeletal muscle and cardiac. All of these variables were also positively related to cardiac AV ratio. In contrast, the second component distinguished among groups treated with the three inhibitors, strongly related to cardiac ejection fraction, atrial and ventricular heart rate, with these variables showing strong positive correlations to cardiac and muscle energy stores (triglycerides and glycogen), as well as liver CYP1A mRNA levels.

Although we were unable to determine the defence mechanism of NAP and PYR in adult zebrafish, we were able to support the complex interaction between normal cardiac and metabolic control with oxidative stress and AhR signalling. NAP toxicity is heavily associated with CYP1A mRNA induction in skeletal muscles, resulting in greater HOAD, GSR, SOD mRNA levels in highly metabolic tissues. This associates with a positive relationship with AV-ratio. Whereas PYR toxicity affects ejection fraction and heart rate, increasing energy stores in skeletal muscles and heart, as well as hepatic CYP1A mRNA levels. In conclusion, acute NAP and PYR exerted cardiorespiratory toxicity, metabolic alterations and mechanisms activated had some similarities with BaP, but BaP is clearly a stronger AhR agonist and much more toxic than either NAP or PYR.

4.2 Strengths and limitations of research

The present study successfully evaluated the acute cardiorespiratory and metabolic acute toxicity of NAP and PYR to adult zebrafish. This work has been the first to characterize the effects of NAP and PYR exposure on adult zebrafish cardiac function *in vivo* using high frequency ultrasound techniques, coupled with swim tunnel respirometry from a toxicological perspective. These techniques coupled with changes of rate limiting enzymes (CS and HOAD), antioxidant response (GSR and SOD) and CYP1A mRNA levels using qRT-PCR in three different tissue types.

One limitation of this study that could have improved the present work was the body burden analysis. We did not evaluate potential metabolites in addition to parent compound. Due to the rapid transformation into more hydrophilic metabolites for excretion, aqueous exposure to NAP and PYR resulted in only trace amounts of the parent compound detected

after 48 h aqueous exposures (renewal at 24 h) in adult zebrafish. Determining NAP and PYR metabolites would have aided in quantifying the biotransformation of the PAHs of interest undergoing in adult zebrafish. Water sample should have also been taken to analyze the concentration of NAP and PYR present in the water, and to quantify the amount actually taken up by zebrafish.

In measuring changes in key metabolizing enzymes, instead of using CS, glycogen phosphorylase, a rate limiting enzyme in glycogen metabolism (Agius, 2015), would have been a better indicator since it is specific to aerobic metabolism compared to CS. In our study we used one of the most commonly used housekeeping gene, G3PDH. Unfortunately, G3PDH also plays an important a role in glycolysis, therefore it may not have been the best choice when determining treatments effects on aerobic metabolism. Instead, multiple housekeeping genes should have been used.

The concentrations that we had exposed our fish to were within the range of what is found in the ambient environment or what is found in crude oil. Although environmentally relevant, the doses were not high enough to alter swimming performance and oxygen consumption. It was apparent from our first study, that the highest PYR concentration (25µg/L) reduced AMR, without altering any other endpoints. In order to get a more accurate change on swimming performance, a more strenuous swim challenge, such as a repeated U_{crit} challenge with minimal recovery time in between would have been needed. However, the concentrations at which we exposed our fish to were enough to initiate changes in energy stores and antioxidant response.

Unfortunately, in the second data chapter, we did not do singular exposures of each pharmacological antagonist. Therefore, unable to conclusively determine if the cardiovascular and metabolic changes were from the inhibitors or binary exposures. However, if we had done the singular exposures, this would have already lowered our power to detect any differences among treatment due to the large number of treatment groups. Our study design analyzed using ANOVA analyses after acute NAP and PYR exposure in adult zebrafish, alone and with inhibitors also lowers the power. Combined with the need to pool liver and heart samples for energy store analysis, our power was further lowered. Future experiments should use a higher number of samples. High Type II error and false negatives likely occurred. Fortunately, principal components analyse was able to explore the data generated in a different way.

4.3 Concluding statement

In conclusion, the present thesis shown that acute NAP and PYR exposure to adult zebrafish differs from each other and from BaP. Both NAP and PYR have some ability be to AhR as agonists. It is assumed that NAP affinity to the AhR is significantly less than PYR, however our research does not support this assumption. Crude oil is a complex mixture of toxicants but many studies have shown that PAHs are the most toxic component to aquatic organisms (Incardona, 2017). The largest fraction of PAHs in crude oil are two-ring NAPs, which have been reported to produce no developmental toxicity in zebrafish (Incardona et al., 2005; 2004) and are poor ligands to the AhR (Barron, et al., 2004). Majority of studies done on PAHs have been done on single compounds and on the most potent AhR ligands, generally being 4 rings or more. However, in our study, we have found that the volatile two-

ring PAH, NAP induced CYP1A mRNA levels and not PYR. Although we found no changes in swimming performance, extensive edema after high NAP exposure was noted during dissection in the current study, consistent with known edematous effects of NAP (USEPA, 2003). In the event of an oil spill, the highest concentration of NAP could potentially cause an entire fish population to undergo full body edema, resulting in a whole-body toxicity. Taken together, due to NAP high abundance and evidence for AhR-mediated toxicity, NAP found in crude oil should be a greater concern to the aquatic environment should be a greater concern.

Incardona et al., (2006) found that PYR produced developmental toxicity in zebrafish by activating the AhR and induce CYP1A expression. However, from our study, adult zebrafish toxicity to PYR differs. PYR failed to induce CYP1A in the heart and skeletal muscle, yet cardiotoxicity still occurred. Cardiovascular ultrasound of PYR exposed zebrafish had a lower contractile rate without changing AV ratio. Swim tunnel analysis revealed that 0.25 µg/L PYR exposure to adult zebrafish resulted in a significant increase in AMR. This indicates that more energy is needed to perform the same amount of exercise. If an oil spill were to occur, fish exposed to PYR would be at a disadvantage because more energy would be allocated in order to find prey or swimming away from predators. As for the population as a whole, less energy would be used for normal growth, function and reproduction, therefore lowering the entire fish populations success.

While the exact mechanism of toxicity was not explained by this study alone, it is clear that cardiovascular and metabolic homeostatic regulation is more complex than expected. The pharmacological antagonists used had cardiovascular effects on their own, separate

from reversing either NAP or PYR effects. PAH exposure cannot be simplified into a linear, singular pathway, often represented by the prototypical PAH, BaP. Future studies should be focused on determining the differences in mechanism of PAHs rather than using one PAH to represent them all.

4.4 Future direction

This study answered questions regarding NAP and PYR acute toxicity in adult zebrafish, but there are many questions yet to be answered. The exact mechanism of toxicity of NAP and PYR are still unknown. Although based on our PCA analysis, we were able to determine that NAP toxicity is more related to oxidative stress and AhR-mediated toxicity than PYR, we were not successful in determining the exact pathway of NAP and PYR toxicity. Previous studies have shown that embryonic PAH exposure in fish can have negative effects on cardiorespiratory effects in adulthood (Hicken et al., 2011). The chronic toxicity of NAP and PYR exposure on adult fish has yet to be researched.

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