COMPARISON OF THE ACUTE EFFECTS OF BENZO[A]PYRENE ON CARDIORESPIRATORY FUNCTION AND FITNESS IN ADULT ZEBRAFISH (*DANIO RERIO*) FOLLOWING I.P. INJECTION OR AQUEOUS EXPOSURE

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By

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants. There are numerous studies reporting developmental cardiac toxicity in multiple fish species due to PAH exposure. However, there are relatively few instances where the effects of acute PAH exposure in adult fish have been characterized. Furthermore, the majority of experiments comparing PAH toxicity with exposure route in adult fish focus on CYP1A gene expression or enzyme activity, while there is a lack of information about the possible pathophysiological effects. Therefore, the overall objective of this thesis was to characterize the sublethal effects of benzo[a]pyrene (BaP), a prototypical PAH, on adult zebrafish (*Danio rerio*) cardiorespiratory function and fitness following acute exposure by two different routes. In the first experiment, adult zebrafish were intraperitoneally (i.p.) injected twice (one injection/24 hr) with increasing concentrations of BaP (0.1, 10, and 1000 μg/kg) and compared to corresponding dimethylsulfoxide (DMSO) controls. In a second set of experiments, adult zebrafish were aqueously exposed to BaP (static, renewal at 24 hr; 16.2 and 162 μg/L) and compared to DMSO controls. Following 48 hr exposure, one group of fish (n=10/treatment group) were subjected to swimming performance tests to assess critical swimming speed (U_{crit}), oxygen consumption rate (MO_2), cost of transport (COT), standard metabolic rate (SMR), active metabolic rate (AMR), and factorial aerobic scope (F-AS). Another group of fish (n=12/treatment group) were subjected to echocardiography following 48 hr BaP exposure to evaluate cardiac function. Following echocardiography analysis, samples were collected for parent compound (BaP) body burden and CYP1A mRNA induction analysis.

48 hr BaP injection resulted in significant sublethal effects on adult zebrafish cardiorespiratory function. Oxygen consumption (MO_2) was increased at three swimming speeds

in injected BaP groups compared to control. In contrast, aqueously BaP-exposed fish showed increased MO_2 only at the single lowest swim speed. COT was also similarly increased for both exposure routes. SMR was elevated with both exposure routes, while AMR remained unchanged. This resulted in a significant decrease in F-AS for all treatment groups compared to corresponding controls with both exposure routes.

Cardiac function was significantly affected by both routes of BaP exposure. Ventricular heart rate was significantly decreased in BaP-exposed fish, both injected and aqueously-exposed. However, stroke volume was decreased only in fish aqueously exposed to BaP, which resulted in significantly reduced cardiac output with that exposure route. In contrast, the ratio of atrial to ventricular heart rate (AV ratio) was increased only in fish i.p. injected with BaP, indicating the possibility of cardiac arrhythmias occurring. Analysis of BaP body burdens in fish tissue allowed for identification of an overlapping dose group between exposure routes, through which comparisons of cardiotoxicity were then made. This comparison revealed slight differences in cardiotoxicity between exposure routes. BaP-injected fish suffered from more severe bradycardia than aqueously exposed fish. Furthermore, cytochrome P4501A (CYP1A) mRNA levels in liver and heart tissue showed more significant increases in injected fish, while skeletal muscle CYP1A was increased only following aqueous exposure.

In conclusion, acute BaP exposure caused metabolic alterations and impaired cardiorespiratory function in adult zebrafish regardless of exposure route. Interestingly, the primary mechanism behind these effects appeared to differ slightly with exposure route. These results suggest that acute BaP exposure may have negative effects on adult fish survivability in the environment. Overall, this work provides valuable insight into the pathophysiogical consequences of acute PAH exposure in adult stage fish.

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A_1 – area one
A_2 – area two
A_3 – area three
AHR – aryl hydrocarbon receptor
AHRR – aryl hydrocarbon receptor repressor
AIP – aryl hydrocarbon interacting protein
AMR – active metabolic rate
ANCOVA – analysis of covariance
ANOVA – analysis of variance
ARNT – aryl hydrocarbon receptor nuclear translocator
ASE – accelerated solvent extraction
AV – atrioventricular
BaP – benzo[a]pyrene
βAR – beta adrenergic receptor
BL – body length
bpm – beats per minute
°C – degrees Celsius
Ca^{2+} – calcium ion
cDNA – complementary deoxyribonucleic acid
cm – centimeter
COT – cost of transport
CSA – cross sectional area
ΔΔCT – change in threshold values; used in real-time reverse transcriptase PCR
CYP – cytochrome P450 monooxygenase
DMSO – dimethyl sulfoxide
d.m. – dry mass
DNA – deoxyribonucleic acid
dpf – days post-fertilization
ECG – electrocardiogram
EDV – end diastolic volume
ESV – end systolic volume
EROD – ethoxyresorufin-O-deethylase
F1 – first filial generation
F-AS – factorial aerobic scope
f_H – heart rate
g – gram
GI – gastrointestinal
GMF – glass microfiber membrane
G3PDH – glyceraldehyde-3-phosphate dehydrogenase
hr – hour
h – ventricular height
HLH – helix-loop-helix
HPLC-FD – high pressure liquid chromatography with fluorescence detection
HSP90 – heat shock protein 90

I_{Kr} – rectifier potassium current
i.m. – intramuscular
i.p. – intraperitoneal
i.v. – intravenous
J – joule
K – condition factor
K^+ – potassium ion
kg – kilogram
l – ventricular length
L – liter
LC50 – median lethal dose
LSD – least significant difference
m – meter
min – minute
mg – milligram
MHz – megahertz
ml – milliliter
mm – millimeter
mm^3 – millimeters cubed
MO_2 – oxygen consumption rate
mRNA – messenger ribonucleic acid
MS-222 – tricaine methanesulfonate
n – number of individuals in a sample (number of replicates)
NAD(P)H – nicotinamide adenine dinucleotide phosphate
NE – norepinephrine
nm – nanometer
NSERC – Natural Sciences and Engineering Research Council of Canada
O_2 – oxygen
p – p-value
PAH – polycyclic aromatic hydrocarbon
PAS – PER/ARNT/Sim
PCR – polymerase chain reaction
pg – pictogram
PLN – phospholamban
psi – pounds per square inch
Q – cardiac output
qPCR – quantitative real-time polymerase chain reaction
RMV – real-time micro visualization
ROS – reactive oxygen species
RT-PCR – real-time polymerase chain reaction
s – second
SA – sinoatrial
SEM – standard error of the mean
SERCA2a – sarcoplasmic reticulum calcium ATPase
SMR – standard metabolic rate
SR – sarcoplasmic reticulum

TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
T_i – time elapsed at the fatigue velocity in critical swimming speed test
T_{ii} – increment time length in critical swimming speed test
U_{crit} – critical swimming speed
µg – microgram
U_i – highest velocity maintained for an entire time interval in critical swimming speed test
U_{ii} – velocity increase per time increment in critical swimming speed test
µl – microliter
µm – micrometer
V – ventricular volume
V_S – stroke volume
VTI – velocity time integral
XRE – xenobiotic response element

CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 Polycyclic aromatic hydrocarbons

1.1.1 Structure and sources

Polycyclic aromatic hydrocarbons (PAHs) are a class of contaminants that are ubiquitous in terrestrial and aquatic environments. Structurally, PAHs consist of two or more fused benzene rings and as many as 100 different PAHs exist, often as complex mixtures in the environment (Mumtaz and George, 1995). Those formed during the combustion of organic matter are considered pyrogenic and typically include higher molecular weight PAHs consisting of four benzene rings or more. In contrast, lower molecular weight PAHs of three benzene rings or less are often present in petroleum sources and said to be petrogenic in nature (Stein et al., 2006). PAHs may be generated from natural sources such as forest fires and volcanoes, but it is anthropogenic sources that are thought to contribute to the majority of PAHs found in the environment today (Kimbrough and Dickhut, 2006). These anthropogenic sources include oil spills, refinery effluent, urban runoff, and automobile exhaust (Canadian Council of Ministers of the Environment, 1999). Of rising concern are the increasing concentrations of PAHs being found in the aquatic environment near areas of growing urban development (Kimbrough and Dickhut, 2006; Stein et al., 2006) which are causing fish to be a target species. Baseline levels of PAHs taken from various locations in the Atlantic Ocean have been reported to range from 32-1400 pg/L total PAHs (Lohmann et al., 2013), while levels of the toxic PAH, benzo[a]pyrene, have been reported as high as 1.872 µg/L near areas of urban development in China (Guo et al., 2007; He et al., 2011; Zhang et al., 2012). Other issues of serious concern are acute events such

as oil spills, where levels of total PAHs have reached 84.8 µg/L in surface water and 189 µg/L in subsurface water during the Deepwater Horizon oil spill (Diercks et al., 2010).

1.1.2 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) is a unique ligand-activated transcription factor belonging to a protein family within the helix-loop-helix (HLH) superfamily of proteins termed the PAS family (PER/ARNT/SIM) (Schmidt and Bradfield, 1996). The AHR is traditionally thought to function in mediating toxicological responses to a variety of environmental contaminants, including but not limited to PAHs, dioxins, and polychlorinated biphenyls. However, there is increasing evidence in mammals that the AHR may play basic biological roles, including in the cardiovascular and immune systems (McMillan and Bradfield, 2007). When inactive and unliganded, the AHR remains bound in the cytosol to heat shock protein 90 (HSP90) and an aryl hydrocarbon interacting protein (AIP) that is also known as hepatitis B virus X-associated protein in mammals (Petrulis and Perdew, 2002). Once it is ligand-bound, the AHR dissociates from its chaperone proteins and translocates into the nucleus where it dimerizes with the aryl hydrocarbon receptor nuclear translocator protein (ARNT). The AHR/ARNT heterodimer then binds to what are termed xenobiotic response elements (XRE) to regulate the transcription of target genes (Carney et al., 2004; See Figure 1.1 for schematic). Target genes of the AHR that are the most widely studied include phase one and two enzymes involved in detoxification processes. Enzymes whose expression are typically induced include cytochrome P450 (CYP) 1A1, CYP1A2, CYP1B1, glutathione S-transferase, NAD(P)H: quinone reductase 1 and 2, and class 3 aldehyde dehydrogenase (Schmidt and Bradfield, 1996; Nebert et al., 2004).

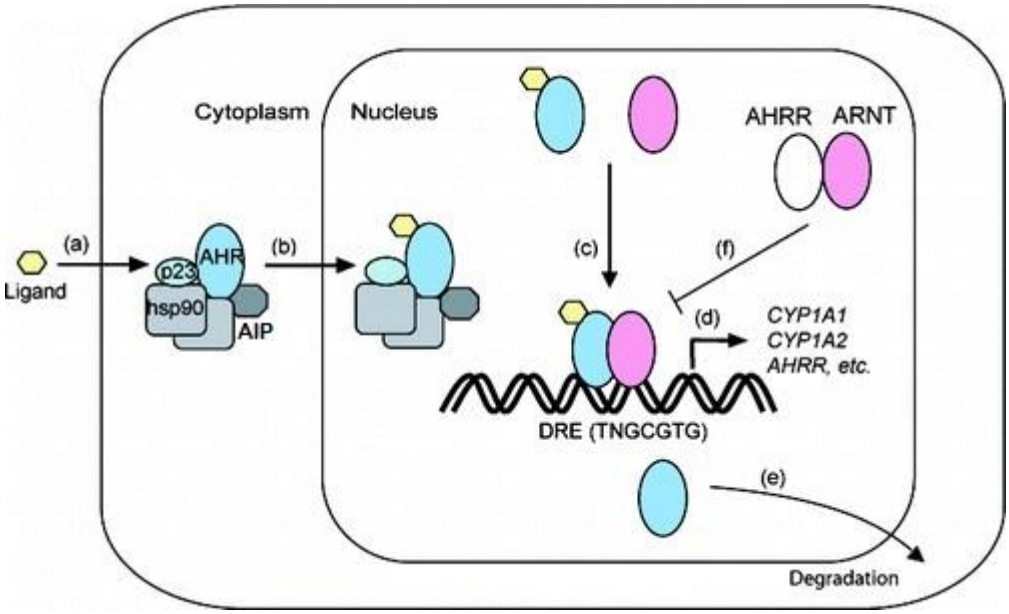


Figure 1.1: Model of the AHR signalling pathway leading to enzyme induction. AHR – aryl hydrocarbon receptor; AHRR – aryl hydrocarbon receptor repressor; AIP – aryl hydrocarbon interacting protein; ARNT – AHR nuclear translocator protein; CYP1A – cytochrome P450 monoxygenase; DRE – dioxin (xenobiotic) response element; HSP90 – heat shock protein 90 (Reprinted (adapted) with permission from (Nguyen and Bradfield, 2008). Copyright (2008) American Chemical Society)

While mammals possess a single AHR, it has been shown in fish that there are at least two AHRs, designated AHR1 and AHR2 (Karchner et al., 2005; Billiard et al., 2006). In zebrafish (*Danio rerio*), three AHRs have been identified, AHR1a, AHR1b, and AHR2 (Karchner et al., 2005). AHR2 (Andreason et al., 2002; Billiard et al., 2006) has been shown to have high affinity for dioxin and dioxin-like compounds, to be transcriptionally active, and to mediate toxic responses to dioxins. In contrast, AHR1a, thought to be similar in structure to the mammalian AHR, has been shown to have little to no binding affinity for dioxin and dioxin-like compounds. Instead it is thought to possibly play a functional role during development (Andreason et al., 2002). AHR1b has been shown to have high affinity for dioxin but not be inducible by dioxin, and so it is speculated to play a role in development similar to AHR1a (Karchner et al., 2005).

1.1.3 Mechanism of action

Toxicity induced by PAHs is thought to occur mainly through AHR-dependent mechanisms as mentioned in the previous section; however evidence also exists for AHR-independent mechanisms. In zebrafish embryos, it was shown that AHR2 knockdown resulted in protection against pericardial effusion following 4- and 5-ring PAH exposure (Van Tiem and Di Giulio, 2011), demonstrating the role of the AHR in developmental cardiac toxicity. However, it has also been reported that lower molecular weight PAHs may cause functional cardiac defects in developing fish embryos through mechanisms independent of the AHR (Incardona et al., 2004). In addition, AHR null mice exposed to PAHs still exhibited genotoxicity (Kandraganti et al., 2003). This was thought to be due to CYPs exerting at least some of their actions via AHR-independent pathways. In support of this, CYP1A1 and CYP1B1 have been reported to be

induced by PAHs in AHR-null knockout mice (Kerzee and Ramos, 2011; Nakatsuru et al., 2004).

Oxidative stress has been put forth as one of the possible mechanisms of PAH toxicity and at least some of this may be independent of AHR. Normal cellular function requires a delicate balance between pro- and anti-oxidant species. When this balance tips towards pro-oxidants, cells are no longer able to neutralize the excess production of reactive oxygen species (ROS) and this can lead to oxidative stress (Di Giulio and Meyer, 2008). PAHs may be metabolized by CYP enzymes to produce quinone metabolites that may undergo redox cycling to produce ROS (Penning et al., 1999). Additionally, PAHs may increase uncoupling of the mitochondrial electron transport chain as well as uncouple CYP redox reactions thereby producing excess ROS (Winston and Di Giulio, 1991). This excess ROS production can lead to acute cellular damage as well as DNA adduct formation and cancer (Miller and Ramos, 2001).

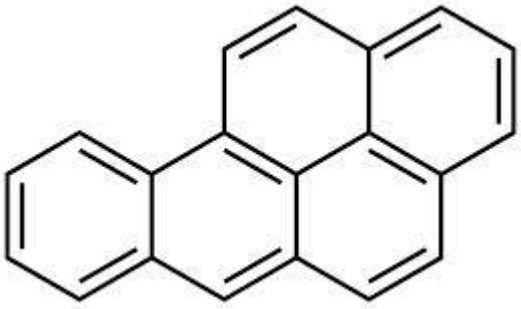


Figure 1.2: Chemical structure of the prototypical PAH, benzo[a]pyrene.

1.1.4 Benzo[a]pyrene

Benzo[a]pyrene (BaP) was chosen as the PAH of study for this thesis because it is a well-studied, toxic PAH that is widely encountered in the aquatic environment. It is classified as a

high molecular weight PAH due to its 5-ringed structure (Figure 1.2). In addition, BaP is a nonpolar, lipophilic compound with a water solubility of 1.62 µg/L at 28°C and a log K_{ow} of 6.04 (May et al., 1983; Canadian Council of Ministers of the Environment, 1999). Despite being highly lipophilic, BaP does not often accumulate to significant amounts in biota due to its rapid metabolism by CYP enzymes, namely CYP1A and CYP1B1 (Miller and Ramos, 2001; Billiard et al., 2002). Parent BaP is also a known agonist of the AHR. Induction of phase one and two enzymes via BaP-AHR binding results in a positive feedback loop, sustaining metabolism of BaP to toxic reactive intermediates (Miller and Ramos, 2001). These unstable BaP metabolites include quinones and epoxides which produce cytotoxic and mutagenic effects primarily through oxidative stress mechanisms and DNA adduct formation, respectively (Miller and Ramos, 2001). Due to these inherent characteristics, Environment Canada has set water quality guidelines for several PAHs, including BaP. The interim water quality guideline for BaP is 0.015 µg/L (Canadian Council of Ministers of the Environment, 1999).

1.2 Cardiac function

1.2.1 Fish cardiac structure and function

The fish circulatory system is a closed system, with blood flowing directly into the heart via the sinus venosus, through the atrium to the ventricle (Figure 1.3), where it is then directed through the bulbous arteriosus towards the gills for oxygenation (Hu et al., 2001). Unlike the mammalian heart, the fish heart consists of only two chambers, a single atrium and a single ventricle. In teleosts, the ventricle may be one of three shapes: saccular, tubular, or pyramidal (Sanchez-Quintana et al., 1995). Saccular and tubular ventricles are generally associated with less active, sedentary teleosts, while pyramidal ventricles are associated with more active,

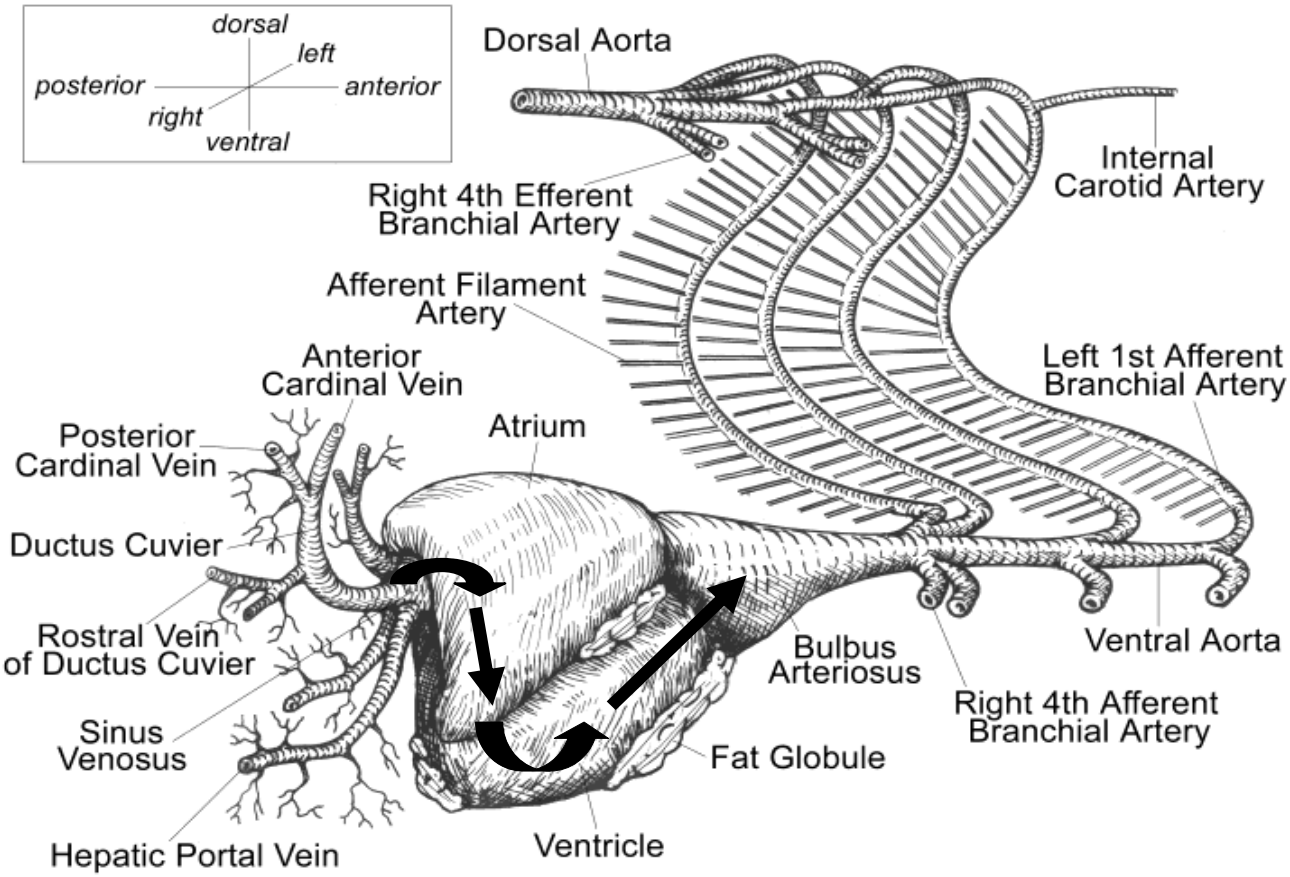


Figure 1.3: The zebrafish heart and major vasculature in the cardiac region. Arrows indicate direction of blood flow through the heart (Hu et al., 2001; reproduced with permission from John Wiley and Sons, Inc.).

pelagic species (Sanchez-Quintana et al., 1995), an example being the zebrafish. Pyramidal ventricles are also associated with what is called a mixed myocardium, consisting of one or more layers of compact tissue (compact layer) and an inner layer termed the spongy layer, while saccular and tubular ventricles consist only of a spongy (trabeculated) layer (Sanchez-Quintana et al., 1995). These two different myocardium types play a significant role in the heart's oxygen supply. Ventricles consisting of both compact and spongy layers receive a supply of oxygenated blood from the gills via the coronary artery in the compact layer, as well as oxygen from venous blood in the spongy layer. In the case of wholly spongy myocardium, the only oxygen supply to the heart is via deoxygenated venous blood (Olson and Farrell, 2006).

When attempting to quantify heart function in fish or any other species, there are a number of parameters that can be measured, including heart rate (f_H), stroke volume (V_S), and cardiac output (Q). Fish heart rate is controlled by the intrinsic rhythm of pacemaker cell depolarization and is also regulated by temperature and autonomic input. Stroke volume, which is the volume of blood ejected per heartbeat, is determined by arterial pressure (afterload), ventricular strength (contractility), and end diastolic volume (preload) (Olson and Farrell, 2006). Total cardiac output can then be calculated:

$$Q = f_H * V_S$$

Cardiac output is an important indicator of overall fish health and metabolism, but can be regulated in response to the needs of the fish by altering either heart rate or stroke volume. For example, during exercise it is possible for cardiac output to increase two- to three-fold (Olson and Farrell 2006). Also, a two-fold increase in cardiac output is possible postprandially compared to fish in the non-fed state (Eliason et al., 2008). The effects of temperature and

hypoxia on fish cardiac output have also been well documented. Heart rate is known to increase following acute temperature changes, generally doubling for a 10°C change (Blank et al., 2002; 2004). Temperature decreases result in bradycardia, but following cold acclimation heart rate may increase (Aho and Vornanen, 2001). Increases in temperature have also been shown to increase contractility thereby increasing ventricular strength and stroke volume (Bailey et al., 1990). Fish hearts have been shown to be relatively tolerant of moderate hypoxic conditions, but in severe hypoxia heart damage and failure can result (Overgaard et al., 2004). In general, moderate aquatic hypoxia results in reflex bradycardia due to increased inhibitory cholinergic tone (Olson and Farrell, 2006).

Fish cardiac function has been evaluated following a number of different kinds of treatments. In zebrafish, heart function was measured following ventricular amputation using micro-electrocardiograms to assess the effect of mechanical manipulation on the heart (Sun et al., 2009). Denvir et al. (2008) examined the effects of norepinephrine and MS-222 on cardiac function in zebrafish embryos, specifically looking at heart rate, myocardial wall velocity in systole and diastole, and wall motion amplitude. They found that norepinephrine significantly increased all of these parameters while MS-222 resulted in a reduction of each. With regard to environmental contaminant exposure and the effects on fish cardiac function and morphology, studies have focused almost exclusively on embryonic exposure scenarios. Toxicology studies assessing the effects of acute PAH exposure on adult fish cardiac function have never before been reported.

1.2.3 High frequency cardiac ultrasound in fish

Many attempts have been made to evaluate fish heart morphology and function using a variety of methods. Characterization of ventricular function in zebrafish embryos has been done using light microscopy followed by analysis using edge detection software in microscopic images (Denvir et al., 2008). However, optical methods such as this are only possible in the embryonic stage when zebrafish embryos are transparent. Therefore alternative methods are necessary when analyzing adult stage fish. Comprehensive analysis of adult zebrafish heart morphology has also been done using scanning electron microscopy and histological analysis (Hu et al., 2001). However, this analysis was done on fixed zebrafish hearts and provides no in vivo or functional information. Invasive methods involving surgical implantation of Doppler flow probes to evaluate blood flow through the ventral aorta have been done on a variety of fish species including rainbow trout (*Oncorhynchus mykiss*) (Gamperl et al., 2011; Keene and Gamperl, 2012) and 6 different species of sunfish (Cooke et al., 2010) thereby providing information on blood velocity, equivalent to cardiac output. Electrocardiograms (ECGs) have also been performed in adult zebrafish to assess cardiac function under normal conditions and in response to pharmacological agents (Milan et al., 2006). A non-invasive method to evaluate fish cardiac function that has proven useful and accurate is ultrasound technology. A standard ultrasound machine has been used to image the four components of the zebrafish heart and also blood velocity through the heart using colour Doppler (Ho et al., 2002). More recently, high frequency ultrasound has proven to be even more useful than standard ultrasound in evaluating zebrafish cardiac function (Sun et al., 2008). This is due to the ability to generate greater spatial resolution, giving clearer images of internal structures and more accurate estimations of blood flow.

1.3 Swim performance in fish

1.3.1 Determination of critical swimming speed

Swim performance testing provides an increasingly accepted method for quantifying the effects of sublethal toxicant exposure on fish (Mager and Grosell, 2011). Many essential fish behaviours depend on adequate swim performance including food acquisition, predator evasion, migration, and reproduction (Drucker, 1996). A toxicant affecting these behaviours could result in consequences at the population and ecosystem level (Sprague, 1971; Plaut, 2001). Many methods for evaluating swim performance have been introduced, the most common being exercise endurance tests using critical swimming speed or U_{crit} (Brett, 1964). It has been suggested that when attempting to examine the sublethal effects of toxicants, U_{crit} may be more useful than traditional EC_{50} values (concentration of a toxicant causing effects in 50% of a test population). This is because U_{crit} allows for quantification of sublethal effects that may influence a population rather than only considering mortality (Hammer, 1995). In order to determine U_{crit}, fish are placed in a swim tunnel and forced to swim against water velocities that are increased in stepwise increments (0.077 m/s (U_{ii}) every 20 minutes (T_{ii}) for this thesis project) until exhaustion. U_{crit} is then calculated from the following equation (Brett, 1964):

$$U_{crit} = U_i + [U_{ii}(T_i/T_{ii})]$$

where U_i is the highest velocity maintained for an entire time interval and T_i is the time elapsed at the fatigued velocity.

1.3.2 Oxygen consumption and metabolic rate

Metabolic rate is often one of the first things an organism will modify in order to adapt to changes in its environment. When determining metabolic rate in fish, oxygen consumption

(MO_2) has become a common and reliable indicator which can be easily measured during swim performance tests (Shingles et al., 2001; McKenzie et al., 2007). MO_2 is defined as the amount of oxygen a fish consumes in mg per fish body weight per hour. Using MO_2 measurements, a number of endpoints can be calculated to determine the metabolic capacity of fish. MO_2 in resting fish (*i.e*. fish held at zero water velocity in the swim tunnel) is referred to as the standard metabolic rate (SMR), and is the minimal maintenance metabolic rate of an unfed fish. In contrast, active metabolic rate (AMR) is the metabolic rate at the maximum water velocity during a U_{crit} test. Factorial aerobic scope (F-AS) is the ratio of AMR to SMR and is thought to indicate aerobic capacity and ability to cope with environmental stressors. Finally, cost of transport (COT) can be calculated using MO_2 values to determine the energetic cost associated with swimming (Shingles et al. 2001; Killen et al. 2007). These parameters can be affected by physiological stressors such as temperature change or hypoxia and also by those more anthropogenic in nature, such as toxicant exposure (Roze et al., 2013; Cucco et al., 2012). It is possible that alterations in these essential physiological processes could result in detrimental effects on individual fish fitness and survival.

1.4 Effects of PAHs on cardiac function, swim performance, and metabolic rate

1.4.1 Effects of PAHs on cardiac function

It has been shown that fish are among the most susceptible species to AHR agonists, particularly during the developmental stages of their life cycle (Incardona et al., 2004; 2006; Billiard et al., 2006). Developmental exposure to AHR agonists elicits cardiovascular-specific toxicity in fish embryos (Heintz et al., 1999; Carney et al., 2004; Hicken et al., 2011; Incardona et al., 2006). Fish embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exhibit a

syndrome known as blue sac disease, resulting in yolk sac edema, pericardial edema, craniofacial malformations, and spinal deformities (Xiong et al., 2008). Developmental exposure to PAH-containing oil mixtures has been shown to cause similar effects (Heintz et al., 1999; Carls et al., 2008). These cardiac deformities are generally thought to be associated with high molecular weight PAHs and to be dependent on AHR activation (Incardona et al., 2011). Alongside and possibly as a result of these developmental cardiac deformities, PAHs have been reported to cause functional cardiac defects in developing fish embryos which are generally thought to be associated with low molecular weight PAHs of 3 rings or less (Incardona et al., 2004; 2009). For example, cardiac arrhythmias as early as 5 days post fertilization (dpf) have been observed in Pacific herring embryos exposed to weathered crude oil (Incardona et al., 2009). In addition, exposure to sublethal concentrations of 3-ringed PAHs resulted in bradycardia and atrioventricular conduction blockade in fish embryos (Incardona et al., 2004; Zhang et al., 2013). Interestingly, short-term exposure to BaP has also been reported to cause not only the expected developmental deformities but functional cardiac defects in embryonic zebrafish, despite BaP being a high molecular weight PAH (Huang et al., 2012). It is clear that embryonic PAH exposure in fish results in cardiovascular-specific toxicity of a developmental and functional nature. What remains unclear are whether the observed adverse functional effects in embryos are the result of or merely coincident with teratogenic PAH effects. Even less understood are the acute effects of PAHs on fully-developed adult fish heart function.

Impaired cardiorespiratory function has been observed following petroleum hydrocarbon exposure in adult common sole, illustrating the possibility of the cardiovascular system as a potential target for PAH toxicity in adult stage fish (Claireaux and Davoodi, 2010). Studies have investigated the effects of various PAHs and PAH-containing oil mixtures in fish to determine

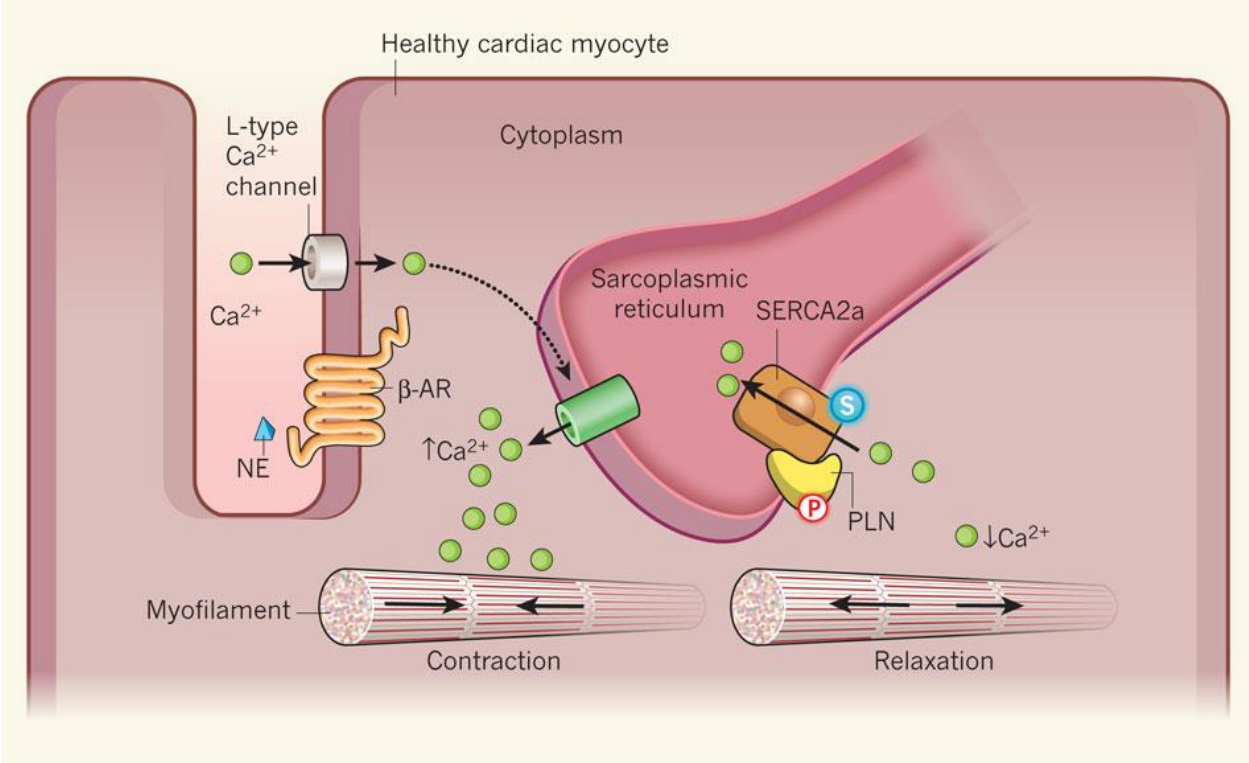


Figure 1.4: Mechanisms of cardiac contraction and relaxation in a healthy cardiac myocyte. Norepinephrine (NE) binds β-adrenergic receptors (β-AR) on the surface of cardiac myocytes, causing increases in intracellular calcium-ion (Ca^{2+}) concentrations resulting in myofilament contraction. Phosphorylation (P) of phospholamban (PLN) releases its inhibitory effect on sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a), allowing it to bind and pump Ca^{2+} into the SR, thereby reducing cellular Ca^{2+} concentrations and causing myofilament relaxation (Shenoy and Rockman, 2011; reproduced with permission from Nature Publishing Group).

the mechanism of functional cardiac toxicity. It was found that compounds in crude oil caused direct effects on ion channels involved in cardiac excitation-contraction coupling as well as cardiac contractility in juvenile tuna (*Thunnus albacares*) hearts (Brette et al., 2014). Studies in zebrafish embryos have also shown altered Ca^{2+} handling in the heart following exposure to phenanthrene, a 3-ring PAH (Zhang et al., 2013). Further investigation revealed inhibition of sarcoplasmic reticulum (SR) calcium (Ca^{2+}) ATPase, or SERCA2a. SERCA2a, encoded by *Atp2a2a* in zebrafish, is responsible for Ca^{2+} reuptake into the SR during diastole (See Figure 1.4 for schematic; Shenoy and Rockman, 2011). SERCA and Ca^{2+} reuptake into the SR are essential for proper relaxation of the ventricle before the next contraction. Alterations in SERCA2a caused by a number of factors, including PAH exposure, have been associated with bradycardia and arrhythmias in embryonic zebrafish (Zhang et al., 2013). Overall, PAHs appear to have specific AHR-mediated toxic effects on fish cardiac development. What are only recently being brought to light are the acute functional effects of PAHs on the fully-developed adult fish heart.

1.4.2 Effects of PAHs on swim performance

It was postulated by Hammer (1995) that critical swimming speed (U_{crit}) depends on several internal and external factors. Internal factors are physiological in nature, such as hematocrit levels, while external factors may include things such as temperature, oxygen levels, or environmental contaminants that may decrease U_{crit} by a known or unknown mechanism. Several studies have been completed examining the effects of various contaminants on swim performance, including selenomethionine, 2,4-dinitrophenol, and complex industrial effluent, all of which were found to significantly decrease U_{crit} following exposure in adult zebrafish (Smolders et al., 2002; Marit and Weber, 2011; Thomas and Janz, 2011). PAHs in the water

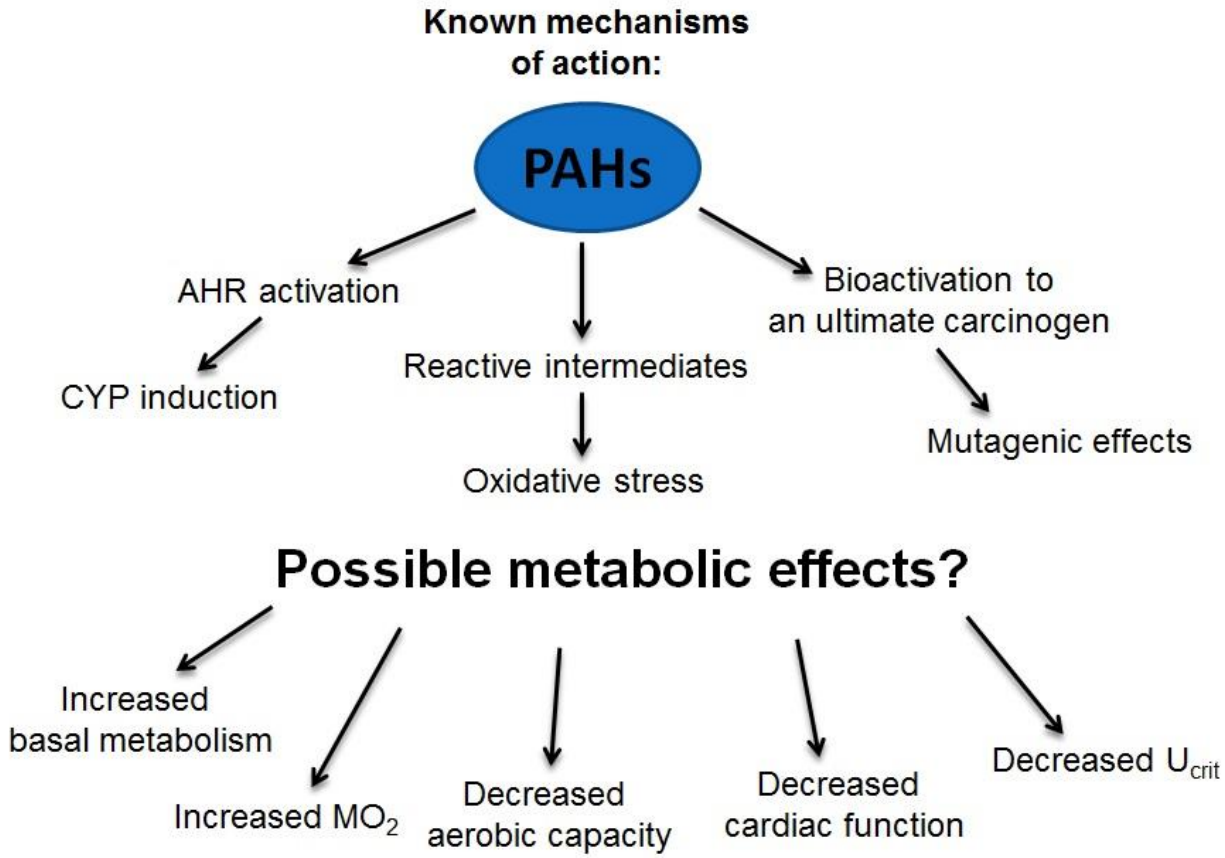


Figure 1.5: Schematic outlining the known mechanisms of action and possible metabolic effects of PAHs. AHR – aryl hydrocarbon receptor; CYP – cytochrome P450; MO_2 – oxygen consumption; PAHs – polycyclic aromatic hydrocarbons; U_{crit} – critical swimming speed

soluble fraction of crude oil have also been reported to cause decreases in swim performance and increased recovery time in Pacific herring (*Clupea pallasii)* (Kennedy and Farrell, 2006). Furthermore, common chub (*Leuciscus cephalus*) exposed to river water polluted with PAHs had a significant reduction in repeat U_{crit} values, where following an initial U_{crit} challenge, fish recovered briefly and then underwent a second U_{crit} challenge (McKenzie et al., 2007). Finally, it has also been reported that impaired U_{crit} in adult fish can result from persistent effects due to early developmental exposure to AHR agonists, including PAHs (Marit and Weber, 2012; Mager et al., 2014). In support of this, Hicken et al. (2011) have also demonstrated that alterations in zebrafish cardiac morphology due to embryonic exposure to crude oil results in decreased cardiac output and swimming performance that persists when fish are reared to adulthood. These studies demonstrate the complex effects that toxicants may have on the physiology of an organism. In addition, it is clear that cardiac function and swimming performance are intimately linked with one another. If PAHs are targeting the cardiovascular system of fishes, the consequences of this may involve physiological behaviours dependent on proper cardiac function, such as swim performance.

1.4.3 Effects of PAHs on metabolic rate

MO_2 in resting fish (*i.e.* fish held at zero water velocity in the swim tunnel) is thought to be a direct measure of basal metabolic rate. Increased metabolic rate has been observed in fish following exposure to a variety of toxicants, including PAHs, as a general stress response (Pacheco et al., 2001; 2002; Tintos et al., 2008). It has also been shown that MO_2 in resting fish increases in response to both TCDD (Marit and Weber, 2012) and petroleum hydrocarbon exposure (Claireaux and Davoodi, 2010). Thus, basal metabolism or SMR appears to increase in

response to toxicant exposure, but may then increase even more as fish are forced to swim. As swimming speed increases, so does MO_2, but environmental factors can alter a fish's aerobic performance (MacKinnon and Farrell, 1992). Ammonia (Shingles et al. 2001) and selenomethionine exposure (Thomas et al. 2013) resulted in decreased AMR and F-AS in rainbow trout and zebrafish, respectively. Additionally, when zebrafish were acutely exposed to β-naphthoflavone (BNF), a model PAH compound, significant decreases in AMR and F-AS were observed while U_{crit} remained unchanged (Gerger et al., 2014). This could indicate that MO_2 and related measurements may be more sensitive indicators of sublethal toxicity than U_{crit}. The known mechanisms of action and possible effects of PAHs on cardiac function, swim performance, and metabolic capacity are summarized in Figure 1.5.

1.5 Exposure route and PAH toxicity

Traditionally, the major routes through which a fish may be exposed to a contaminant either in the field or in the laboratory are dietary exposure, aqueous exposure, intramuscular (i.m.) injection, intravenous (i.v.) injection, or intraperitoneal (i.p.) injection. Each method possesses unique advantages and disadvantages. For example, i.m. injection of BaP in African catfish (*Clarias gariepinus*) was found to have poorer absorption and reduced biotransformation compared to i.p. injection (Karami et al., 2011). This may be due to the reduced bioavailability of compounds injected through the muscle, while i.p. injection introduces compounds directly into the peritoneal cavity where they typically enter the hepatic circulation to be rapidly metabolized (Rozman and Watkins, 2003). In contrast, i.v. injection introduces a compound directly into circulation which can result in rapid absorption and toxicological effects occurring quicker than desired. However, i.v. injection is relatively uncommon in aquatic toxicity tests due

to the difficulty of injecting smaller organisms with this method. I.p. injection provides a middle ground between i.m. and i.v. injection in that it is absorbed relatively quickly and is easily performed. Overall, injection of compounds is a useful method in laboratory toxicity testing to ensure all test compound is successfully delivered to the test organism and to minimize the potential impacts of other confounding factors. Despite their utility, injection methods do not simulate environmental exposure scenarios and other methods of exposure are necessary to replicate real world conditions.

Dietary and aqueous exposure are the two most common ways that fish are exposed to PAHs in the aquatic environment (Lee et al., 1972). There are numerous studies examining potential differences in the toxicity of compounds, including BaP, between these and other exposure routes. The majority of adult fish studies comparing BaP toxicity with exposure route focus on CYP1A gene expression or enzyme activity (Levine et al., 1994; Van Veld et al., 1997; Boleas et al., 1998; Ortiz-Delgado et al., 2005; Costa et al., 2011; Karami et al., 2011). CYP1A induction is a valuable biomarker for assessing exposure to AHR agonists in fish. Furthermore, CYP1A induction following BaP exposure appears to differ between tissues types with exposure route. Fish exposed to dietary BaP had significant induction of CYP1A-associated staining in the liver (Ortiz-Delgado et al., 2005) and gut mucosal epithelium (Van Veld et al., 1997; Ortiz-Delgado and Sarasquete., 2004), and a significant increase in ethoxyresorufin-o-deethylase (EROD) activity, an enzyme assay specific for CY1A activity, in the intestine (Costa et al., 2011). Hepatic EROD activity was increased with i.p. injection of BaP in gizzard shad (*Dorosoma cepedianum*; Levine et al., 1994) and juvenile turbot (*Scophthalmus maximus*; Boleas et al., 1998). Adult zebrafish i.p. injected with BaP also showed elevated CYP1A mRNA expression in the vasculature, specifically in the mesenteric artery (Bugiak and Weber, 2009).

Finally, aqueous exposure may cause different patterns of CYP1A induction. Aqueous BaP exposure resulted in increased CYP1A-associated staining in the liver, gills, and heart of gilthead seabream (*Sparus aurata*; Ortiz-Delgado et al., 2005) and overall increased CYP1A-associated staining of the vasculature in mummichog (*Fundulus heteroclitus*; Van Veld et al., 1997). It is clear that exposure route contributes to different CYP1A induction patterns in various fish tissues. What remains unclear is the effect of BaP exposure route on whole-organism physiology. Gene expression can be a useful indicator of exposure, but changes in expression of genes such as CYP1A do not necessarily indicate whether a toxic response is taking place. Measuring body residues are considered the gold standard for assessing exposure. Therefore, a goal of this thesis project was to connect the results of BaP body residues with CYP1A expression and functional, physiologically relevant effects to better understand the dose-response relationship. Furthermore, another goal of this thesis was to compare how this might differ between i.p. and aqueous exposure routes.

1.6 Zebrafish as a model species for cardiovascular toxicology studies

Zebrafish are a useful model organism for many areas of research. A great deal is known about normal zebrafish morphology and physiology. This allows them to be a useful model for toxicology studies where it is necessary to determine deviations from normal (Hill et al., 2005). Zebrafish have also been shown to be sufficiently sensitive to AHR agonists at early life stages (Heintz et al., 1999; Carney et al., 2004; Hicken et al., 2011). In addition, adult zebrafish are being utilized more and more frequently as models for cardiac physiology and toxicology studies (Heideman et al., 2005). Normal zebrafish heart morphology has been documented in detail (Hu et al., 2001) and cardiac function evaluated in vivo as well (Ho et al., 2002; Sun et al., 2008).

Zebrafish have also been used more recently in swim performance testing and produced consistent results (e.g. Marit and Weber, 2011; Thomas and Janz, 2011). A wealth of molecular and biological information is also known regarding zebrafish since the zebrafish genome has been completely sequenced and annotated. Zebrafish are also small in size, allowing relative ease of handling and requiring reduced housing space. For all of these reasons zebrafish are an ideal model for this study.

1.7 Importance of understanding sublethal PAH effects

It is becoming clear that fish are among the most sensitive aquatic species to AHR agonist exposure and that this sensitivity may extend past the developmental stages of their life cycle. This is of increasing concern because often concentrations of PAHs in the environment may not reach levels resulting in immediate fish mortality, but instead remain at concentrations sufficient to cause sublethal toxicity (Diercks et al., 2010; Zhang et al., 2012; Lohmann et al., 2013). This sublethal toxicity may then manifest as effects on physiological functions essential for survival, such as cardiac function, metabolic rate, and swim performance. Changes to these essential physiological functions may ultimately result in reduced fitness and survivability in affected fish, potentially resulting in population scale effects. This is of great importance due to the potential environmental and economic impacts that may result from declines in fish populations. A goal of this thesis is to provide insight into not only the mRNA expression changes with acute BaP exposure but also the physiological consequences in adult fish, and how these sublethal effects may be affected by exposure route. This will provide valuable information for extrapolating effects observed in laboratory studies to environmental exposure scenarios.

1.8 Research objectives and hypotheses

1.8.1 Hypotheses

The essential hypothesis of this thesis was that acute BaP exposure will have negative effects on adult zebrafish cardiorespiratory function and fitness. Specific hypotheses included:

1) Acute BaP exposure will impair cardiac function in adult zebrafish in the form of decreased cardiac output.

2) Acute exposure to BaP will impair aerobic metabolism in adult zebrafish by altering oxygen consumption and metabolic rate.

3) Adult zebrafish will have decreased U_{crit} as a result of impaired aerobic metabolism and reduced cardiac function.

4) Acute BaP exposure will result in increased CYP1A mRNA induction while *Atp2a2a* expression will be decreased in BaP-exposed fish compared to controls.

5) I.p. injection will achieve greater BaP body burdens, alter CYP1A and *Atp2a2a* mRNA expression to a greater extent and exert greater adverse physiological effects compared to aqueous BaP exposure.

1.8.2 Objectives

The unifying objective of this thesis was to characterize the sublethal effects of acute BaP exposure on adult fish cardiorespiratory function and fitness using two different routes of exposure. Specific objectives to test the above hypotheses included:

1) To assess the effects of 48 hr BaP exposure via i.p. injection or aqueous exposure on adult zebrafish cardiac function, metabolic rate, and swim performance.

2) To determine the effects of 48 hr i.p. injection or aqueous BaP exposure on tissue-specific CYP1A or *Atp2a2a* mRNA expression in adult zebrafish.

3) To measure parent BaP body burdens in adult zebrafish following i.p. injection or aqueous exposure to BaP for 48 hr.

4) To compare the effects of similar BaP body burdens on cardiac function, metabolic rate, swim performance, and mRNA expression in adult zebrafish following 48 hr i.p. injection or aqueous exposure to BaP.

CHAPTER 2

2.0 METHOD DEVELOPMENT

2.1 Optimizing benzo[a]pyrene analysis in adult zebrafish

It was necessary for this project to develop a novel method for extracting and analyzing BaP in adult zebrafish tissues due to the small mass of the zebrafish body. Extraction of BaP was done in fish tissues consisting mainly of head and muscle (i.e. carcass with abdominal contents removed) using accelerated solvent extraction (ASE). This was done using a method adapted from previous work which determined PAH content in mussel tissue using ASE followed by high pressure liquid chromatography (HPLC) (Yusa et al., 2005). Initially in this project, ASE was done using clean Ottawa sand (Sigma-Aldrich, ON, Canada) to fill the stainless steel cells following sample input and prior to extraction. However, this yielded low percent recoveries when spiked with known amounts of standard. Inert glass wool (Sigma-Aldrich, ON, Canada) was then tested as a cell filler instead and percent recoveries determined. Glass wool provided superior percent recoveries of standard and was sufficient to maintain pressure during the extraction process. Therefore, inert glass wool was chosen for use in extraction of BaP from experimental samples (Table 2.1).

Table 2.1: Percent recovery of benzo[a]pyrene (BaP) standard following accelerated solvent extraction (ASE) and then high pressure liquid chromatography (HPLC) in adult zebrafish tissue using clean Ottawa sand or inert glass wool as cell fillers. Results are presented as mean ± SEM following 2-3 trial runs per cell filler type.

ASE Cell Filler	Recovery of Standard (%)
Clean Ottawa Sand	58.7 ±1.8
Inert Glass Wool	128 ± 8.6

2.2 Preliminary assessment of *in vivo* cardiac function in adult zebrafish

In order to evaluate adult zebrafish cardiac function *in vivo* using a non-invasive method, techniques for high frequency cardiac ultrasound were adapted from rodents to zebrafish. Cardiac function measurements were carried out using a VEVO 770 high frequency ultrasound machine (VisualSonics, Markham, ON, Canada) equipped with B-mode imaging and pulsed-wave Doppler (VisualSonics, 2008). Initial experiments were performed to determine which method for determining stroke volume (V_S) provided the most consistent results, as V_S can be calculated using either Doppler or B-mode imaging. A comparison of V_S measurements, standard errors, and coefficients of variation for both methods is provided in Table 2.2.

Pulsed-wave Doppler is one method for determining V_S in subjects using high frequency cardiac ultrasound. Briefly, individual measurements of velocity are taken over time because blood flow is not constant due to the pulsatile nature of the cardiac cycle. These individual velocities are then summed and integrated over time, giving the velocity time integral (VTI) in units of millimeters (mm) for zebrafish (Figure 2.1). Additionally, the cross sectional area (CSA) in mm^2 of the valve or artery through which VTI is measured is then taken. When VTI and CSA are measured at the same time point, the product of both gives V_S in mm^3:

$$V_S = \text{VTI} * \text{CSA}$$

An alternative method to calculate V_S using high frequency cardiac ultrasound is through B-mode imaging using Simpson's rule of discs. In this method, the ventricle is divided into cylindrical disks whose volumes are added together with the volume of an apical disk shaped like a bullet (Figure 2.2 and 2.3). Unlike mammals, zebrafish do not have a hollow ventricular

chamber, so instead the areas of three different short axis views are traced along the outer edges of the ventricle and measured (A_1, A_2, A_3). The ventricular length (l) of one long axis view is then measured and divided by the number of short axis views (3) to give the height (h = l/3) of each ventricular disk. All of these values are measured at both systole and diastole. Using these values, end systolic and diastolic volumes in units of mm^3 (equivalent to µl) are calculated for the fish ventricle using:

$$V = (A_1+A_2)h + ((A_3h)/2) + (\pi/6(h^3))$$

End systolic volume is then subtracted from end diastolic volume to give V_S.

Table 2.2: Stroke volume (V_S) measurements in adult zebrafish determined using either pulsed-wave Doppler or B-mode imaging during high frequency cardiac ultrasound experiments. Results are mean ± SEM of n=10-12 fish per method.

Method	V_S (mm^3)	Coefficient of Variation (%)
Pulsed-wave Doppler	0.53 ± 0.09	52.5
B-mode Imaging	0.26 ± 0.02	21.9

Both methods of V_S determination provided estimates that were reasonably close to each other (i.e. within the same order of magnitude). During these initial practice trials and when comparing the results of both methods, it became evident that B-mode imaging and calculation of V_S using Simpson's rule of discs provided more consistent, less variable results than pulsed-wave Doppler, as indicated by the lower coefficient of variation (Table 2.2). The most likely

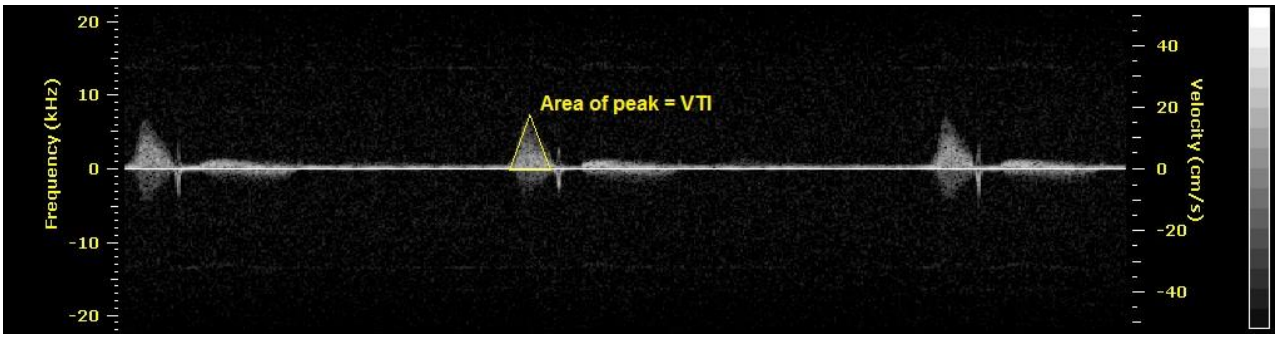


Figure 2.1: A representative pulsed-wave Doppler scan obtained from an adult zebrafish heart *in vivo* using high frequency ultrasound. Velocity time integral (VTI) measurements were taken by measuring the areas of individual peaks using VisualSonics software (Markham, ON).

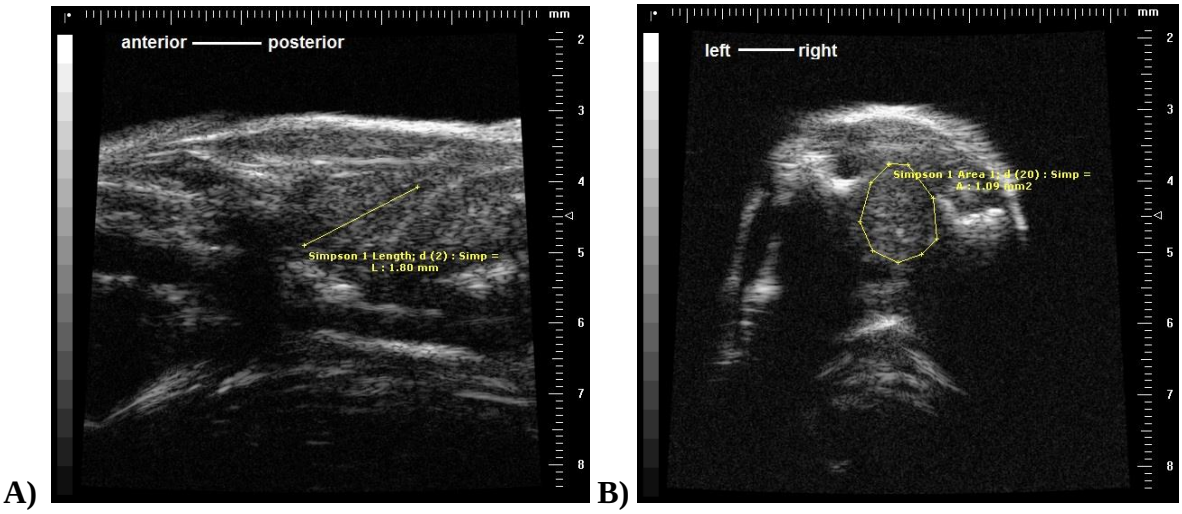


Figure 2.2: High frequency B-mode ultrasound scans of an adult zebrafish heart *in vivo*. Long axis (A) and short axis (B) views were taken in order to determine ventricle length (l) and areas (A_1, A_2, A_3), respectively, for determination of stroke volume (V_S) using VisualSonics software (Markham, ON).

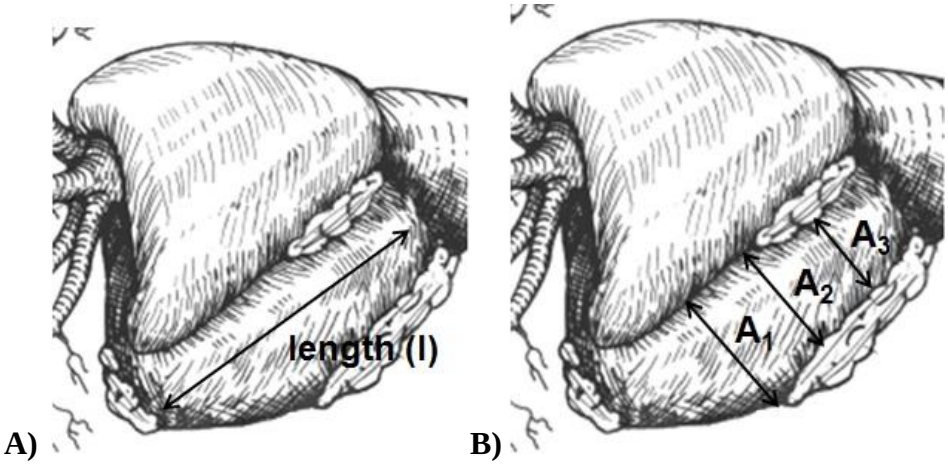
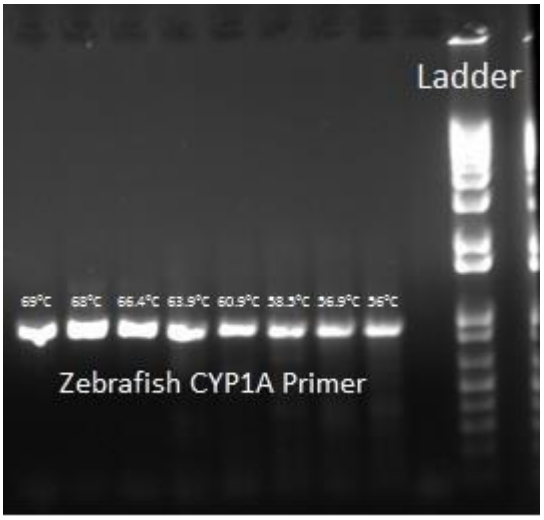


Figure 2.3: Representative diagrams illustrating measurements of the adult zebrafish heart taken *in vivo* using high frequency cardiac ultrasound. Long axis (A) and short axis (B) measurements were taken in order to determine ventricle length (l) and areas (A_1, A_2, A_3), respectively, for determination of stroke volume (V_S) (Hu et al., 2001; reproduced with permission from John Wiley and Sons, Inc.).

reason for this discrepancy is the CSA measurement required for pulsed-wave Doppler. In order to properly obtain this measurement, a clear view of the valves through which flow is measured is necessary. However, due to the nature of this technique and the small size of the zebrafish heart, the required level of detail was not obtainable and so approximations of the valve location had to be made when taking CSA measurements. In contrast, despite requiring four different views and corresponding measurements for each, B-mode imaging provided clear images sufficient to obtain accurate and consistent measurements of the ventricle. Therefore, B-mode imaging and Simpson's rule of discs were used to determine V_S in all subsequent experiments in adult zebrafish using high frequency cardiac ultrasound.

2.3 Primer validation for real time fluorescence qPCR

Target genes of interest for this thesis project were adult zebrafish cytochrome P4501A (CYP1A) and *Atp2a2a*. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as a housekeeping gene (internal control) and its expression was determined for each run of a gene of interest in every sample. CYP enzymes are commonly used to assess AHR agonist exposure and CYP1A in particular has been shown in multiple instances to be significantly induced in fish following BaP exposure (Van Veld et al., 1997; Ortiz-Delgado et al., 2005; Bugiak and Weber, 2009). CYP1A was therefore chosen as a target gene of interest in order to assess the exposure and distribution of BaP with different exposure routes. *Atp2a2a*, which encodes for the calcium reuptake channel SERCA2a in zebrafish, was also chosen as a target gene of interest to gain insight into the mechanistic effects on BaP specifically on heart function. Primers for G3PDH and CYP1A were previously developed for zebrafish in this laboratory (Bugiak and Weber, 2009) and these were used for this thesis. The primer for zebrafish *Atp2a2a* was chosen based on

A) 

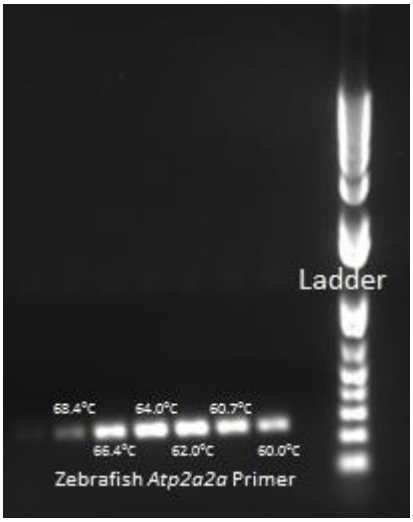
B) 

Figure 2.4: Agarose gel images of PCR fragments from primer validation at different annealing temperatures for CYP1A (A) and *Atp2a2a* (B) in adult zebrafish tissue.

Table 2.3: Gene-specific primer sequences and GenBank accession numbers used for quantitative real time PCR.

Target	Accession #	Sequence (5'-3')
G3PDH	NM_001115114	F: AGCACTGTTCATGCCATCAC R: TACTTTGCCTACAGCCTTGG
CYP1A	NM_131879	F: TGCCGATTTCATCCCTTTCC R: TTCGGTCTTCGCAGTGGTTGA
Atp2a2a	NM_200965.1	F: ATTTACTTGTGCGGATTCTTCTAC R: CACGATGTCTTTGGCTTTGA

Table 2.4: Primer efficiencies at 61°C for primer pairs chosen for each gene of interest.

Gene	Slope	Efficiency (%)
G3PDH	-3.436	95.5
CYP1A	-3.343	99.1
Atp2a2a	-3.457	94.7

the previously reported results from Zhang et al. (2013). Primer sequences and GenBank accession numbers can be found in Table 2.3.

Validation experiments were conducted to determine optimal annealing temperatures for both target genes to facilitate running target genes and the housekeeping gene on one plate. This was done to reduce variability between plates. It was found that all genes produced a single band of expected size and of sufficient product at an annealing temperature of 61°C and this temperature was therefore used to run all samples on all plates (Figure 2.4).

Primer efficiencies were calculated at an annealing temperature of 61°C for each gene of interest (Table 2.4). Briefly, serial dilutions of a single sample were used in real time fluorescence qPCR and then threshold value versus sample dilution was plotted on a logarithmic scale. The slope of the plot for each gene was then used to calculate efficiencies for each gene of interest using the equation:

$$\text{Efficiency (\%)} = (10^{(-1/\text{slope})} - 1) * 100$$

Efficiencies for all primers were found to range from 94-99%. Based on these optimal efficiencies, a single melting curve during real time qPCR, and a single product at the chosen annealing temperature, these primers were then used in all subsequent experiments for analysis of gene induction in adult zebrafish.

CHAPTER 3

3.0 METHODS

3.1 Chemicals

Benzo[a]pyrene (BaP), dimethyl sulfoxide (DMSO), and ethyl 3-aminobenzoate methanesulfonate (MS-222) were all purchased from Sigma-Aldrich (Oakville, ON, Canada). Aquacalm was obtained from Syndel Laboratories (Vancouver, BC, Canada). Dosing solutions consisted of BaP dissolved directly in DMSO.

3.2 Test species

Adult zebrafish were purchased from Aquality Tropical Fish Wholesale (Mississauga, ON, Canada) and acclimated for at least two weeks prior to experiment commencement in four 30 L holding tanks with particulate, charcoal, and ammonia biofilters. Fish were held at a density of 50 fish per tank and kept on a 14 hr light: 10 hr dark photoperiod prior to use in experiments. Dechlorinated municipal water was maintained at 28°C and an 80% water change was performed every 2-3 days. Fish were fed twice daily with Nutrafin basic flake food (Hagen Inc., Montreal, QC, Canada), but not during the last 24 hr of exposure or during subsequent swim performance or cardiac function testing.

3.3 Xenobiotic exposures

3.3.1 Intraperitoneal injection

All methods used in this thesis were approved by the University of Saskatchewan's Animal Research Ethics Board (Protocol #20120084) and adhered to the Canadian Council on Animal Care guidelines for humane animal use. For intraperitoneal (i.p.) injection exposures

(Experiment 1), adult zebrafish (0.3 – 0.8 g) were randomly selected from holding tanks and then anaesthetized with 120 mg/L MS-222 until unresponsive to a tail pinch test. Fish were then injected into the i.p. cavity with 5 µl of test solution per gram of fish using an ultrafine needle attached to a glass 25-µl Hamilton syringe. Test solutions consisted of vehicle control (DMSO) or three increasing doses of BaP (0.1, 10, and 1000 µg/kg). Following injection, fish recovered individually in an aerated beaker containing clean water until transferred to an exposure beaker (4 fish/2 L beaker) for the duration of the experiment. Fish received two injections total, once every 24 hr for a 48 hr exposure period. In one set of experiments, following exposure fish were subjected to swim tunnel testing (n=10 fish/treatment) with concurrent oxygen consumption measurement, then euthanized with an overdose of MS-222 (400 mg/L) upon completion of swim testing. In a parallel set of experiments, following exposure fish were subjected to high frequency cardiac ultrasound testing (n=12 fish/treatment). Following completion of cardiac ultrasound testing, fish were euthanized and tissues (liver, muscle, and heart) taken and stored in RNAlater (Life Technologies) at -80°C for subsequent CYP1A or *Atp2a2a* mRNA analysis using real time RT-PCR. The remaining carcass minus the gastrointestinal (GI) tract was saved and stored at -80°C for analysis of parent compound residues (benzo[a]pyrene) using high pressure liquid chromatography with fluorescence detection (HPLC-FD). See Figure 3.1 for schematic.

3.3.2 Aqueous exposure

For aqueous BaP exposure (Experiment 2), adult zebrafish (0.3 – 0.8 g) were randomly selected from holding tanks and then placed in static aerated glass beakers (4 fish/2 L beaker) containing vehicle control (0.1% DMSO), 16.2, or 162 µg/L BaP with water and BaP renewal at 24 hr, for a total 48 hr exposure period. Following aqueous exposure, fish were subjected to

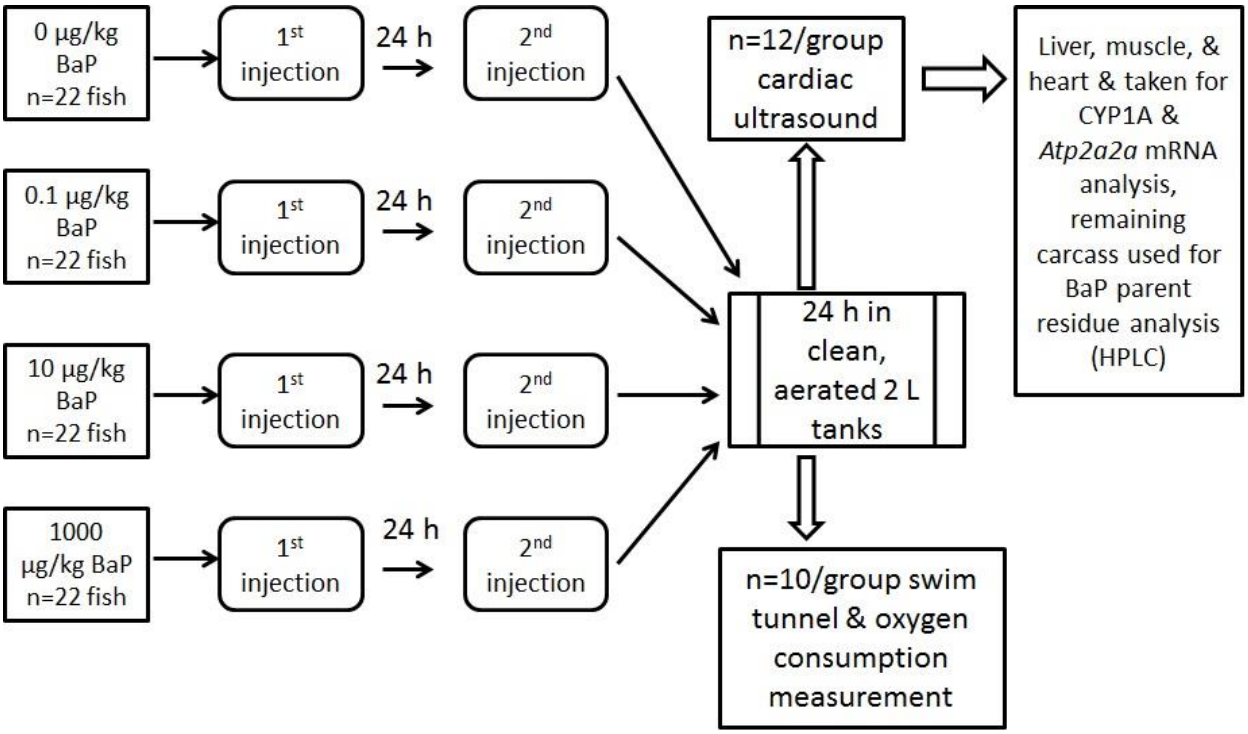


Figure 3.1: Schematic outlining the experimental design of Experiment 1. Adult zebrafish were injected (intraperitoneal) twice over 48 hr with the indicated doses of benzo[a]pyrene (BaP) and then subjected to cardiac ultrasound or swim tunnel testing. CYP1A – cytochrome P4501A; HPLC – high pressure liquid chromatography

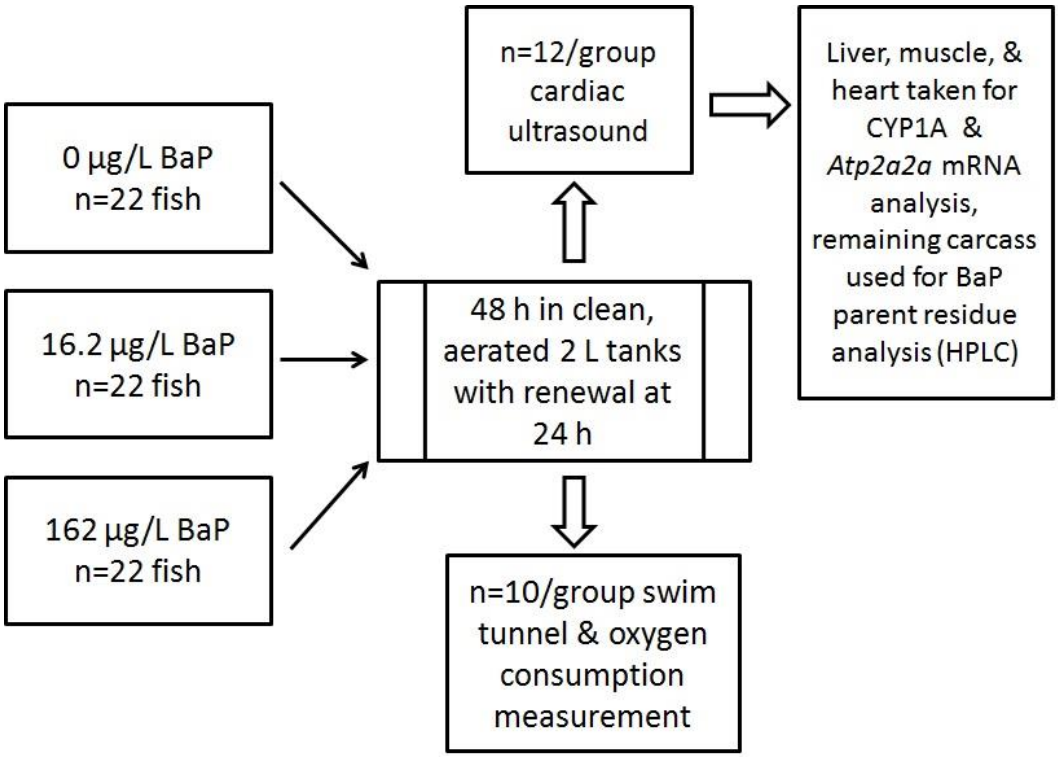


Figure 3.2: Schematic outlining the experimental design of Experiment 2. Adult zebrafish were aqueously exposed to the indicated concentrations of benzo[a]pyrene (BaP) for 48 hr with renewal at 24 hr. Following exposure, fish were subjected to cardiac ultrasound or swim tunnel testing. CYP1A – cytochrome P4501A; HPLC – high pressure liquid chromatography

swim tunnel (n=10 fish/treatment) and cardiac ultrasound (n=12 fish/treatment) testing in the same manner as described for the i.p. injection protocol, with the same sample types saved for subsequent CYP1A or *Atp2a2a* mRNA and parent compound residue analysis. See Figure 3.2 for schematic.

3.4 Benzo[a]pyrene body burden analysis

Whole fish tissue samples (consisting mainly of head, muscle, and skin) minus the GI tract were freeze dried using a Virtis Genesis 25ES freeze drier and then stored at -20°C until extraction. GI tract tissues were removed to ensure that only the amount of BaP absorbed by the fish was measured and that samples were not contaminated by GI tract contents. Extractions were carried out using a Dionex ASE 200 Accelerated Solvent Extractor. Samples were weighed and placed into methanol-rinsed 1 ml stainless-steel cells, with the remaining space filled with inert glass wool to maintain pressure during extraction. Cells were fit with a cellulose filter at both ends before being sealed and then situated in the carousel of the ASE 200 system. The conditions were as follows: oven temperature of 125°C with 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 resuspended in 1.8 ml acetonitrile. Samples were then syringe-filtered through Whatman GMF 0.45 µm filters into labeled 2 ml amber HPLC vials. An Agilent 1260 Infinity High Pressure Liquid Chromatography system coupled with Fluorescence Detection (HPLC-FD) was then used to analyze the BaP concentration in the prepared samples according to the Agilent

method for PAH analysis in soil (Volk and Gratzfeld-Huesgen, 2011) with some modifications. An aliquot of 10 µl was injected into an Agilent PAH Pursuit column maintained at 25°C. The runtime was set for 25 minutes 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 minutes. BaP eluted at ~12-15 minutes with a constant excitation wavelength of 260 nm and an emission wavelength of 420nm. Recovery of BaP from blank fish samples spiked with a known amount averaged 122% ± 6.6. The limit of detection for this protocol was 0.626 µg/kg dry mass.

3.5 *In vivo* high frequency cardiac ultrasound

Cardiac function measurements were carried out using a VEVO 770 high frequency ultrasound machine (VisualSonics, Markham, ON, Canada) equipped with B-mode imaging and pulsed-wave Doppler according to methods adapted from rodents (VisualSonics, 2008) to fish in this laboratory. Aquacalm (metomidate) was used for all cardiac ultrasound experiments instead of the traditional MS-222, which has been shown to produce higher cortisol levels in sedated fish compared to Aquacalm (Small, 2003). Prior to ultrasonography, fish were anaesthetized with Aquacalm at 20 mg/L in clean aerated water. Anaesthetized fish were then transferred to a holding dish containing a layer of 3% agarose gel with a groove fashioned in which the fish was placed ventral side up with the aid of minutien pins (Fine Science Tools, Vancouver, BC, Canada). The holding dish was filled with aerated water containing 20 mg/L Aquacalm to maintain anaesthesia throughout ultrasonography. Temperature has been shown to have a significant effect on cardiac function (Blank et al. 2002; 2004), therefore all ultrasound experiments were conducted at zebrafish housing temperature (28°C ± 0.5°C) using a recirculating water bath (VWR International, Mississauga, ON, Canada).

A RMV 708B scanhead (22-83 MHz) was used to obtain short and long-axis views of the zebrafish ventricle in B-mode and Simpson's rule of disks used to calculated ventricular volume (Mercier et al. 1982; VisualSonics, 2008). In this method, the ventricle is divided into cylindrical disks whose volumes are added together with the volume of an apical disk shaped like a bullet. Unlike mammals, zebrafish do not have a hollow ventricular chamber, so instead the areas of three different short axis views were traced along the outer edges of the ventricle and measured (A_1, A_2, A_3). Next, the ventricular length (l) of one long axis view was measured and divided by the number of short axis views (3) to give the height (h = l/3) of each ventricular disk. All of these values were measured at both systole and diastole using VisualSonics software (Markham, ON). Using these values, end systolic and diastolic volumes in units of mm^3 (equivalent to µl) were each calculated for the fish ventricle using:

$$V = (A_1+A_2)h + ((A_3h)/2) + (\pi/6(h^3)) \quad \text{(Mercier et al., 1982)}$$

End systolic volume was subtracted from end diastolic volume to give stroke volume (V_S) for each fish. Ventricular and atrial heart rates were calculated by counting the number of heart beats per 10 second ultrasound video loop and multiplying by six in order to convert to beats per minutes (bpm). Cardiac output (Q) was then calculated using ventricular heart rate (f_H) and V_S:

$$Q = f_H * V_S$$

To determine whether cardiac arrhythmias were occurring, atrial heart rate was divided by ventricular heart rate for each individual fish to give the atrioventricular (AV) ratio, a measure of cardiac conduction:

$$AV\ Ratio = Atrial\ f_H / Ventricular\ f_H$$

3.6 Swimming performance and oxygen consumption

A 170 ml swim tunnel respirometer (Loligo Systems, Tjele, Denmark) was used for swim testing. The swim tunnel was submerged in a 20 L buffer tank supplied with clean, aerated, dechlorinated municipal water and kept at 28°C by a 20 L recirculating water bath (VWR International, Mississauga, ON, Canada). Water velocities were calibrated using constant temperature anemometry according to manufacturer's instructions. Oxygen consumption rate was calculated for each fish during swimming performance trials using AutoResp™ 1 software (Loligo Systems, Denmark). For U_{crit} determination, a fish was placed in the swim tunnel and acclimated at the lowest speed (0.02 m/s) for 90 min. Following acclimation, water velocity was increased in stepwise increments (0.077 m/s (U_{ii}) every 20 min (T_{ii})) until the fish completely fatigued (stopped swimming for a minimum 25 seconds). U_{crit} was then calculated:

$$U_{crit} = U_i + [U_{ii}(T_i/T_{ii})] \quad \text{(Brett, 1964)}$$

where U_i was the highest swimming velocity maintained for an entire time interval and T_i was the time elapsed at the fatigue velocity. U_{crit} values were then converted from cm/s to body lengths (BL)/s to account for slight variations in fish size.

3.7 Determination of COT, SMR, AMR, and F-AS

Standard metabolic rate (SMR) was calculated by extrapolating consumption of oxygen back to a water velocity of zero using a plot of swim speed (m/s) versus MO_2 (mg O_2/kg/h). This was done using a nonlinear, curve fitting regression analysis (Thomas et al. 2013) with GraphPad Prism software (La Jolla, CA, USA). Cost of transport (J/kg/m) was calculated by multiplying MO_2 (mg O_2/kg/s) by an oxycaloric value of 14.1 J/mg O_2 and then dividing by the corresponding swimming speed (m/s) (Videler, 1993). Active metabolic rate was determined for individual fish using the MO_2 values at the maximum swimming velocity in the U_{crit} test.

Factorial aerobic scope (F-AS) was calculated as AMR/SMR (Shingles et al. 2001; Killen et al. 2007; Thomas et al. 2013).

3.8 Real time fluorescence qPCR

Real time RT-PCR was carried out to determine the expression levels of CYP1A and *ATP2a2a* in adult zebrafish tissues. Total RNA was extracted from liver, heart (pooled, 4 per sample), and skeletal muscle samples with TRIzol (Life Technologies, Burlington, ON, Canada) according to manufacturer's instructions. Quantity (A260) and quality (A260:280; purity > 1.7 for all acceptable samples) were assessed using a NanoDrop apparatus and software (Thermo Fisher Scientific). cDNAs were synthesized using iScript cDNA synthesis kits (BioRad, Mississauga, ON, Canada). Real time rt-PCR was performed in a Chromo4 Multicolor Real-Time PCR Detection System (BioRad) using SSoAdvanced Universal SYBR Green Supermix (BioRad) with a reaction volume of 20 µl. Expression of zebrafish glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was determined for every PCR run for every sample as an internal control (housekeeping gene). G3PDH and CYP1A primers (Bugiak and Weber, 2009) and *Atp2a2a* primers (Zhang et al., 2013) were all obtained from Life Technologies (Table 1). Primer efficiencies ranged from 94-99% at an annealing temperature of 61°C. All samples were run in duplicate.

3.9 Statistical analyses

In initial analyses sex was included as an additional factor in two-way ANOVAs, but we found $p > 0.05$ for sex in all analyses of all endpoints measured. Therefore, sexes were pooled and data analyzed instead by one-way ANOVA followed by Fisher's least significant difference (LSD) test for the majority of endpoints measured. In the case of oxygen consumption (MO_2)

and cost of transport (COT), data were analyzed using a one-way ANOVA at each speed followed by Fisher's LSD test using Bonferroni-corrected p-values. For cardiac output and stroke volume measurements, data were analyzed using a one-way analysis of covariance (ANCOVA) using body mass as a covariate followed by Fisher's LSD test where appropriate. For gene expression data, all values were expressed as ΔΔCT values first using expression of the housekeeping gene (G3PDH) as an internal correction for all genes of interest. Data were then normalized to control group expression of the gene of interest to calculate a fold-change in expression with treatment. All gene expression data were log-transformed to achieve normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). Exposure route comparisons were done using two sample t-tests or one-way ANCOVAs (with body weight as covariate) as appropriate. All statistical analyses were carried out using SYSTAT 13 software (Chicago, IL, USA). A p-value < 0.05 was considered significant. All results are presented as mean ± standard error of the mean (SEM).

CHAPTER 4

4.0 RESULTS

4.1 Zebrafish benzo[a]pyrene body burdens

Whole body concentrations of BaP in adult zebrafish i.p. injected with 0, 0.1, 10, and 1000 µg/kg BaP were 3.85, 7.42, 14.2, and 68.6 µg/kg dry mass (d.m.), respectively. Although BaP concentrations in fish increased as the dose of BaP increased, only the highest dose group of 1000 µg/kg produced BaP concentrations in adult fish that were significantly increased from control ($p<0.001$; Table 4.1). Aqueous BaP concentrations of 0, 16.2, and 162 µg/L produced whole body concentrations in adult zebrafish of 6.32, 62.2, and 158 µg/kg d.m., respectively. Both aqueous concentrations of BaP produced whole body concentrations significantly higher than those observed in control fish (16.2 µg/l, $p<0.01$; 162 µg/L, $p<0.001$; Table 4.1).

4.2 Mortalities and morphometrics

Following BaP i.p. injection and subsequent swim tunnel or cardiac ultrasound testing, there were no statistically significant differences in fish body mass, body length, or condition factor among the different exposure groups (Table 4.1). Some mortalities were observed with BaP injection, however, these results were not statistically significant. Slight differences in body mass between control groups (Table 4.1) are due to the aqueous controls being slightly younger than those used for i.p. injection, however all zebrafish used were mature, fully developed adult fish. Fish that were aqueously exposed to BaP had significant differences in body length ($p<0.001$) and condition factor ($p<0.001$) compared to control (Table 4.1). This was due to skewed sex ratios within treatment groups, which as mentioned previously in the statistical

Table 4.1: Parent benzo[a]pyrene (BaP) body burdens (µg/kg, dry mass), mortalities, and morphometrics of adult zebrafish i.p. injected or aqueously exposed to BaP or solvent control for 48 hr. Data are mean ± SEM with n=10-12 for BaP analyses and n=28-32 for morphometrics. Percent mortality was calculated as the percent per exposure unit (n=4 fish per unit) with n=8 independent exposure trials per treatment group.

Nominal BaP Dose	Fish BaP (µg/kg d.m.)	Mortalities (%)	Body Weight (g)	Body Length (cm)	Condition Factor (K)
Intraperitoneal Injection					
Control	3.85 ± 1.4	0 ± 0	0.665 ± 0.05	3.13 ± 0.05	2.1 ± 0.08
0.1 µg/kg	7.42 ± 3.0	9.38 ± 4.6	0.592 ± 0.04	3.05 ± 0.05	2.0 ± 0.10
10 µg/kg	14.2 ± 8.4	12.5 ± 4.6	0.580 ± 0.04	3.05 ± 0.05	2.0 ± 0.10
1000 µg/kg	68.6 ± 22***	9.38 ± 4.6	0.645 ± 0.05	3.15 ± 0.04	2.0 ± 0.10
Aqueous Exposure					
Control	6.32 ± 2.7	0 ± 0	0.404 ± 0.02	2.78 ± 0.04	1.8 ± 0.05
16.2 µg/L	62.2 ± 8.5**	0 ± 0	0.440 ± 0.02	2.98 ± 0.04***	1.6 ± 0.06**
162 µg/L	158 ± 16***	0 ± 0	0.371 ± 0.02	2.89 ± 0.04*	1.5 ± 0.05***

Condition factor (K) = (weightX(100/length^3)) (Nash et al., 2006).
*p<0.05, **p<0.01, ***p<0.001 when compared to control group following Fisher's LSD after one-way ANOVA.

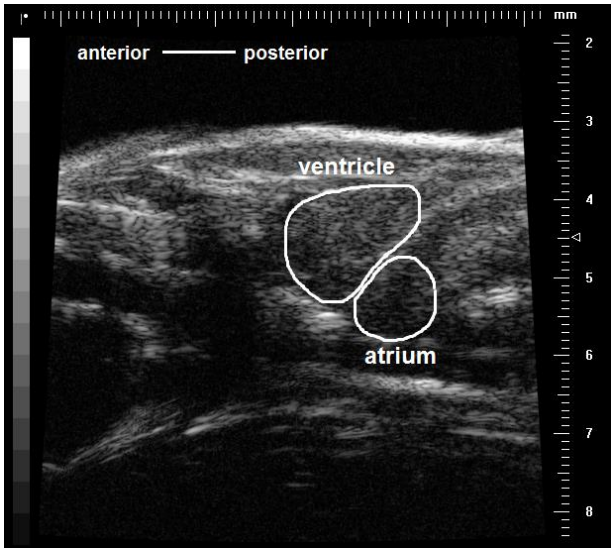
analyses methods section of this thesis had no statistically significant effect on any endpoint measured. No mortalities were observed in adult zebrafish following aqueous exposure to BaP.

4.3 Cardiac function analysis

Aqueous BaP exposure appeared to have a more pronounced effect on adult zebrafish cardiac function than i.p. injection. Representative B-mode echocardiograms in long and short axes of the zebrafish heart are shown (Figure 4.1) and these images were used to calculate ventricular end systolic, end diastolic, and stroke volumes. Cardiac output was significantly decreased with both aqueous BaP concentrations ($p<0.01$, $p<0.001$; Figure 4.2B) while i.p. injection caused no statistically significant change in cardiac output (Figure 4.2A). Ventricular heart rate was significantly decreased with the highest BaP dose for both routes of exposure ($p<0.05$ for i.p. injection and aqueous exposure; Figures 4.3A, B, respectively), while atrial heart rate remained unchanged with both routes of BaP exposure (Figure 4.3C, D). The ratio of atrial to ventricular heart rates (AV ratio) was significantly elevated in fish injected with 10 and 1000 µg/kg BaP ($p<0.05$, $p<0.01$; Figure 4.3E). Alternatively, aqueous BaP exposure failed to cause a statistically significant change in the AV ratio in adult zebrafish (Figure 4.3F).

Stroke volume was significantly decreased with only aqueous BaP exposure (162 µg/L, $p<0.05$; Figure 4.4B) while i.p. injection resulted in no change (Figure 4.4A) compared to corresponding control groups. In addition, end diastolic volume and end systolic volume were both significantly decreased with aqueous BaP exposure (162 µg/L, $p<0.001$ for both endpoints; Figure 4.4D, F) compared to controls, but remained unchanged with i.p. injection (Figure 4.4C, E).

A)



B)

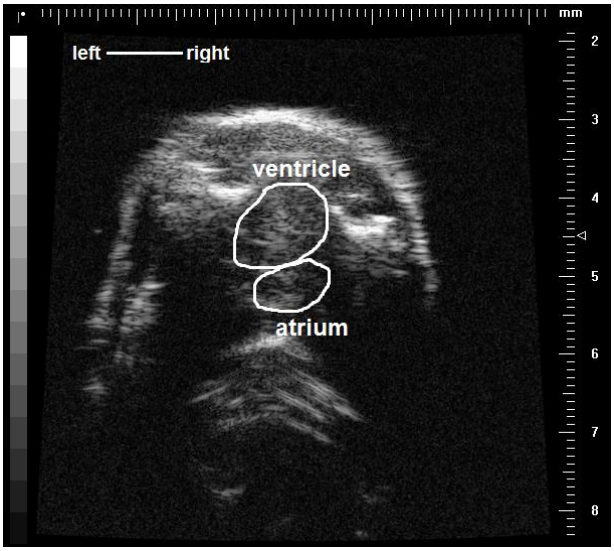


Figure 4.1: High frequency B-mode ultrasound scans of a control adult zebrafish heart in an upside-down position. A representative long axis view (A) and short axis view (B) was taken for each zebrafish (n=12 fish/treatment group) following 48 hr exposure to benzo[a]pyrene or solvent control.

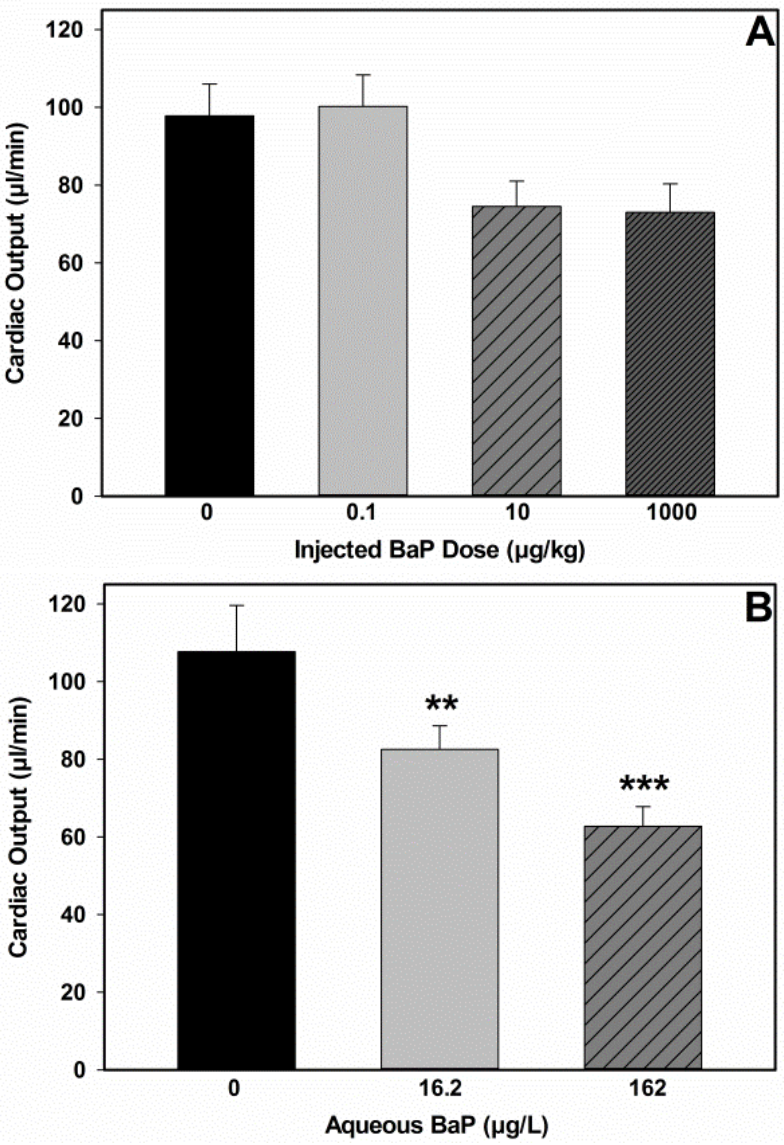


Figure 4.2: Cardiac output of adult zebrafish following intraperitoneal (i.p.) injection (A) or aqueous exposure (B) to benzo[a]pyrene or solvent control for 48 hr. Data are mean ± SEM of n=10-12 fish for i.p. injection and n=12 for aqueous exposure. **p<0.01, ***p<0.001 in Fisher's LSD after one-way ANCOVA with body weight as a covariate compared to corresponding controls.

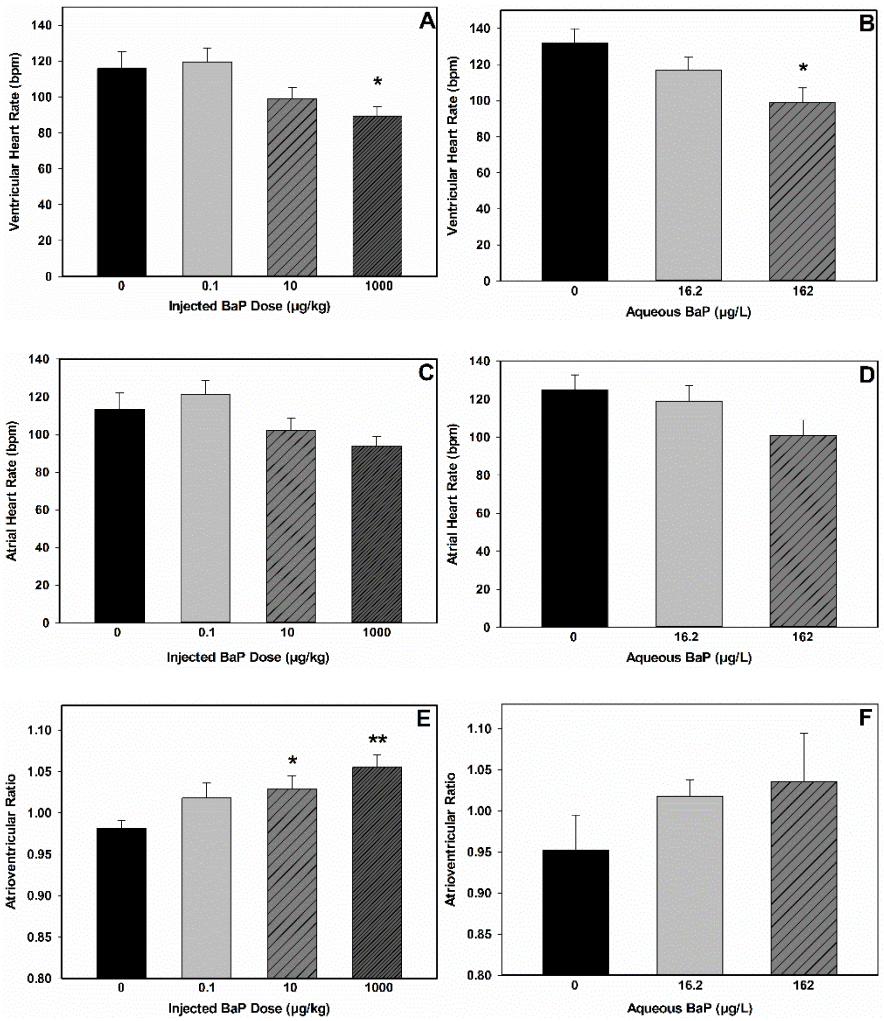


Figure 4.3: Ventricular heart rate (A, B), atrial heart rate (C, D) and atrioventricular (AV) ratio (E, F) of adult zebrafish following intraperitoneal (i.p.) injection (left) or aqueous exposure (right) to benzo[a]pyrene or solvent control for 48 hr. Data are mean ± SEM of n=10-12 fish for i.p. injection and n=12 for aqueous exposure. *p<0.05, **p<0.01 in Fisher's LSD after one-way ANOVA compared to the corresponding control group.

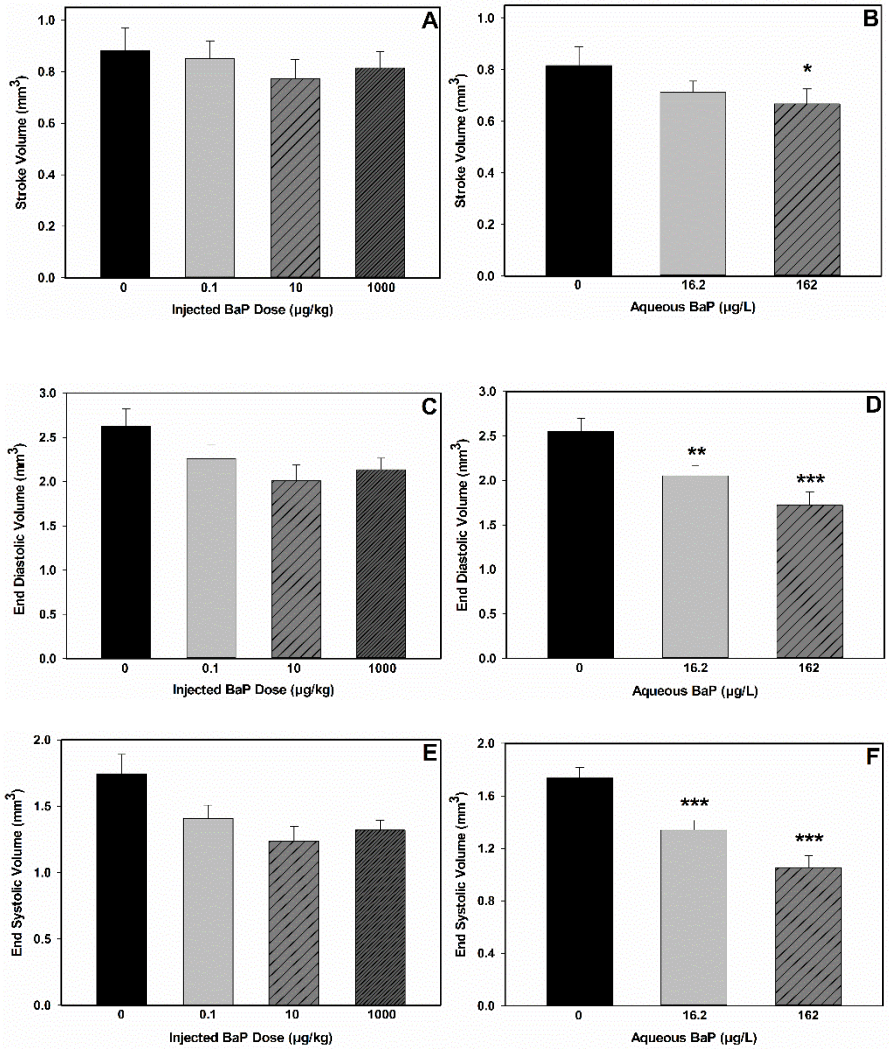


Figure 4.4: Stroke volume (A, B), end diastolic volume (C, D), and end systolic volume (E, F) of adult zebrafish following intraperitoneal (i.p.) injection (left) or aqueous exposure (right) to benzo[a]pyrene or solvent control for 48 hr. Data are mean ± SEM of n=10-12 fish for i.p. injection and n=12 for aqueous exposure. *$p<0.05$, **$p<0.01$, ***$p<0.001$ in Fisher's LSD after one-way ANCOVA with body weight as a covariate, all compared to the corresponding control group.

4.4 Swim performance and oxygen consumption

MO_2 of control and BaP-exposed zebrafish was increased as swim speed was increased, regardless of exposure route (Figure 4.5A, B). Adult zebrafish i.p. injected with 10 and 1000 µg/kg BaP showed elevated MO_2 at the lowest swim speed of 0.02 m/s ($p<0.01$). Fish injected with 0.1 µg/kg showed elevated MO_2 at 0.17 m/s ($p<0.01$), while all three doses had increased MO_2 at 0.25 m/s ($p<0.01$; Figure 4.5A). Fish that were aqueously exposed to 162 µg/L BaP showed elevated MO_2 only at the lowest swim speed of 0.02 m/s ($p<0.001$; Figure 4.5B).

COT of control and BaP-exposed zebrafish decreased as swim speed was increased, regardless of exposure route (Figure 4.5C, D). The injection doses of 10 and 1000 µg/kg BaP caused elevated COT at the swim speeds of 0.02 m/s and 0.25 m/s ($p<0.01$), while 0.1 µg/kg increased MO_2 at 0.17 m/s ($p<0.01$; Figure 4.5C). Aqueous BaP exposure resulted in elevated COT only at the lowest swim speed of 0.02 m/s (162 µg/L, $p<0.01$; Figure 4.5D). Regardless of exposure route, BaP failed to significantly alter swimming performance (U_{crit}) in adult zebrafish (Figure 4.6A, B).

4.5 SMR, AMR, and F-AS

Metabolic capacities of adult zebrafish were significantly altered with both routes of BaP exposure. SMR was significantly elevated compared to control fish with all injection doses ($p<0.05$, $p<0.01$; Figure 4.7A) as well as with aqueous BaP exposure (162 µg/L, $p<0.01$; Figure 4.7B). AMR was not significantly affected by either route of BaP exposure (Figure 4.7A; B). Alternatively, F-AS was significantly decreased in fish exposed to BaP. Fish i.p. injected with BaP showed decreased F-AS at all doses ($p<0.05$; Figure 4.7C). F-AS in fish aqueously exposed

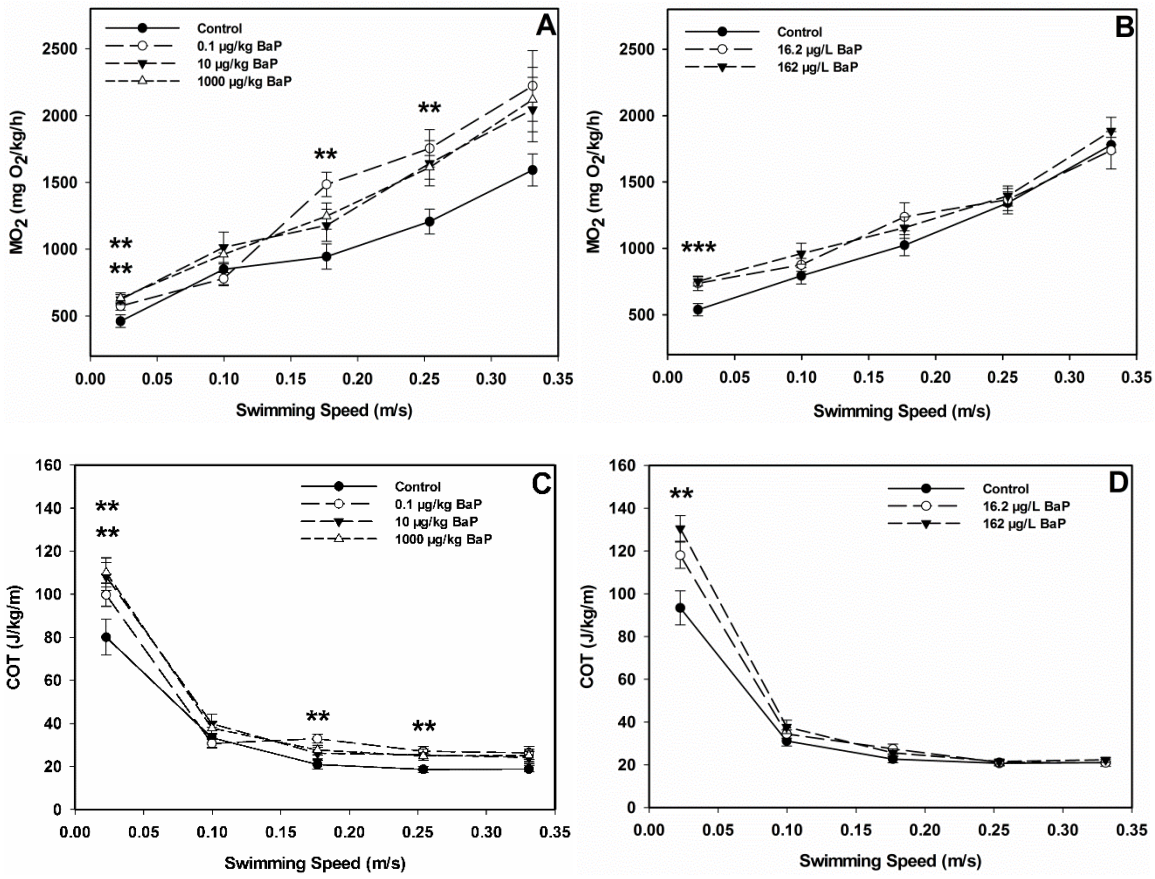


Figure 4.5: Oxygen consumption rates (MO_2) (A, B) and cost of transport (COT) (C, D) as a function of swimming speed in adult zebrafish following intraperitoneal (i.p.) injection (left) or aqueous exposure (right) to benzo[a]pyrene or solvent control for 48 hr. Data are mean ± SEM of n=8-10 fish for i.p. injection and n=10 for aqueous exposure. **p<0.01, ***p<0.001 in Fisher's LSD using Bonferroni-corrected p-values after one-way ANOVA at each swim speed compared to corresponding control.

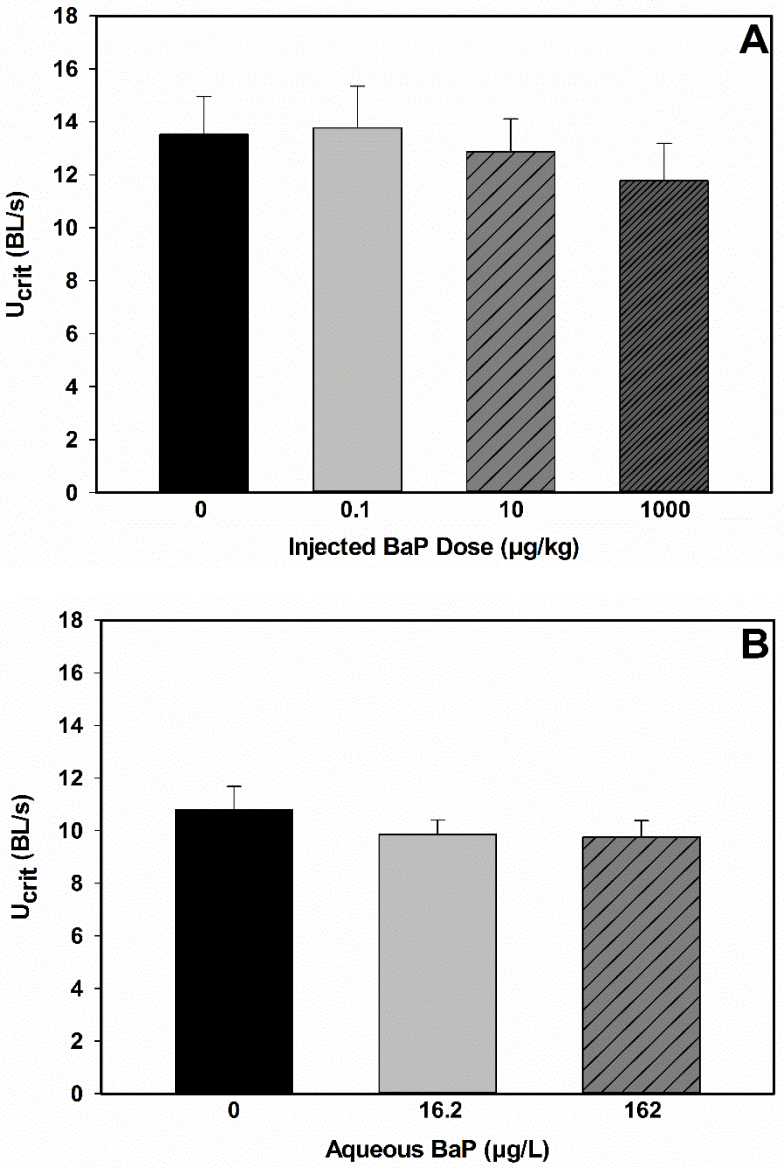


Figure 4.6: Critical swimming speed (Ucrit) of adult zebrafish exposed to benzo[a]pyrene or solvent control via intraperitoneal injection (A) or aqueous exposure (B) for 48 hr. Data are mean ± SEM of n=8-10 fish for i.p. injection and n=10 for aqueous exposure. No significant differences between treatment groups and corresponding controls after one-way ANOVA.

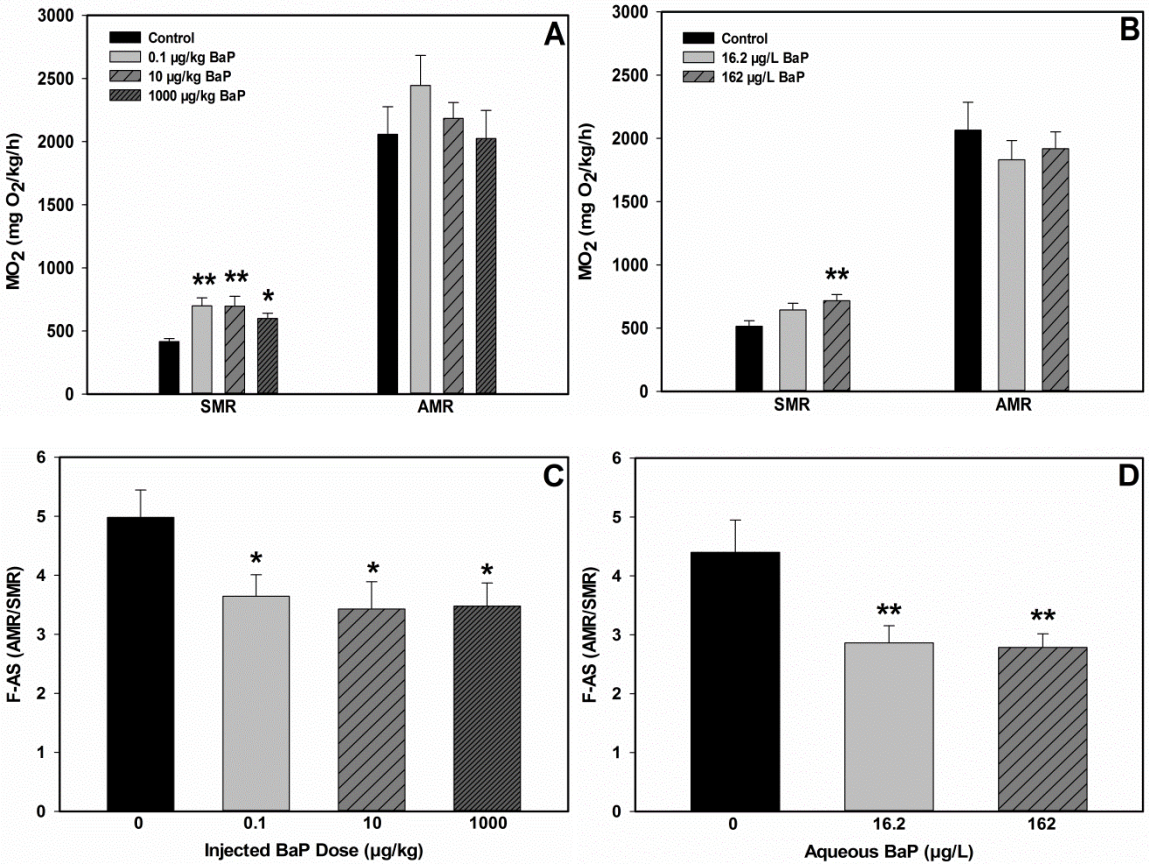


Figure 4.7: Standard metabolic rate (SMR), active metabolic rate (AMR) (A, B), and factorial aerobic scope (F-AS) (C, D) of adult zebrafish following intraperitoneal (i.p.) injection (left) or aqueous exposure (right) to benzo[a]pyrene or solvent control for 48 hr. Data are mean ± SEM of n=8-10 fish for i.p. injection and n=10 for aqueous exposure. *$p<0.05$, **$p<0.01$ in Fisher's LSD after one-way ANOVA compared to corresponding control.

to BaP was also significantly decreased at both exposure concentrations compared to control ($p<0.01$; Figure 4.7D).

4.6 mRNA abundances of CYP1A and *Atp2a2a*

CYP1A mRNA abundance was determined in adult zebrafish liver, skeletal muscle, and heart following 48 hr BaP exposure. *Atp2a2a* mRNA abundance was determined solely in heart tissue. CYP1A abundance was significantly increased in liver ($p<0.01$) and heart ($p<0.001$) compared to control fish following i.p. injection of 1000 µg/kg BaP, while expression remained unchanged in skeletal muscle (Figure 4.8A). Similarly, 1000 µg/kg BaP significantly elevated heart *Atp2a2a* mRNA abundance compared to control fish ($p<0.001$; Figure 4.8C).

Aqueous BaP exposure caused a greater magnitude of up-regulation of CYP1A mRNA abundance than i.p. injection. The highest aqueous concentration of 162 µg/L BaP significantly induced CYP1A in all three tissues analyzed ($p<0.001$). In addition, 16.2 µg/L BaP significantly up-regulated CYP1A in heart and skeletal muscle in adult zebrafish ($p<0.05$; Figure 4.8B). Interestingly, heart *Atp2a2a* mRNA abundance was not significantly changed with aqueous BaP exposure (Figure 4.8D).

4.7 Comparison of benzo[a]pyrene effects at similar body burdens

Analysis of benzo[a]pyrene body burdens in adult zebrafish allowed for comparisons to be made between the physiological and gene expression endpoints that were measured for each exposure route. The i.p.-injected dose of 1000 µg/kg and aqueous concentration of 16.2 µg/L produced comparable body burdens of approximately 65 µg BaP/kg dry mass (d.m.) in adult zebrafish (Table 4.1). Endpoints were then compared between exposure routes at this similar 65 µg BaP/kg d.m. using two sample t-tests or one-way ANCOVAS as appropriate (Table 4.2). The

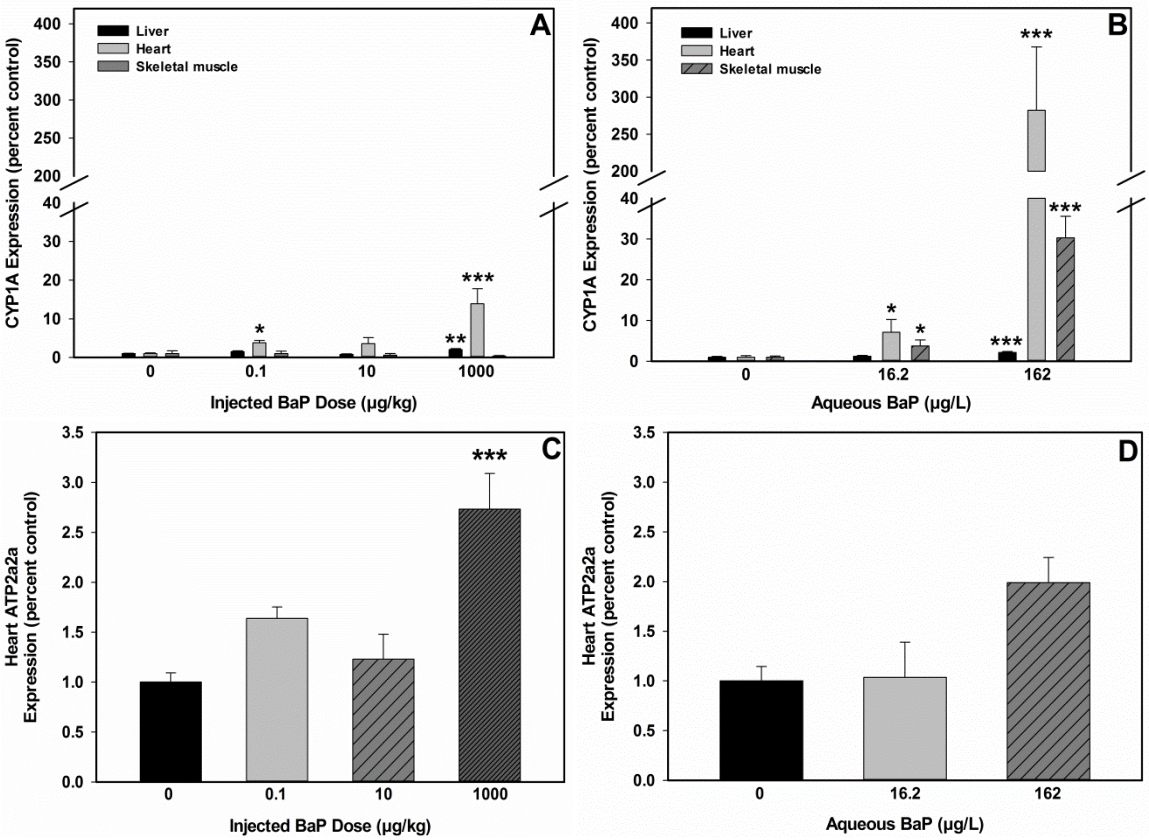


Figure 4.8: CYP1A (A, B) mRNA expression (fold change relative to control) in adult zebrafish liver (black bars), heart (light grey bars), and muscle (dark grey striped bars) and heart *Atp2a2a* (C, D) mRNA expression (fold change relative to control) following intraperitoneal (i.p.) injection (left) or aqueous exposure (right) to benzo[a]pyrene or solvent control for 48 hr. Data are mean ± SEM of n=10-12 for i.p. injection and n=12 for aqueous exposure for liver and skeletal muscle samples. Heart samples are pooled (n=4 hearts/sample) for a total n=3/treatment group. *p<0.05, **p<0.01, ***p<0.001 in Fisher's LSD after one-way ANOVA compared to corresponding control.

Table 4.2: Comparison of the effects of similar benzo[a]pyrene (BaP) body burdens (~65 µg/kg dry mass) on selected endpoints measured in adult zebrafish following 48 hr intraperitoneal (i.p.) injection or aqueous exposure to BaP. Data are mean ± SEM with n=8-12 fish/exposure route.

Endpoint	I.p. Injection	Aqueous Exposure
SMR (mg O_2/kg/h)	599.4 ± 41.0	643.7 ± 52.8
F-AS (AMR/SMR)	3.48 ± 0.39	2.87 ± 0.29
Cardiac output (µl/min)	73.0 ± 7.3	82.5 ± 6.1
Heart rate (bpm)	89.3 ± 5.1**	117 ± 7.3
CYP1A liver (fold change relative to control)	2.04 ± 0.25*	1.23 ± 0.19
CYP1A heart (fold change relative to control)	13.9 ± 3.9	7.11 ± 3.1
CYP1A skeletal muscle (fold change relative to control)	0.343 ± 0.17**	3.73 ± 1.5
Atp2a2a (fold change relative to control)	2.73 ± 0.36*	1.04 ± 0.35

*$p<0.05$, **$p<0.01$ compared to aqueous exposure group in a two-sample t-test.

metabolic endpoints SMR and F-AS were both statistically similar between exposure routes. In addition, there was no statistically significant difference in cardiac output between i.p. injection and aqueous exposure to BaP. However, ventricular heart rate was significantly different between exposure routes. I.p. injection of BaP resulted in a significantly larger decrease in ventricular heart rate (bradycardia) in adult zebrafish than aqueous BaP exposure ($p<0.01$; Table 4.2). Induction of mRNA expression in zebrafish tissue was also significantly different between exposure routes. CYP1A mRNA in the liver was more significantly induced with i.p. injection ($p<0.05$; Table 4.2) while aqueous BaP exposure resulted in greater CYP1A mRNA induction in skeletal muscle ($p<0.01$; Table 4.2). Interestingly, CYP1A in the heart remained statistically unchanged between exposure routes, however, heart *Atp2a2a* mRNA was induced to a significantly greater extent with i.p. injection compared to aqueous BaP exposure ($p<0.05$; Table 4.2).

CHAPTER 5

5.0 DISCUSSION

5.1 Project rationale and summary

Polycyclic aromatic hydrocarbon (PAH) contamination in aquatic environments is becoming a common occurrence due to growing urbanization and industrialization of the natural landscape. PAHs are thought to cause toxicity primarily by activating the aryl hydrocarbon receptor (AHR), however there is also evidence indicating their potential to cause AHR-independent toxicity as well. Because of their inherent sensitivity to AHR agonists, fish are an aquatic species of particular concern. There are numerous studies focused on developmental PAH exposure in fish, but relatively few instances where the effects of acute PAH exposure on fully developed adult fish have been characterized. Furthermore, the majority of adult fish studies comparing benzo[a]pyrene (BaP) toxicity with exposure route focus on cytochrome P4501A (CYP1A) gene expression or enzyme activity (Levine et al., 1994; Van Veld et al., 1997; Boleas et al., 1998; Ortiz-Delgado et al., 2005; Costa et al., 2011; Karami et al., 2011), while there is a lack of information about the possible effects on physiological endpoints. Therefore, the purpose of this thesis was to link the potential sublethal effects of BaP following intraperitoneal (i.p.) injection to the environmentally-relevant aqueous exposure route. Specific physiological endpoints that were examined included cardiac function, aerobic capacity, and swim performance. In addition, CYP1A gene expression alongside parent compound body burdens were analyzed to quantify BaP exposure in adult zebrafish.

The most significant finding of the current study was that acute BaP exposure in adult zebrafish resulted in significant negative effects on cardiovascular function and aerobic capacity

regardless of exposure route. Furthermore, this study demonstrated notable differences in these endpoints between i.p. injection and aqueous BaP exposure. Surprisingly, aqueous BaP exposure resulted in more severe effects on fish cardiac function and aerobic capacity compared to i.p. injection. The mechanism of cardiotoxicity between exposure routes also appeared to differ; with aqueous exposure having affected both heart rate and stroke volume, while only heart rate appeared to be affected by i.p. injection of BaP. Despite the significant effect of BaP on fish oxygen consumption (MO_2) and aerobic capacity, critical swimming speed (U_{crit}) remained unchanged regardless of exposure route. The different pathophysiological effects of BaP with exposure route were reinforced by differing patterns of CYP1A and *Atp2a2a* mRNA expression between zebrafish that underwent i.p. injection or aqueous exposure.

5.2 Effects of intraperitoneal injection of benzo[a]pyrene

I.p. injection of BaP resulted in significant pathophysiological effects in adult zebrafish following a 48 hr exposure period. In the present study, BaP-injected fish had elevated MO_2 and cost of transport (COT) compared to controls, which is in agreement with several studies where both of these parameters increased following exposure to toxicants, including PAHs (Shingles et al., 2001; Davoodi and Claireaux, 2007; McKenzie et al., 2007; Thomas et al., 2013). In general, increases in MO_2 and COT are considered indicative of greater energy requirements in fish, whether for routine energy maintenance or for forced increases in activity such as a U_{crit} test. Standard metabolic rate (SMR) was also increased with i.p. injection of BaP compared to controls, indicating that basal metabolism of BaP-exposed fish was elevated in response to toxicant exposure. This is consistent with previous studies which found increases in basal metabolism as a general stress response in fish exposed to PAHs and PAH-containing petroleum

distillate products (Pacheco et al., 2001; 2002; Tintos et al., 2008). This increase in basal metabolism in the present study may also represent increased energy requirements to maintain homeostasis, despite increased energy allocation to eliminate toxicants and/or repair toxicant-induced tissue damage (Calow, 1991; Bains and Kennedy, 2004). Since active metabolic rate (AMR) remained unchanged with BaP exposure, factorial aerobic scope (F-AS) was significantly decreased at all injection doses, indicating a reduction in aerobic capacity and ability to cope with environmental stressors. It has been shown that common sole (*Solea solea*) exposed to petroleum hydrocarbons exhibited reduced aerobic capacity and thus were unable to adequately cope with hypoxic conditions compared to unexposed fish (Davoodi and Claireaux, 2007). In contrast, in the present study adult zebrafish subjected to U_{crit} challenge tests following BaP injection demonstrated no significant differences when compared to control fish. A different kind of swim challenge may be needed to better assess the effects of BaP on swim performance, such as repeat U_{crit} or burst swimming tests. Indeed, a significant reduction was observed in repeat U_{crit} values in chub while initial U_{crit} remained unchanged following exposure to polluted river water (McKenzie et al., 2007). Exposure to dehydroabietic acid also resulted in reduced repeat U_{crit} performance in sockeye salmon (*Oncorhynchus nerka*) despite performing similar to control fish in an initial U_{crit} challenge (Jain et al., 1998). Furthermore, it may be that under normal housing conditions (i.e. well-fed with little variability in environmental conditions), BaP-exposed zebrafish are able to adequately cope with swim challenge tests. Based on the results of this and other studies, it warrants further investigation whether BaP-exposed fish are able to perform as well as unexposed fish when faced with environmental stressors such as hypoxia or acute temperature changes.

Cardiac function was significantly affected by acute i.p. injection of BaP in adult zebrafish. I.p. injection of BaP caused a significant decrease in ventricular heart rate (bradycardia), an effect that has been reported in fish embryos exposed to BaP (Huang et al., 2012; Incardona et al., 2011) and more recently in adult zebrafish exposed to the AHR agonist and model PAH, β-naphthoflavone (Gerger et al., 2014). In addition, in the present study BaP injection caused an increase in the ratio of atrial to ventricular heart rates (AV ratio) in adult zebrafish, a result found to be characteristic of AV conduction blockade and subsequent cardiac arrhythmias (Zipes, 1997). In fish, heart rate is primarily controlled by the intrinsic rhythm of pacemaker cell depolarization in the atrium (Olson and Farrell, 2006). AV block is due to impairments in the cardiac conduction system and typically results in a normal atrial heart rate but occasional failure of the ventricles to depolarize (Ramos et al., 2003). Since the ventricular bradycardia in BaP-injected fish is accompanied by a normal atrial heart rate compared to control fish, this lends further support to the occurrence of AV block in BaP-injected zebrafish. This is in agreement with a previous study which reported the occurrence of AV block in zebrafish embryos exposed to various PAHs (Incardona et al., 2004). In the present study, these results suggest an effect of BaP on cells in the AV node, which under normal circumstances transmit the electrical impulse generated in the sinoatrial (SA) node to the ventricles resulting in contraction (Ramos et al., 2003). This finding is consistent with a recent study by Brette et al. (2014) which examined the effects of crude oil on cardiac excitation-contraction coupling in juvenile tuna *(Thunnus albacares*) hearts. It was found that crude oil exposure affected the rectifier potassium current (I_{Kr}) resulting in prolongation of the action potential, likely by blocking K^+ channel pores in cardiomyocytes. Action potential prolongation is associated with bradycardia as well as severe arrhythmias in human patients (Kannankeril et al., 2010) and may potentially explain the effects

observed in this thesis with i.p injection of BaP. Despite the bradycardia and AV block associated with injection of BaP, stroke volume and cardiac output remained unchanged in BaP-injected fish when compared to controls. Since cardiac output is the product of heart rate and stroke volume, the reduction in heart rate coupled with no change in stroke volume was not sufficient to significantly alter cardiac output.

5.3 Effects of aqueous benzo[a]pyrene exposure

The effects of aqueous BaP exposure on zebrafish metabolism and swim performance followed a trend comparable to i.p. injection. Similar to i.p. injection, 48 hr aqueous BaP exposure resulted in elevated MO_2, COT, and basal metabolism (SMR), indicative of increased energy requirements in BaP-exposed fish compared to controls. In addition, F-AS was significantly decreased in fish aqueously exposed to BaP, suggesting that these fish had reduced aerobic capacity and ability to cope with environmental challenges (Davoodi and Claireaux, 2007). Despite these significant effects on aerobic capacity and metabolic rate, when fish aqueously exposed to BaP were subjected to swim performance tests, U_{crit} remained unchanged compared to unexposed fish, echoing the same trend observed for i.p. injection of BaP. Different types of swim performance tests or environmental challenges may be necessary to adequately determine whether the sublethal pathophysiological effects observed in these experiments translate into negative effects on zebrafish fitness.

Acute aqueous exposure to BaP resulted in notable effects on adult zebrafish cardiac function that differed slightly from those caused by i.p. injection. Aqueous BaP exposure resulted in ventricular bradycardia similar to i.p. injection which may be due to the effect of BaP on K^+ channels in the heart as discussed above. Interestingly, while i.p. injection of BaP resulted

in a significant increase in the AV ratio of exposed fish compared to controls, it remained statistically insignificant in aqueously-exposed fish. However, upon closer examination the magnitude of increase in the AV ratio between exposure routes is similar but the standard error associated with aqueous exposure is higher, rendering the results statistically insignificant. Therefore, it is possible that a Type II statistical error prevented detection in aqueously-exposed zebrafish of arrhythmias similar to those associated with i.p. injection of BaP. More importantly, while i.p. injection failed to elicit a significant reduction in cardiac output, aqueous BaP exposure resulted in a significant dose-dependent decrease in cardiac output after 48 hr. This is due to the reduced ventricular heart rate combined with a more notable reduction in stroke volume observed in aqueously-exposed zebrafish.

Changes in stroke volume are dependent upon end diastolic volume (preload), contractility, and arterial pressure (afterload) (Olson and Farrell, 2006). Since stroke volume is the difference between end diastolic volume (EDV) and end systolic volume (ESV), it is important to examine these two parameters to determine what is likely causing the change in stroke volume. In the present study, aqueous BaP exposure resulted in a reduction in both EDV and ESV, however the decrease in EDV was of greater magnitude than that of ESV, resulting in an overall reduction in stroke volume. Decreases in EDV are indicative of reduced ventricular filling, which may be due to reduced venous pressure or ventricular stiffness (Olson and Farrell, 2006). Ventricular stiffness is often attributed to chronic hypertension and subsequent fibrosis (Grossman et al., 1974) and is less likely to occur following a 48 hr exposure period. Therefore, it is more likely that venous pressure was decreased in BaP-exposed fish, resulting in the observed reduction in EDV. This is consistent with several studies which have reported the vasculature as a responsive target organ to BaP and other AHR agonists in fish. Significant

induction of CYP enzymes (Van Veld et al., 1997; Ortiz-Delgado et al., 2005; Bugiak and Weber, 2009) as well as those involved in prostaglandin synthesis, the cyclooxygenase (COX) enzymes (Bugiak and Weber, 2009), has been reported in fish vasculature following BaP exposure. It should be noted that EDV and ESV are not independent of each other, and changes in one often induce compensatory changes in the other (Klabunde, 2011). The reduction in ESV following BaP exposure is likely a compensatory mechanism following the reduction in EDV in an unsuccessful attempt to maintain adequate stroke volume, since stroke volume ultimately decreased with BaP exposure. It is unlikely that contractility was significantly affected by aqueous BaP exposure, since ESV should have instead increased if this were the case, resulting in a reduced stroke volume, but this was not observed. In summary, the effects of 48 hr aqueous BaP exposure on adult zebrafish included ventricular bradycardia, which was also observed with i.p. injection, in addition to a more notable effect of reduced EDV that may be explained by negative effects on preload and the vasculature.

5.4 Comparison between intraperitoneal injection and aqueous benzo[a]pyrene exposure

In the current study, analysis of BaP body burdens allowed for identification of overlapping doses between exposure routes. The injection dose of 1000 µg/kg and aqueous concentration of 16.2 µg/L both produced body burdens of approximately 65 µg BaP/kg dry mass (d.m.) in adult zebrafish (Table 4.1). Select major endpoints were then compared between exposure routes at 65 µg BaP/kg d.m. using two sample t-tests (Table 4.2). In the present study, both i.p. injection and aqueous exposure to BaP resulted in significant induction of CYP1A mRNA in adult zebrafish after a 48 hr exposure period. This is consistent with the literature where it is accepted that AHR agonists cause CYP1A induction in multiple species and tissue

types (Billiard et al., 2002; Nebert et al., 2004). However, different levels of CYP1A expression were observed in the liver, heart, and skeletal muscle at body burdens of 65 µg/kg d.m. between exposure routes. CYP1A expression was more significantly induced in the liver following i.p. injection, while skeletal muscle CYP1A expression was only induced in aqueously-exposed fish. I.p. injection often results in contaminants first entering the hepatic circulation where they may be more rapidly metabolized (Rozman and Watkins, 2003). This is consistent with the greater hepatic CYP1A induction observed in this study as well as several others utilizing i.p. injection of BaP (Lemaire et al., 1990; Levine et al., 1994; Boleas et al., 1998; Karami et al., 2011). Furthermore, it is unlikely that parent BaP injected through the i.p. cavity would come into contact with skeletal muscle without initial biotransformation from CYP enzymes in other organs. In contrast, aqueous BaP resulted in higher CYP1A expression in skeletal muscle than liver, which is in agreement with studies that have shown greater CYP1A expression in extrahepatic tissues with aqueous BaP exposure (Costa et al., 2011; Rey-Salgueiro et al., 2011).

Overall, CYP1A was significantly induced in the heart with both routes of exposure, implying the presence and metabolism of parent BaP in this tissue which is consistent with the pathophysiological effects seen in the heart in this study. Surprisingly, at 65 µg/kg d.m. BaP, all metabolic and cardiac physiological endpoints remained statistically unchanged between exposure routes with the exception of heart rate; BaP injection resulted in more significant bradycardia than aqueous exposure. Although not statistically significant, at a body burden of 65 µg BaP/kg d.m., i.p. injection also resulted in a 13-fold induction in heart CYP1A expression compared to control while aqueous BaP caused a 7-fold induction. This 6-fold greater CYP1A induction may explain the greater bradycardia observed with i.p. injection compared to aqueous BaP, because it implies more parent BaP present and undergoing metabolism in the heart. In

addition to notable differences between heart CYP1A expression, i.p. injection of 1000 µg/kg BaP resulted in a significant induction of *Atp2a2a* mRNA expression while aqueous exposure failed to cause a significant change. This increased expression is in contrast with a previous study that reported decreased mRNA expression of *Atp2a2a* following exposure to phenanthrene, a 3-ringed PAH, in zebrafish embryos (Zhang et al., 2013). These conflicting results may represent differences in PAH effects between developmental and adult fish exposure scenarios, as well as between BaP and phenanthrene. Interestingly, another recent study saw increased *Atp2a2a* expression in the larval descendants of zebrafish exposed to pyrolytic PAHs, but this was accompanied by an increase in heart rate (Lucas et al., 2014). Rather than being a direct effect of AHR gene activation, the observed induction of *Atp2a2a* in the present study may instead be a compensation mechanism in response to the bradycardia caused by acute BaP injection. Indeed, increases in SERCA2a (aka *Atp2a2a*) have been shown to alleviate symptoms associated with heart failure in the rat model (Maier et al., 2005).

When considering the effects of 48 hr BaP exposure without focusing on the overlapping dose of 65 µg/kg d.m., it is apparent that both routes of exposure were sufficient to cause sublethal toxicity in adult zebrafish. I.p. injection of BaP resulted in significant bradycardia with evidence of arrhythmias. However, the effects of aqueous exposure were of overall greater magnitude than those caused by i.p. injection. Aqueous BaP exposure caused a significant dose-dependent reduction in cardiac output due to bradycardia as well as decreased stroke volume. The decreased stroke volume with aqueous BaP was due to a significant reduction in EDV, which, as discussed above, are likely linked to reduced preload and reduced venous pressure after 48 hr aqueous BaP exposure, but not after 48 hr i.p. injection. Significant induction of CYP1A was observed in the zebrafish vasculature following aqueous BaP exposure in other

studies, indicating the presence of a transcriptionally active AHR (Van Veld et al., 1997; Ortiz-Delgado and Sarasquete, 2004; Bugiak and Weber, 2009). Typically, aqueous exposure results in toxicants being absorbed through the gills and introduced directly into the systemic circulation where the vasculature is the primary organ that is initially exposed (Rozman and Watkins, 2003). The results observed in this study are therefore consistent with the toxicokinetics associated with aqueous exposure in fish.

The more significant cardiovascular effects associated with aqueous BaP exposure may be attributed to either the specific way in which BaP is being taken up and distributed compared to i.p. injection, or it may simply be that more BaP bioaccumulated with aqueous exposure resulting in a higher exposure dose. Comparing the effects at 65 µg/kg d.m. allows us to distinguish between these two hypotheses. While CYP1A expression is significantly changed with exposure route, the differences between pathophysiological effects at similar body burdens are minimal. With the exception of greater bradycardia with i.p. injection, all other metabolic and cardiac endpoints remained unchanged between exposure routes. This indicates that it is a higher dose of BaP due to increased bioaccumulation that is responsible for greater cardiotoxicity observed with aqueous exposure. Disadvantages of waterborne exposure to PAHs typically include the potential for photodegredation (Kot-Wasik et al., 2004) as well as difficultly attaining concentrations of lipophilic contaminants (such as BaP) in amounts sufficient to facilitate adequate uptake in biota. However, previous studies have shown that dose-dependent responses to PAHs at concentrations above the water solubility limit can be obtained in static, small volume exposures (Incardona et al., 2011). Therefore, in the present study water concentrations of 16.2 and 162 µg/L were chosen as 10 and 100 times higher than the reported water solubility limit of BaP which is 1.62 µg/L at 28°C (May et al., 1983). This was done to ensure adequate

uptake while remaining near reported environmental concentrations of PAHs (Diercks et al., 2010; Zhang et al., 2012; Lohmann et al., 2013). Despite the lipophilic nature of BaP, aqueous exposure resulted in higher body burdens, greater CYP1A induction, and more significant physiological effects in most cases than i.p. injection, demonstrating the usefulness of aqueous exposure as an environmentally relevant option to evaluate sublethal effects in fish. I.p. injection also proved sufficient to facilitate uptake of BaP, however, as mentioned previously in this thesis, at similar body burdens BaP had slightly different cardiotoxic effects with i.p. injection. Results examining physiological endpoints with i.p. injection should therefore be interpreted carefully, as it does not simulate an environmental exposure.

5.5 Study strengths and deficiencies

The present study successfully evaluated the effects of acute BaP exposure on adult zebrafish cardiac function and fitness using two different routes of exposure. This work coupled with my previous study (Gerger et al., 2014) has been the first to characterize the effects of PAH exposure on adult zebrafish cardiac function *in vivo* using high frequency ultrasound techniques. Other studies have also utilized high frequency ultrasound to measure adult zebrafish cardiac function, however ours is the first to use this technique from a toxicological perspective. Furthermore, our study used parent compound body burdens, which are considered the gold standard for evaluating toxicant exposure, in addition to CYP1A mRNA induction in different tissue types. These techniques together allowed for a high level of certainty when discussing the observed effects of BaP in exposed zebrafish.

One aspect that could have improved the present work was analysis of BaP metabolites in addition to parent compound body burdens. This would have aided in determining how much biotransformation BaP was undergoing in adult zebrafish. Furthermore, due to the nature of the

images obtained using high frequency ultrasound in fish, it was necessary to measure the outer circumference of the whole ventricle. Therefore, included in the volume measurements are both the spongy and compact layers of the heart. As a result, these volumes cannot be compared to mammalian heart function values. Fish ultrasound also necessitates the use of an anesthetic for obvious reasons, and by their nature anesthetics have an effect on heart function. However, because the same technique and anesthetic was used for all control and BaP-exposed fish, we can be confident that the effects observed are a result of BaP-exposure.

5.6 Conclusions

In conclusion, the present thesis has shown that acute BaP exposure has significant sublethal toxicity in adult zebrafish. In particular, zebrafish aerobic capacity and cardiovascular function were negatively affected by BaP exposure. A comparison of these sublethal effects revealed notable differences between exposure routes. Aqueous BaP exposure resulted in significant effects on fish heart rate and stroke volume, resulting in reduced cardiac output. In contrast, only zebrafish heart rate was affected after i.p. injection of BaP. Both exposure routes elicited tissue-specific changes in CYP1A expression, indicative of different toxicokinetics between exposure routes. These results offer valuable insight into not only the mRNA expression changes with acute BaP exposure but also the physiological consequences, and how these may or may not be affected by exposure route. Furthermore, the information provided in this thesis can aid in extrapolating effects seen in laboratory injection studies to environmental exposure scenarios. While the exact mechanism of toxicity cannot be elucidated from this study alone, it is becoming clear that the cardiovascular system is an important target organ for PAH toxicity in fish. Proper cardiac function is paramount to fish survival, and if the sublethal effects of acute PAH exposure persist following short exposure windows, this could have vastly detrimental

effects on individual fish fitness and survivability. Future studies should focus on determining the mechanism of BaP cardiotoxicity in adult fish, as well as investigating the persistence of these effects once exposure has been terminated.

5.7 Future work

While this study answered important questions regarding PAH toxicity in adult fish, there are many more to be answered. In particular, the exact mechanism of toxic action of PAHs is still mostly unknown. Determining whether or not the effects seen in this study are AHR- and/or CYP1A-dependent is an important question to answer. Additionally, it has been shown that embryonic PAH exposure in fish results in negative effects on heart function and aerobic capacity once fish are reared to adulthood (Hicken et al., 2011). Even further, the effects of PAH exposure can still be seen in the F1 progeny of exposed fish (Corrales et al., 2014). Whether the effects seen with acute exposure in the present study are persistent or transient following cessation of exposure is another direction that future studies should follow.

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