

Swim Performance as an Effective, Environmentally Relevant Measure of Sublethal Toxicity in
Zebrafish (*Danio rerio*)

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Abstract

Examination of the swimming capabilities of fish is increasingly being considered as an effective method for determining sublethal toxicity. Acute toxicant exposure is known to cause decreases in swim performance in fish but less is known about how developmental exposure can cause persistent effects that hinder swimming. In addition, little is known about how triglyceride levels fluctuate during fish swimming upon both acute and developmental exposure to toxicant. In this thesis, two studies, one acute and one developmental, were carried out using two different toxicants in order to address these issues.

In order to examine acute effects, adult zebrafish (*Danio rerio*) were exposed to ethanol vehicle or increasing concentrations of 2,4-dinitrophenol (DNP), a mitochondrial electron transport chain uncoupler, for a 24 h period. Following exposure, fish were placed in a swim tunnel for critical swimming speed (U_{crit}) determination and swim motion analysis. Whole body triglyceride levels were then determined. U_{crit} was decreased in a concentration dependent manner in both the 6 mg/L and 12 mg/L DNP exposure groups, with 6 mg/L DNP being considered sublethal and 12 mg/L approaching the LC_{50} . A decrease in tail beat frequency was observed and is likely the main cause for the decrease in U_{crit} in the DNP exposure groups. Triglyceride levels were elevated in a concentration dependent manner in the DNP exposure groups. This increase in triglyceride stores may be due to a behavioral adaption limiting swimming capabilities or due to a direct toxic action of DNP on lipid catabolism.

The second study examined whether developmental 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure would cause persistent toxic effects. Zebrafish embryos were exposed to dimethyl sulfoxide control or increasing concentrations of TCDD between 2-4 days post

fertilization (dpf). At 5 dpf, cytochrome P450 1A (CYP1A) activity was determined. Fish were raised to 90 dpf with mortalities and deformities being recorded at 5 dpf, 10 dpf, and 90 dpf. At 90 dpf, fish were placed in swim tunnel and U_{crit} , swimming motion, and aerobic scope (oxygen consumption rate during exercise minus oxygen consumption rate during rest) were determined. Following swimming, some fish were used for whole body triglyceride analysis while others were used for histological examination. U_{crit} was shown to be decreased in the two highest sublethal TCDD exposure groups (0.1 and 1 ng/L) but not in the lowest TCDD exposure group (0.01 ng/L). The exact cause of the decrease in U_{crit} is not known, but may be linked to the observed decrease in dorsal aorta diameter, an inability to mobilize triglyceride stores, behavioral adaptations limiting swimming, decreased body length, or a combination of these factors. This TCDD related defect in swimming ability is not due to any increases in gross deformity or mortality rates, nor does it appear that CYP1A induction is required to mediate the toxic effects. Thus, it appears that examination of swim performance may serve as an effective measure of both sublethal acute and developmental toxicities.

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List of Abbreviations

- A- atrium
- AHR- aryl hydrocarbon receptor
- ANCOVA- analysis of covariance
- ANOVA- analysis of variance
- ARNT- aryl hydrocarbon receptor nuclear translocator
- ATP- adenosine triphosphate
- BA- bulbus arteriosus
- BL/s- body lengths per second
- CCV- common cardinal vein
- CFM- craniofacial malformation
- COX- cyclooxygenase
- CYP- cytochrome P450 monooxygenase
- DA- dorsal aorta
- DMSO- dimethyl sulfoxide
- DNP- dinitrophenol
- dpf- days post fertilization
- DRE- dioxin response element
- EROD- ethoxyresorufin-*O*-deethylase
- Hsp90- 90 kDa heat shock protein
- hpf- hours post fertilization
- LC₅₀- concentration causing 50% lethality of a group
- LC₁₀- concentration causing 10% lethality of a group

LSD- least significant difference

M_{O_2} - mean oxygen consumption rate

MS-222- tricaine methane sulphonate

n- number of individuals in a sample (number of replicates)

p- p-value

PCB- polychlorinated biphenyl

PCE- pericardial edema

PCDD- polychlorinated dibenzodioxin

PCV- posterior cardinal vein

r- Pearson correlation coefficient

SC- spinal curvature

SEM- standard error of the mean

SV- sinus venosus

TCDD- 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

U_{crit} - critical swimming speed

V- ventricle

v/v- volume of solvent per volume of diluent

w/v- weight of solute per volume of solvent

YSE- yolk sac edema

1.0 Introduction

Historic environmental regulations are based primarily on examining mortality of fish exposed to a particular toxicant. More recently, reproductive endpoints have been included in these regulations and other sublethal toxicities such as behavior are increasingly being considered. Therefore, this thesis will examine sublethal toxicity using other potentially ecological relevant endpoints, namely swim performance and energy usage.

1.1 Sublethal, Lethal, and Developmental Toxicity

Sublethal toxicity refers to a detectable negative change in some functional, biochemical, or molecular aspect of a toxicant-exposed individual that does not lead to death. This toxicity can result in detrimental effects to the species or may have no effect at all. This is in contrast to lethal toxicity, which refers to a toxicant eventually causing mortality in the examined individual or group of individuals. Developmental toxicity refers to the exposure of a specimen to a toxicant during key stages in early development before complete differentiation into particular tissues (Westernhagen, 1988). If the exposure is both sublethal and developmental, the detrimental effects have the potential to be irreversible.

Many environmental contaminants are present in smaller amounts than would normally cause acute toxicity and death. Some sublethal toxicity end points include but are not limited to monitoring immune function (McMurry *et al.*, 1995), genotoxicity (McBee *et al.*, 1987), and reproduction (Kendall *et al.*, 1990). In addition, there are a whole host of behavioral endpoints

that may be affected by sublethal toxicant exposure. Though not immediately apparent, sublethal toxicity has the potential to decrease health in a large number of fish sufficient to cause population level effects.

Early developmental stages tend to be the most sensitive to toxicants (Westernhagen, 1988). Development is characterized by rapid change both on a biochemical and physiological level, requiring an elegant interplay of gene upregulation and downregulation, signaling cascade stimulation or attenuation, as well as a host of transcriptional, translational, post-translational, and epigenetic factors (Rogers and Kavlock, 2001). Activation and inactivation of any of these processes follow a particular pattern and timeline. Interruption of this timeline by toxicant exposure can lead to severe developmental effects that are often irreversible (i.e. teratogenic effects). If the toxicant concentration is high enough then the effect is often death. However, at sublethal concentrations, developmental toxicant exposure may cause measureable side effects that persist to adulthood.

1.2 Toxicants and Energy Utilization

The bioenergetics of an ecosystem refers to the transfer and utilization of energy amongst the different organisms that make up that system (Nisbet *et al.*, 2000; Kelly and Janz, 2008). Lipids serve as the main source of energy stores in fish and are responsible for mediating the over winter survival of fish populations (Lemly, 1993). Several studies have examined the role of triglyceride levels in overall fish health (Henderson *et al.*, 1988; Brown and Taylor, 1992). Many have examined effects of whole effluent toxicity on energy stores since effluents are the main source of pollutants in aquatic ecosystems (Chapman, 2000; Smolders *et al.*, 2003). Most

studies indicate that triglyceride levels are decreased in polluted environments (Munkittrick and Dixon, 1988; Levesque *et al.*, 2002) as the fish species are devoting stored energy to deal with detoxification processes (Calow, 1991; Benton *et al.*, 1994; Adams, 1999) as well as to tissue damage and cellular repair (Campbell *et al.*, 2003). In fact, serum triglyceride levels have been shown to be highly negatively correlated with contaminant levels in fish (Adams *et al.*, 2000). In contrast, a few studies have indicated higher triglyceride levels in fish isolated from polluted sites relative to control sites (Kelly and Janz, 2008; Driedger *et al.*, 2009). A laboratory study showed that exposure of zebrafish to the fungicides tebuconazole and tricyclazole also increased whole body triglyceride levels (Sancho *et al.*, 2009; Sancho *et al.*, 2010). This suggests that the role of triglycerides in response to toxicants is more complex than originally thought.

1.3 Swim Performance in Fish

Examination of swim performance serves as an important ecologically relevant measurement of several different factors. Swim performance allows for the evaluation of such aspects as predator evasion, food acquisition, and overall survival (Hammer, 1995). With respect to food acquisition, decreased consumption would lead to a decrease in the energetic stores of the fish (i.e. a decrease in triglyceride and glycogen levels) (Weber *et al.*, 2003). In addition, fish spawning activities can require a return to natal spawning areas, a migration that can be hundreds of kilometers long for salmonids. Such an endeavor requires a high fitness level in several fish species and a decrease in this swimming fitness may lead to a lower number of fish returning to their breeding ground (Veldhoen *et al.*, 2009). Salmonids sublethally exposed to crude oil were less likely to return to native spawning grounds (Heinz *et al.*, 2000) and there

has recently been a decrease in returning salmon to the British Columbia Fraser River system (Hanson *et al.*, 2008; Veldhoen *et al.*, 2009). A decrease in the fish's potential for swimming performance may provide an explanation for the reduced numbers in both instances. Therefore, along with measurements of reproductive fitness, measurements of fish swim performance may serve as an ecologically relevant method of determining population level effects.

1.3.1 Critical Swimming Speed

There are several methods for measuring the swimming capabilities of fishes, the most common and easiest being determination of the critical swimming speed (U_{crit}) developed by Brett in 1964. For this method, fish are introduced to a swim tunnel and are forced to swim against a water current at known velocities. The flow velocity is increased by a prescribed increment (U_{ii}) for a prescribed amount of time (T_{ii}). At the end of the time interval, the flow velocity is increased again by the prescribed increment and the fish swims for the prescribed time period. After several intervals and flow increases, the fish becomes fatigued and can no longer swim against the current. Critical swimming speed can now be calculated (Brett, 1964 and 1967):

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

where U_i is the highest velocity maintained by the fish for the entire time interval and T_i is the time elapsed at the fatigue velocity. Thus, U_{crit} becomes the highest sustained velocity plus the fraction of the velocity maintained at the fatigue velocity. Hammer (1995) suggested that U_{crit} may be more informative than LC_{50} values (concentration of toxicant causing 50% lethality of group) on a toxicological and ecological fitness basis. Whereas LC_{50} tests examine effects of acute exposures to toxicants and look at one endpoint (death), U_{crit} takes into account both the

chronic and sublethal effects of these toxicants. The sum of all non-acute effects is far more ecologically relevant to polluted habitats and has relevance to effects seen at the population level.

When looking at the effects of bleached kraft pulpmill effluent on swimming performance of juvenile coho salmon, Howard (1975) found that critical swimming speeds were significantly decreased in fish exposed acutely during adulthood to effluent concentrations that were 20% of the LC₅₀. After aerobic fermentation, lower concentrations of effluent showed no toxicity while higher concentrations of fermented effluents still showed decreased swim performance. Howard suggested that this might be the result of dyes and lignin materials that are unaffected by aerobic fermentation. However, it may also have been the result of persistent organic compounds such as polychlorinated dibenzodioxins (PCDDs) which would remain after treatment. When examining whole effluent toxicity on a particular measurable end point, it becomes very difficult to pinpoint the compound responsible for the adverse effect as the effluent may contain numerous toxicants (Smolders *et al.*, 2003).

Most studies have examined acute effects of toxicants during the adult stage of fish. The mitochondrial electron transport chain uncoupler, rotenone, was shown to cause a decrease in the critical swimming speed of trout when exposed acutely for 96 hours (Cheng and Ferrell, 2007). Ammonia can also significantly decrease swimming performance in fish (Tudorache *et al.*, 2008). Since ammonia is a byproduct of fish metabolism, its levels must be monitored to ensure it is not contributing to declines in swim performance while examining the toxicant effects with critical swimming speed. Other studies have also reported decreases in U_{crit} upon exposure of fish to various environmental contaminants such as sodium pentachlorophenate, cyanide, organophosphate pesticides, and metals (Webb and Brett, 1973; Kovacs and Leduc, 1982; Cripe

et al., 1984; Beaumont *et al.*, 1995; DeBoek *et al.*, 2006). The majority of these studies examine acute effects at relatively high levels of toxicant, levels that are most relevant at spill sights or sights of extensive industrial use. Few studies have examined the effects that either long term or developmental exposure may have in affecting fish swimming capabilities. For example, acute effects of pulpmill effluent in juvenile salmon were shown to be reversible once the fish was transferred to a clean medium (Howard, 1975). However, this reestablishment of control U_{crit} values may not be possible in fish exposed long term to harmful chemicals. Low level exposure at a developmentally sensitive life stage may also result in long term effects that translate to persistent alterations in swimming capabilities in adulthood. Examining the link between developmental toxicity and adult physiological performance will be a major topic of this thesis.

1.3.2 Swim Motion

While U_{crit} is a good measure of swim endurance, it does not provide information on how the fish is swimming. Swim motion analysis is a complimentary method of examining swim performance to U_{crit} . This type of analysis does not look at the maximum sustained speed or overall endurance, instead it focuses on the way in which the fish swim. Ohleberger *et al.* (2006) found that the body form differences could account for differences in swimming efficiencies between species. Those fish with better streamlining had a more “optimum” body form for efficient swimming. Traditionally, there are four classification modes that define the kinematics of fish that use their bodies and caudal fins for propulsion (Webb, 1975). These four modes are distinguished based on the length of the propulsive wave that travels down the body during

steady swimming and the percentage of the body that undergoes lateral undulatory movements (Webb, 1975).

- (1) Anguilliform mode is characterized by whole body participation in the undulatory movement with a wavelength of a least one-half the body length and is usually more than a full body length. An example would be eel species (*Anguilla anguilla*).
- (2) Subcarangiform mode is characterized by more than half the wavelength maintained within the body length. The wave amplitude increases towards the posterior of the fish. Examples include *Salmonidae* family (salmon and trout) as well as *Cyprinidae* family (minnows and carp) and zebrafish (*Danio rerio*).
- (3) Carangiform mode is characterized by body and caudle fin usually thrown into a wave, with up to one half-wavelength within the length of the body and an increasing wave amplitude over the posterior. Examples are many and include the mackerels (suborder *Scombridae*).
- (4) Ostraciform/Thunniform is characterized by a rigid body that is not thrown into a wave. Propulsion is via caudal fin oscillation only. Examples include tunas (suborder *Scombridae*).

Most small fishes, including zebrafish (*Danio rerio*; the test species used in this study), are considered to be subcarangiform swimmers (Plaut and Gordon, 1994). This general assumption has not been completely validated, but will be assumed to be true for the purpose of this thesis. When examining the swimming metabolism of zebrafish, Plaut and Gordon (1994) found that zebrafish were capable of swimming at rapid speeds (40 cm/s, 14 body lengths/s) for long periods of time (up to two hours) and that this swimming level is maintained aerobically without significant fatigue. Despite predominantly aerobic swimming, lactate buildup (an

anaerobic metabolism product) was significantly increased when compared to resting zebrafish. These relatively high swimming speeds (when compared to larger fish) can be explained by shorter contraction times and higher tail-beat frequencies in small fishes (Wardle, 1975). The wild type zebrafish U_{crit} was 56.0 ± 4.8 cm/s or 15.5 body lengths/s which is the fastest reported data for any small fish (Plaut, 2000). These speeds are much higher than those reported earlier in an earlier zebrafish study (4.2 – 6.55 body lengths/s; Fuiman and Webb, 1988).

Though more recent techniques for measuring kinematics make use of x-ray photography to look at curvature of the vertebrae during swimming (Donley and Dickson, 2000), a simple measurement of caudal fin height and curvature can be just as effective (Bainbridge, 1963). Tail beat frequency and tail beat amplitude are also appropriate measurements when examining swim performance since they are direct determinants of the swimming speed of the fish (Bainbridge, 1957). Both of these measurements will be used with U_{crit} in this thesis to characterize toxicant effects on zebrafish swimming.

1.3.3 Triglycerides and Swim Performance

Triglycerides are an important energy source for fishes as a whole (Weber *et al.*, 2003; Bennett *et al.*, 2007) and are especially important during endurance swimming (Chatelier *et al.*, 2006; Magnoni and Weber, 2007) since locomotion accounts for a large portion of the expended energy budget (Boisclair and Sirios, 1993). Lipids serve a very important role in determining the health of fish populations and are important for overwinter survival, reproductive performance, dealing with environmental stress, and maintaining overall fitness (Adams, 1999). Smolders *et al.* (2003) examined the effects of whole effluent toxicity on zebrafish lipid levels and found that

the change in lipid concentration was very sensitive to toxicant exposure. Exposure to 75 and 100% effluent caused dose-dependent decreases in lipid budget within the first week of a 28-day exposure. In fact, it appears that many things that alter fitness also alter swimming ability in fish. Chatelier *et al.* (2006) found that varying the fatty acid composition of the fish diet can alter critical swimming speeds. When food availability was altered in a previous study so that the condition factor of fish ($\text{weight}/\text{length}^3$, a commonly used indicator of fish ‘fatness’) was decreased, U_{crit} was also decreased (Lapointe *et al.*, 2006). This highlights the important link between energy stores and physiological performance. Thus, the depletion of triglyceride stores is likely to affect the swimming performance of fish and in turn, may have effects at a population level. Therefore, this thesis seeks to examine the relationship between depleted energy stores caused by toxicant exposure and swim performance.

1.3.4 Metabolic Rate and Swimming Performance

Metabolic rate, measured as the mean oxygen consumption rate (MO_2), increases as critical swimming speed increases (Blank *et al.*, 2007; Ohlberger *et al.*, 2007). Thus, fish with higher U_{crit} values tend to have higher MO_2 values. Metabolic rates can be altered by changes in condition factor (Lapointe *et al.*, 2006), fatty acid composition (Chatelier *et al.*, 2006), temperature (Claireaux *et al.*, 2006; Ohlberger *et al.*, 2007), and toxicant exposure (Hammer, 1995). A previous study examining acute effects of the mitochondrial electron transport chain uncoupler, rotenone, on MO_2 showed that peak active oxygen uptake in juvenile rainbow trout was impaired (Cheng and Farrell, 2007). In addition, critical swimming speed was reduced at ≥ 3 $\mu\text{g}/\text{L}$ rotenone. Since rotenone is a protonophore that abolishes the proton gradient in the

mitochondria, electron transport results in the generation of heat rather than the production of adenosine triphosphate (ATP; Nedergaard and Cannon, 2003). Therefore, rotenone would deplete ATP and decreased swimming ability is a logical consequence.

Aerobic scope is an effective method for examining the metabolic capacity of fish. It is determined by subtracting the basal metabolic rate or the resting metabolic rate from the maximal metabolic rate during activity. Thus, it represents the ability of the fish to perform oxygen-consuming functions above the minimal metabolic requirements (Djawdan *et al.*, 1997; Bohdansky *et al.*, 2005; Killen *et al.*, 2007). A reduced aerobic scope could signify a compromised ability to respond to stressors in a physiological manner (Killen *et al.*, 2007) and effects of toxicants on aerobic scope have never been reported.

Polychlorinated biphenyl (PCB) exposure in European eel (*Anguilla anguilla* L.) resulted in significantly lower metabolic rates while swimming long distances (Ginneken *et al.*, 2009). During an 800 km migration test, it was found that PCB exposure significantly reduces oxygen consumption rate at the 400 km mark. Since critical swimming speed and swim motion analysis were not measured in this eel study, it is not known whether PCBs altered the efficiency of metabolism or whether the fish were swimming differently to compensate for increased metabolic demand from the toxicant exposure. Metabolic rates have also been linked with a few swim motion endpoints. For example, bluefin tuna had a higher metabolic rate during swimming tests and also had higher tail beat frequencies and shorter stride lengths (length traveled with one tail beat) than yellowfin tuna (Blank *et al.*, 2007).

Only a select group of studies have examined oxygen consumption during swim tests (Plaut and Gordon, 1994; Plaut, 2000) and toxicant exposure (Smolders *et al.*, 2002) in zebrafish. Being a small fish species with a high respiratory rate, zebrafish have a relatively high metabolic

rate that can vary between 0.60-1.54 mL O₂ g⁻¹ h⁻¹ (Plaut and Gordon, 1994). When exposed to effluent from an industrial plant for 28 days, Smolders *et al.* (2002) found that oxygen consumption increased after 14 days when compared to controls and continued to rise the following week. Their measurements were obtained in the absence of any endurance tests such as U_{crit} determination. Therefore, a goal of this thesis was to examine how oxygen consumption is changed by toxicant exposure in zebrafish during U_{crit} measurements.

1.4 Zebrafish as a Model Fish Species in Toxicology

Zebrafish (*Danio rerio*) are used extensively to study developmental toxicity, especially developmental cardiovascular and neurological toxicity (for reviews see Hill *et al.*, 2005; Goldstone and Stegeman, 2006). However, critical swimming speed and swim motion analysis have been less extensively studied in zebrafish than in other fish species (Plaut, 2000). Zebrafish are genetically, morphologically, and developmentally well characterized. Their upkeep and maintenance are relatively inexpensive and non-intensive. They are capable of producing a large amount of embryos continuously and reliably with the optimal clarity, transparency, and accessibility of the embryos allowing for easy developmental monitoring and genetic manipulation. Their rapid maturation (~100 days post fertilization or dpf) allows for adult scale tests within a reasonable time frame and their sequenced genome makes molecular studies more feasible. In addition, the availability of many transgenic and mutant zebrafish lines enables effective monitoring of various genes and development of specific organs (i.e. vasculogenesis; for reviews see Carven *et al.*, 2000; Weinstein, 2002; Lawson and Weinstein, 2002).

1.5 Zebrafish Cardiovascular System

1.5.1 Development

Zebrafish cardiovascular development begins with the formation of a primitive heart tube composed of both cardiomyocytes and endothelial cells at around 24 hours post fertilization (hpf) (Nemer and Nemer, 2001). This heart tube begins beating shortly after and cardiac looping of this tube, which places the atrium alongside the ventricle, takes place at 36 hpf (Dooly and Zon, 2000; Goldstone and Stegeman, 2006). Hatching is complete at 48 hpf and is followed by further development including erythropoiesis. It is important to note that the critical time period for developmental toxicity in the zebrafish cardiovascular system appears to be between 0 hpf and 96 hpf (i.e. within the first five days of life). A hypothesis of this thesis is that exposure during this sensitive developmental time frame will result in irreversible sublethal toxic effects that persist into adulthood.

1.5.2 Normal Anatomy and Function in Adulthood

Fish circulation is characterized by a unidirectional flow meaning that blood is pumped via the ventral aorta to the gills before oxygenated blood enters the systemic circulation via the dorsal aorta (Figure 1.1; Hu *et al.*, 2001). Pumping is accomplished via one atrium and one ventricle. Blood collects at the sinus venosus via large veins such as the common cardinal vein and is channeled into the atrium. From the atrium blood is pumped into the ventricle which then pumps the blood into the highly elastic bulbus arteriosus. The bulbus arteriosus provides a

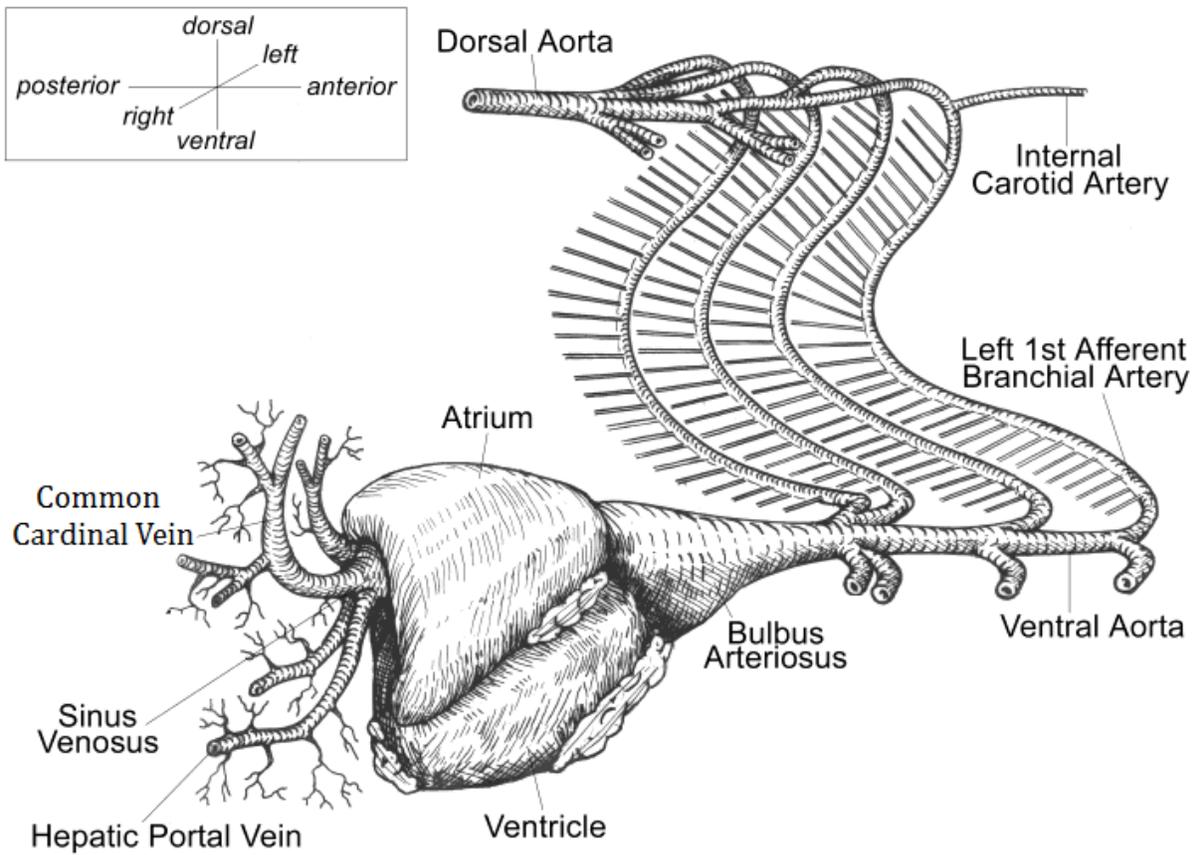


Figure 1.1. Diagram of the adult zebrafish heart. A diagrammatic representation of the adult zebrafish heart is presented. (Figure from Hu *et al.*, 2001).

pressure dampening effect similar to the mammalian aorta, allowing for the gradual release of blood into the systemic circulation. Blood is channeled to the gills via the ventral aorta and is oxygenated before being delivered to the peripheral tissues. Aerobic swimming is dependent on the continual supply of oxygen. Thus, a healthy cardiovascular system is essential for endurance swimming in fish.

1.6 DNP Toxicity

1.6.1 Chemistry, Environmental Sources, Fate, and Concentrations

Dinitrophenols are a purely synthetic class of organic compounds. They are found in several forms but the term usually refers to 2,4-dinitrophenol (DNP) (Harris and Cocoran, 1995) (Figure 1.2). They are used commercially for several different processes including dye and explosive manufacturing, as a photographic developer, as an antifungal in wood treatment and in several other pest control management strategies (Harris and Cocoran, 1995). In the environment, they are found in 61 of the sites that the US Environmental Protection Agency considers a priority for national clean up. DNP enters air, water and soil in several different ways. Primarily, DNP environmental release is during its manufacture and use. However, other anthropogenic sources include automobile exhaust, creation due to reaction of air pollutants, through spill sites and ground water seepage from contaminated landfills. Once in an aquatic system, bacterial processes are responsible for the majority of the break down (Harris and Cocoran, 1995). Environmental concentrations of DNP can be variable with the largest

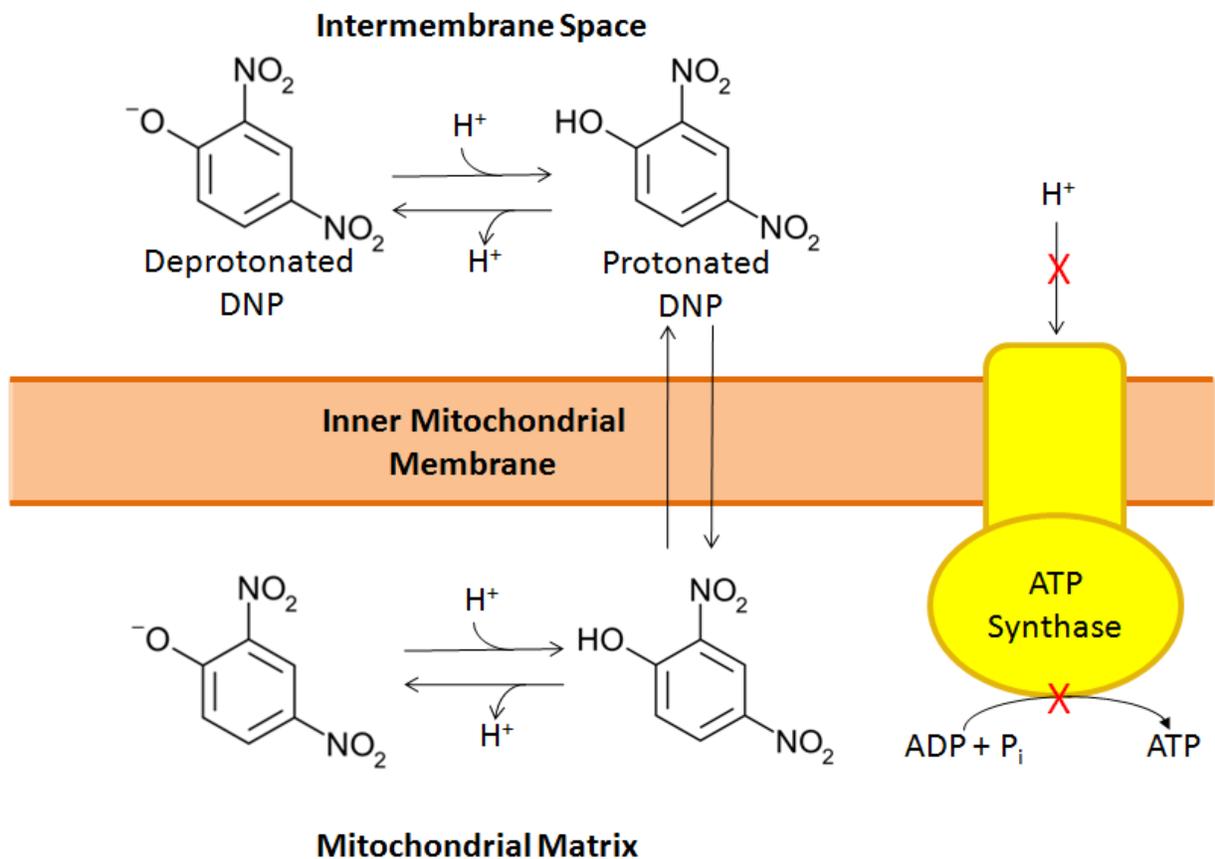


Figure 1.2. Mode of Action of 2,4-Dinitrophenol Toxicity. The protonophore activity of 2,4-dinitrophenol (DNP) is due to the hydroxyl group which can readily accept protons in the acidic intermembrane space of the mitochondria and transport them across the inner mitochondrial membrane, thus abolishing the proton gradient (indicated by the red Xs) that is otherwise used to drive production of ATP from the ATP synthase.

concentrations (up to 3 mg/L in waste water and 30 mg/L in ground water) observed in spill sites or sites where DNP was either used or manufactured (Harris and Cocoran, 1995).

1.6.2 Known Mechanisms of DNP Toxicity

In living cells, DNP acts as a proton ionophore that transports hydrogen ions across the inner mitochondrial membrane, bypassing the ATP synthase channel (Blakie *et al.*, 2006). This collapses the proton motive force in the mitochondria and uncouples the transport of electrons from the production of ATP. The energy that is lost is given off as heat (Figure 1.2).

1.6.3 DNP, Energy Usage, and Swimming Effects

Since DNP is a protonophore, its primary effect is on energy homeostasis. By uncoupling oxidative phosphorylation, it disrupts normal production of cellular energy by the mitochondrial electron transport chain. Partial uncoupling by DNP not only increases metabolic rate, but also increases fatty acid β -oxidation and results in a loss of adipose tissue (Harper *et al.*, 2001; Blaikie *et al.*, 2006). This treatment was a desired effect when DNP was tested as an obesity treatment (Cutting *et al.*, 1933). In mammals, short term exposure to DNP resulted in reduced strength and decreased endurance during exercise tests (MacBryde and Taussig, 1935), an expected result of having decreased ATP levels. In rainbow trout acutely exposed to DNP both ventilation volume and oxygen consumption increased since tissue oxygen requirement increased in order to generate more ATP (McKim *et al.*, 2009).

However, the environmental impacts of DNP are not as well understood. While the increased metabolic rate effects in humans may have been seen as a beneficial treatment of obesity, it may be detrimental to the health of aquatic species. Many fish species require increased lipid levels in order to survive the winter, when their metabolisms slow down and they are not capable of easily acquiring food. If DNP decreases the lipid stores in fish as it does in humans, this may disrupt the overall health of a fish population simply because the fish would not have enough energy to survive the winter. Although rotenone, a toxicant that also interferes with mitochondrial electron transport, was shown to decrease critical swimming speed of juvenile rainbow trout when exposed in an acute, sublethal manner (Cheng and Farrell, 2007), DNP effects on swimming have not been examined in any previous study. Furthermore, no previous study has examined toxicant effects on both swimming and energy utilization. Finally, because swim endurance testing and swim motion analyses were new techniques to this laboratory, acute DNP effects in adult zebrafish were used to validate our methods before the next series of experiments examining developmental toxicity were performed.

1.7 TCDD Toxicity

1.7.1 Chemistry, Environmental Sources, Fate, and Concentrations

Dioxins are persistent organic environmental pollutants with a high chemical stability and lipophilicity (Shlu *et al.*, 1988). The term ‘dioxin’ actually refers to a group of chlorinated aromatic compounds such as PCDDs and polychlorinated dibenzofurans, but may also include dioxin-like PCBs (Rappe, 1984). All of these chemicals are referred to as halogenated aromatic

hydrocarbons. Dioxins are not used in any industry however they are an unwanted byproduct of some industrial processes such as smelting, chlorine bleaching of paper pulp, the manufacturing of some herbicides and pesticides, and are present in car exhaust (Hites, 1990). They are also produced naturally during volcanic eruptions and forest fires.

PCDDs have the general structure shown in Figure 1.3A. Chlorination substitution can occur at any of the aromatic hydrogens and leads to a total of 75 possible congeners, not all of which have toxic effects. The most toxic of the congeners was found to be 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) which contains four chlorine substitutions in the lateral positions, is the model dioxin used for examining a plethora of toxic effects (Figure 1.3B), and is the standard by which toxicity of similar acting compounds are measured (Frakes *et al.*, 1993).

In many cases, aquatic environments serve as the major route of distribution for a host of man-made environmental contaminants, including PCDDs (Carvan *et al.*, 2000). Due to their high lipophilicity and stability, PCDDs tend to deposit in organic matter in sediments and fatty tissues of fish and can thus attain concentrations that are much greater than the PCDD concentration in the aquatic medium. Fish accumulate these environmental contaminants primarily by biomagnification in the food chain (Frakes *et al.*, 1993). Developmentally, fish eggs become exposed to dioxins via maternal transfer from the exposed female (King-Heiden *et al.*, 2005). Dioxin is concentrated in the eggs, which have a large amount of lipids, and is therefore passed on via the 'body burden' of the mother. Dioxins have been shown to cause an array of toxic effects to the reproductive and immune systems in fish as well as various developmental malformations all of which can lead to carcinogenesis and death (Peterson *et al.*, 1993).

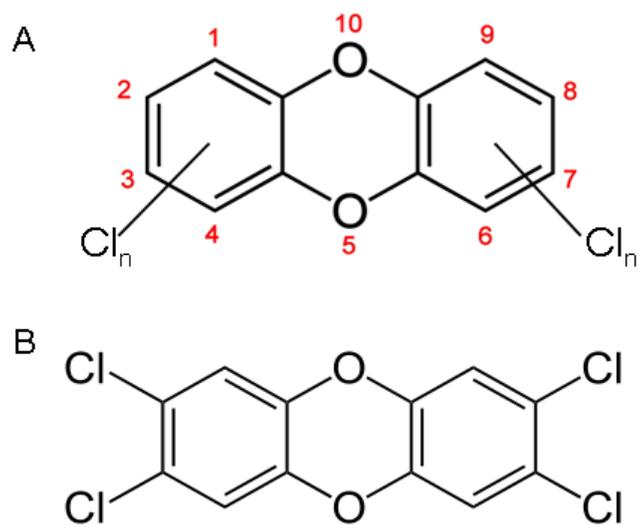


Figure 1.3. Chemical structure of dioxins. The chemical structures of a general polychlorinated dibenzodioxin (A) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (B) are presented.

1.7.2 Molecular Mechanisms of TCDD

The mechanism for dioxin-mediated toxicity appears to be dependent on binding to the aryl hydrocarbon receptor (AHR) in both mammalian (Fernandez-Salaguero *et al.*, 1996; Mimura *et al.*, 1997) and fish (Prasch *et al.*, 2003) species. In most studies, TCDD, the most toxic PCDD known, is used as the model dioxin (for a review see Schmidt and Bradfield, 1996). In the cell, TCDD binds to cytosolic AHR which is bound to and stabilized by chaperones (including the 90 kDa heat shock protein Hsp90). This complex translocates to the nucleus where AHR dimerizes with the AHR nuclear translocator (ARNT), then dissociates from Hsp90 (90 kDa heat shock protein) and associated chaperones. The AHR/ARNT heterodimer binds to dioxin response elements (DRE, also known as xenobiotic response elements) and upregulates the transcription of many different genes containing this regulatory element (Figure 1.4). Many of the genes activated by the AHR/ARNT heterodimer are involved in xenobiotic metabolism and include a subunit of glutathione-S-transferase, quinone oxidoreductases, an aldehyde dehydrogenase, and several cytochrome P450 monooxygenases (CYPs), the most prevalent being CYP1A isoforms (Paulson *et al.*, 1990; Favreau and Pickett., 1991; Asman *et al.*, 1993). Therefore, CYP1A activity and expression has become a standard biomarker for characterizing exposure to AHR agonists.

Fish are known to have at least three aryl hydrocarbon receptor genes encoding AHR: *ahr1* (a and b) and *ahr2*. While both *ahr1* genes appear to be novel vertebrate receptors exclusive to fish (Andreason *et al.*, 2002), it appears that AHR2 is required for TCDD-mediated developmental toxicity (Prasch *et al.*, 2003; Teraoka *et al.*, 2010; Clark *et al.*, 2010). Independent knockdown studies have shown that both CYP1A and ARNT1 induction are

required for TCDD to elicit developmental cardiovascular toxicity, namely circulation failure, edema, and brain blood vessel malformation (Teraoka *et al.*, 2003; Antkiewicz *et al.*, 2006; Teraoka *et al.*, 2010). However, contradictory results have been presented by Carney *et al.* (2004) who found that antisense knockdown of CYP1A in zebrafish offered no protection against TCDD-mediated cardiovascular toxicity. This group suggests instead that TCDD exerts developmental toxicity via CYP1A-independent mechanisms. In support of this hypothesis, a recent study reported that offspring of TCDD-exposed zebrafish showed no increase in CYP1A expression despite the presence of TCDD-associated larval deformities (King-Heiden *et al.*, 2009).

In contrast, Dong *et al.* (2002) pharmacologically inhibited zebrafish CYP1A using two different antagonists and found that they protected against TCDD-mediated blood flow inhibition and dorsal midbrain apoptosis. If CYP1A is necessary for toxicity, one hypothesis suggests that TCDD uncouples CYP1A monooxygenase function with subsequent free radical generation and increased oxidative stress (Figure 1.5; Schlezinger *et al.*, 1999 and 2000).

Moreover, cyclooxygenase-2 (COX-2) has recently been shown to be associated, along with increased CYP1A expression, with the deformed cardiac phenotype caused by AHR agonists (Bugiak and Weber, 2010) or to be necessary for TCDD-mediated vascular changes in the mid-brain (Teraoka *et al.*, 2009). Therefore, further studies examining how CYP1A activity relates to TCDD-induced developmental cardiovascular deformities are warranted to help clarify discrepancies before moving on to examining the role of other AHR-responsive genes.

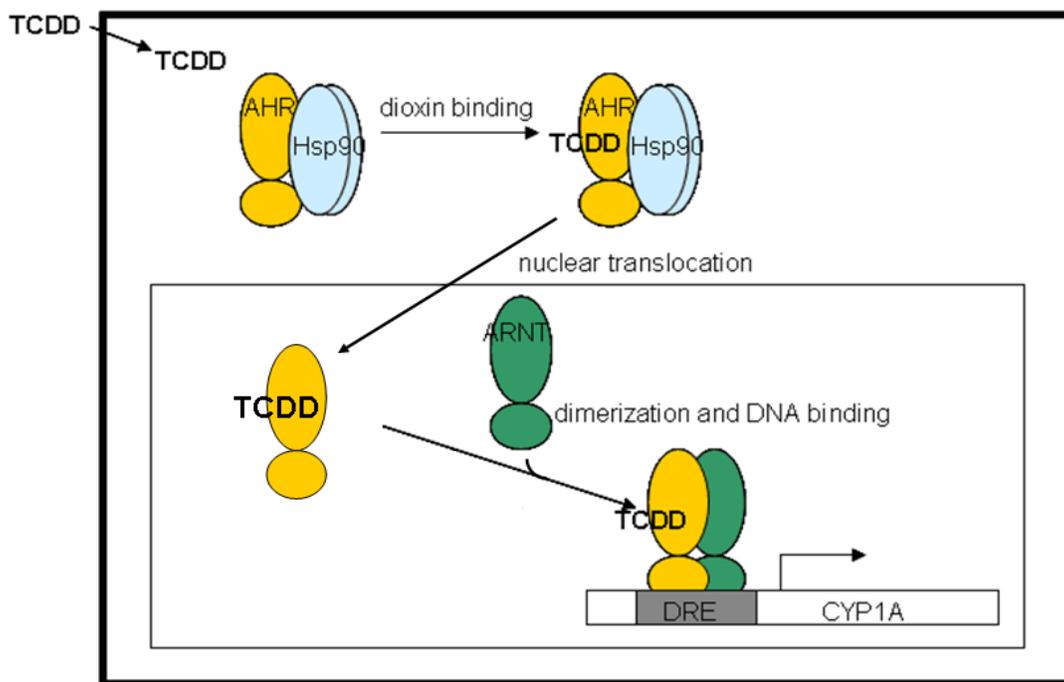


Figure 1.4. Schematic diagram of TCDD activation of the AHR. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) binding to the aryl hydrocarbon receptor (AHR) leads to its translocation to the nucleus, dimerization with aryl hydrocarbon receptor nuclear translocator (ARNT), and upregulation of cytochrome P450 1A (CYP1A) via the dioxin response element (DRE). Hsp90- 90 kDa heat shock protein. (Figure modified from Schmidt and Bradfield, 1996).

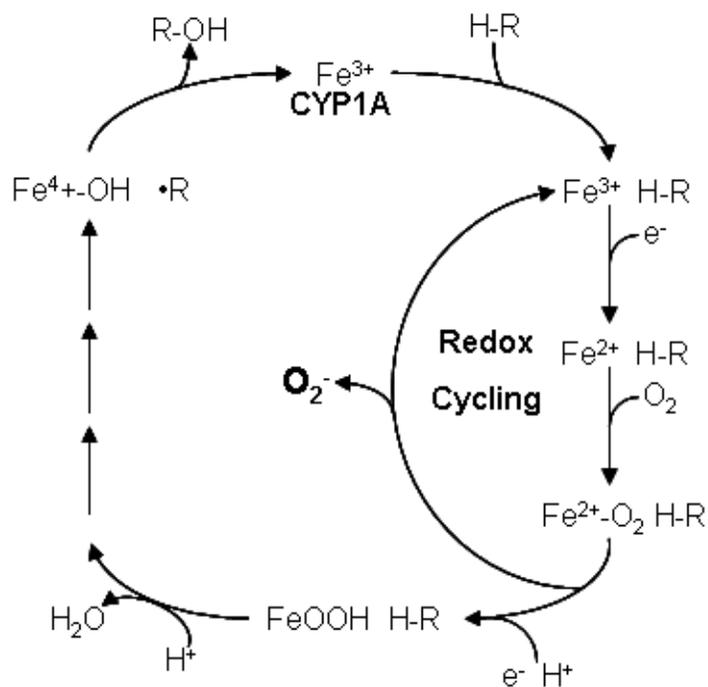


Figure 1.5. Schematic representation of TCDD mediated oxidative stress. The diagram depicts the heme prosthetic site on cytochrome P450 1A (CYP1A). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; denoted here as H-R) binds to the active site of the enzyme but this chlorinated compound is too stable to allow for oxidation. As a result, the CYP-mediated oxidation is uncoupled and a superoxide radical is released in a continuous cycle (redox cycling), causing oxidative stress. (Figured modified from Schlezinger *et al.*, 2000).

1.7.3 TCDD-Mediated Developmental Cardiovascular Toxicity in Fish

Blue sac disease is characterized by edema, hemorrhaging, cardiovascular abnormalities, spinal deformities, and craniofacial deformities in fish exposed developmentally to AHR agonists such as TCDD (Elonen *et al.*, 1998). Many species of teleost fish show these characteristic signs of early life stage TCDD toxicity including: lake herring (*Coregonus artedii*), white sucker (*Catostomus commersoni*), northern pike (*Esox lucius*), fathead minnow (*Pimephales promelas*), channel catfish (*Ictalurus punctatus*), medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), and rainbow trout (*Oncorhynchus mykiss*) (Elonen *et al.*, 1998; Hornung *et al.*, 1999). Of these species, rainbow trout appears to be one of the most sensitive to TCDD while zebrafish appears to be one of the most insensitive (Elonen *et al.*, 1998). Despite their relative insensitivity, zebrafish do display all classical signs of blue sac disease and remain the most commonly used fish species for mechanistic TCDD toxicity studies.

Toxicity testing information shows that the developmental LC₅₀ for zebrafish is between 2.3-2.9 ng/g TCDD, and the LC₁₀ is 1.2-2.1 ng/g (Elonen *et al.*, 1998). There are two things to note however; the concentrations refer to the amount of TCDD in the egg itself, and the observed effect endpoint was a decrease in survival. Dose-response curves involving other endpoints, such as cardiovascular development, have been somewhat unclear. In most cases, it appears that TCDD developmental cardiovascular toxicity is an ‘all-or-none’ event or at least has a very steep dose-response curve when looking at gross malformations. In contrast, sublethal cardiovascular toxicity has not been well studied and requires evaluation of more than just gross external deformities.

1.7.4 Vascular and Peripheral TCDD Effects

Hornung *et al.* (1999) examined the temporal effects of lethal doses of TCDD on cardiovascular dysfunction in rainbow trout. Blood flow first ceased in the brachial arteries of the gills and was significantly reduced in the sub-intestinal and vitelline vein. Severe edema manifested in the yolk sac while only mild edema was seen in the pericardium. The extent of the yolk sac covered by functional blood vessels was reduced in TCDD-exposed rainbow trout sac fry compared to control fry. Vitelline vein branching, which carries nutrients extracted from the yolk sac, was shown to decrease, likely contributing to reduced blood perfusion to the yolk sac and reduced yolk sac absorption. Developmental toxicity in zebrafish appears to be very similar. Reduced common cardinal vein growth prior to hatching appears to be the first sign of toxicity followed by the aforementioned reduction in blood flow to various tissues including the head and trunk (Goldstone and Stegeman, 2006; Figure 1.6). This reduction in blood flow to the peripheral vessels leads to vascular bed failure and fin rot (Belair *et al.*, 2001), followed by pericardial edema and death (Henry *et al.*, 1997).

TCDD caused a decrease in erythrocyte number and erythrocyte flow rate in the peripheral vessels with virtually no erythrocyte movement seen by 96 hpf (Belair *et al.*, 2001). Thus, TCDD disrupts events in the formation of erythrocytes (erythropoiesis) leading to TCDD-induced anemia (Belair *et al.*, 2001). Reduction in peripheral blood flow occurs in embryos exposed to TCDD anytime prior to 72 hpf (Carney *et al.*, 2006). This indicates that the critical events for TCDD induced disruption of erythropoiesis occur between 72 and 96 hours post fertilization. Similar results were seen with mesencephalic vein (the main collection vessel of blood directed towards the head) blood flow, which decreased in a concentration-dependent

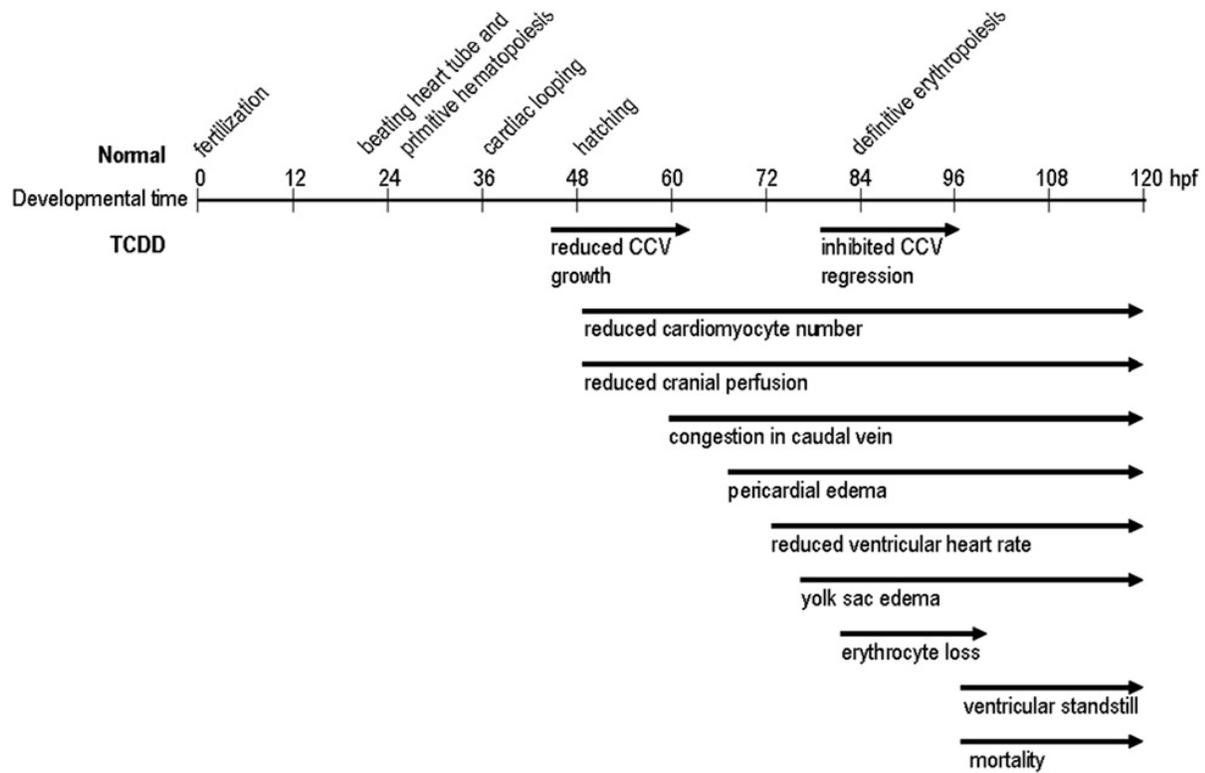


Figure 1.6. Temporal timeline for TCDD toxicity in zebrafish. Normal development and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) mediated toxic effects are depicted. Timecourse shows central cardiac effects as well as peripheral vascular effects (Goldstone and Stegeman, 2006). CCV-common cardinal vein.

manner after TCDD exposure (Dong *et al.*, 2002). This specific TCDD toxic effect was AHR- and CYP1A dependent (Dong *et al.*, 2004) although other studies using other vascular beds (Carney *et al.*, 2006) suggest otherwise. Therefore, it is unclear whether the discrepancies regarding CYP1A involvement in TCDD developmental toxicity are the result of technical differences between laboratories or representative of fundamental differences in toxic response among different vascular beds.

1.7.5 Central Cardiac TCDD Effects

Hearts from TCDD-exposed zebrafish embryos were elongated with the ventricle positioned anterior to the atrium indicating that the normal looping process that places the ventricle and atrium side by side did not take place (Antkiewicz *et al.*, 2005) creating a shorter, thicker-walled ventricle (Bugiak and Weber, 2010). This was quantified by an increase in the sinus venosus to bulbus arteriosus distance indicating that these hearts did not undergo normal looping and compaction compared to controls (Antkiewicz *et al.*, 2005). This looping process may be mediated partially by regression of the common cardinal vein (CCV; Bello *et al.*, 2004), which provides all the initial venous return to the heart (Isogai *et al.*, 2001). TCDD both blocks the regression of the CCV as well as decreases CCV area, thus inhibiting dorsal movement of the heart (Bello *et al.*, 2004).

Lethal doses of TCDD decrease ventricular stroke volume and cardiac output in zebrafish due to a decrease in end-diastolic volume (Carney *et al.*, 2006; Bugiak and Weber, 2010). This is mostly due to a decreased ability of the ventricle to relax but may also be caused by increased peripheral resistance causing a decrease in peripheral blood flow return to the heart (Carney *et*

al., 2006). A decrease in cardiac myocyte number upon lethal exposure to TCDD is also seen but it may be a secondary effect to reduced cardiac output since heart function and growth are tightly linked (Hove *et al.*, 2003) or it may be the result of TCDD-induced alterations in genes that control proliferation (Puga *et al.*, 2000).

Only a few studies have examined sublethal developmental TCDD exposure in zebrafish. Bugiak and Weber (2010) looked at sublethal toxicity and effects on cardiovascular development. They found subtle effects in zebrafish surviving to 10 dpf but it is not known if these defects would persist into adulthood or lead to mortality prior to adulthood. Another study found that the offspring produced by adult zebrafish exposed to sublethal amounts of TCDD throughout their growth to adulthood had higher mortality and deformity rates relative to control (King-Heiden *et al.*, 2009). Therefore, one hypothesis of this thesis is that developmental exposure to TCDD will result in cardiovascular deformities that will persist later in life and in turn cause persistent impairment in swimming ability.

1.7.6 TCDD and Energy Stores

TCDD is known to cause chronic wasting in several mammalian species which is characterized by the loss of fat stores (Schmidt and Bradfield, 1996). In past studies a successive decrease in feeding behavior in TCDD-treated rats (TCDD-induced anorexia) was suggested as the cause for the observed decrease in weight (Potter *et al.*, 1986). Subsequent studies examining the mechanistic role of TCDD in wasting and energy imbalance showed reduced gluconeogenesis via inhibition of phosphoenol-pyruvate carboxykinase and pyruvate kinase (Gorski *et al.*, 1990; Weber *et al.*, 1991). TCDD-treated rats also showed reduced insulin-like

growth factor receptor activity (Croutch *et al.*, 2005). Furthermore, activation of the AHR by TCDD was shown to decrease adipogenesis (lipid synthesis) in mouse embryonic fibroblasts (Alexander *et al.*, 1998), suggesting an effect in lipid tissue. Low doses of dioxin also downregulated the expression of a whole battery of genes responsible for energy homeostasis, particularly those involved with cholesterol metabolism and lipogenesis (Sato *et al.*, 2008). Finally, adipose cell tissue cultures showed a decrease in glucose transportation upon exposure to TCDD denoting disruption of energy homeostasis in this cell type (Enan *et al.*, 1992).

In contrast to adipose tissue in animals, the liver responds in a different fashion upon exposure to TCDD and instead a fatty liver is most often observed. When examining the lipid profiles of rats exposed to TCDD, Albro *et al.* (1978) found that a sublethal dose of TCDD caused a temporary increase in liver triglyceride and free fatty acid levels while a lethal dose of TCDD caused a large increase in liver cholesterol and free fatty acid levels. Related to these alterations, the lipid profiles of workers exposed sublethally to TCDD showed elevated plasma lipids, which was coincident with elevated atherosclerotic plaques (Pelclova *et al.*, 2002). Thus it appears that a sublethal exposure to TCDD results in elevated triglycerides in the livers of rats and elevated plasma lipids in humans.

The effects of TCDD on fish energy levels have not been fully characterized. While lethal levels of TCDD have been reported to cause a similar wasting syndrome in fish as mammals (Kleeman *et al.*, 1988), the molecular mechanisms have not been fully elucidated. Contrary to this earlier report, a recent study reported that PCB-exposed eels did not lose as much weight when engaged in prolonged migration (800 km) as control eels (Ginneken *et al.*, 2009). For this thesis, it is hypothesized that energy stores will be decreased by TCDD exposure and that this will impair swim performance in fish.

1.8 Hypotheses

1. Acute, sublethal exposure to the oxidative phosphorylation uncoupler, 2,4-dinitrophenol, will decrease swim performance in zebrafish.
2. Acute, sublethal exposure to 2,4-dinitrophenol will decrease triglyceride levels in zebrafish.
3. Developmental exposure to sublethal concentrations of TCDD will result in decreased swim performance in subadult zebrafish including effects on swim motion and critical swimming speed.
4. Aerobic scope during swim testing will be decreased in subadult zebrafish exposed to TCDD during early development.
5. Structural cardiovascular abnormalities will be detected in both larval zebrafish (grossly evident) and subadult zebrafish (histologically evident only) after developmental TCDD exposure.
6. Subadult zebrafish developmentally exposed to TCDD will have decreased triglyceride levels when compared to control fish.
7. CYP1A induction in zebrafish will be associated with sublethal TCDD-mediated defects in cardiovascular development, swim performance, and energetics.

1.9 Research Objectives

1. Acutely expose adult zebrafish to increasing concentrations of 2,4-dinitrophenol and observe effects on swim performance by measuring critical swimming speed and swim motion endpoints.
2. Determine whole body triglyceride levels in adults following acute exposure to several concentrations of 2,4-dinitrophenol.
3. Expose larval zebrafish to vehicle or increasing concentrations of TCDD between 2-4 dpf and raise zebrafish to 90 dpf. Determine differences in mortalities and deformities in larval (10 dpf) and sub-adult (90 dpf) zebrafish exposed to TCDD from 2-4 dpf.
4. Examine swim performance in sub-adult zebrafish by measuring swim performance and swim motion end points.
5. Examine aerobic scope during swim testing in sub-adult zebrafish exposed to TCDD between 2-4 dpf.
6. Determine whole body triglyceride levels in sub-adult zebrafish exposed to TCDD between 2-4 dpf.
7. Determine CYP1A activity in 5 dpf larvae after exposure to TCDD from 2-4 dpf.

2.0 Acute Exposure to 2,4-Dinitrophenol Alters Zebrafish Swim Performance

2.1 Introduction

Examination of swimming capabilities in fish is emerging as an effective method for monitoring the sublethal effects of toxicants on the overall health of fish populations (Hammer, 1995). Swimming performance can be linked to several ecologically relevant factors such as predator evasion, food acquisition, migratory behavior, and overall survivability (Hammer, 1995). The most common and simplest method for measuring swim performance in fish is determination of the critical swimming speed (U_{crit} ; Brett, 1964). Sublethal concentrations of the mitochondrial electron transport chain uncoupler, rotenone, were shown to decrease U_{crit} after an acute 96 hour exposure (Cheng and Ferrell, 2007). The metabolic byproduct ammonia can also significantly decrease swimming performance in fish (Tudorache *et al.*, 2008). Therefore its levels must be monitored when performing static exposure tests as build up of ammonia may skew effects caused by other toxicants. Other studies have reported decreases in U_{crit} upon exposure of fish to various other environmental contaminants such as sodium pentachlorophenate, cyanide, organophosphate pesticides and metals (Webb and Brett, 1973; Waiwood and Beamish, 1978; Kovacs and Leduc, 1982; Cripe *et al.*, 1984). Importantly, acute exposure to pulp mill effluent was shown to decrease U_{crit} in juvenile salmon, but this decrease was reversible once the fish were removed to clean water (Howard, 1975).

In addition to effects on swim endurance (i.e. U_{crit}), toxicant exposure may also affect the way the fish swims. While more recent techniques for measuring kinematics make use of x-ray photography to look at curvature of the vertebrae during swimming (Donley and Dickson, 2000),

a simple measurement of caudal fin height and curvature from images obtained using a high speed camera can be just as effective (Bainbridge, 1963). For swim motion analyses, both tail beat frequency and tail beat amplitude are important measurements since they are direct determinants of fish swimming speed (Bainbridge, 1957; Blank *et al.*, 2007). No previous study has examined effects of toxicant exposure on swim motion.

Lipids serve as a marker of overall fish energy storage (Weber *et al.*, 2003) and are also the main source of energy for fish muscle during endurance swimming (Chatelier *et al.*, 2006; Bennett *et al.*, 2007; Magnoni and Weber, 2007). Lapointe *et al.* (2006) found that when food availability is altered, U_{crit} decreased indicating the important link between energy stores and physiological performance. Even varying the type of fatty acid in the fish diet is able to alter critical swimming speeds (Chatelier *et al.*, 2006). When examining toxic exposures in fish, most studies indicate that triglyceride levels are decreased in polluted environments (Munkittrick and Dixon, 1998; Levesque *et al.*, 2002) as the fish species are devoting stored energy to deal with detoxification processes (Calow, 1991; Benton *et al.*, 1994; Adams, 1999) as well as repair of tissue and cellular damage (Campbell *et al.*, 2003). When zebrafish (*Danio rerio*) were exposed to toxic effluent, it was found that the change in lipid concentration was the most sensitive endpoint out of the indices examined (Smolders *et al.*, 2003). However, no study has yet linked decreases in swim performance with decreases in triglyceride levels in response to toxicant exposure.

Like rotenone, 2,4-dinitrophenol (DNP) is a mitochondrial electron transport chain uncoupler which acts as a protonophore that decreases the mitochondrial proton gradient by transporting hydrogen ions across the inner membrane and thus inhibits ATP synthesis (Blaikie *et al.*, 2006). DNP is a purely synthetic organic compound used commercially as an intermediate

in the production of dyes, explosives, and several pesticides, or produced as a secondary product of automobile exhaust and reactive air pollutants (Harris and Cocoran, 1995). Environmentally, DNP is found in 61 of the sites that the United States Environmental Protection Agency considers a priority for national clean up (Harris and Cocoran, 1995). DNP enters the air, water, and soil primarily from manufacturing releases, spill sites and ground water seepage from landfills (Harris and Cocoran, 1995). In mammals, sublethal mitochondrial uncoupling by DNP increases β -oxidation of fatty acids and leads to a loss in adipose tissue (Cutting *et al.*, 1933; Harper *et al.*, 2001; Blaikie *et al.*, 2006). However, the role of DNP in affecting the energy stores and swimming capabilities of fish has not previously been investigated.

Therefore, it was hypothesized that acute exposure to DNP in fish will decrease the swim endurance, affect the manner in which fish swim and decrease energy stores. In order to investigate this hypothesis, adult zebrafish were used as a model species because of their ease in handling and robust swimming ability (Plaut, 2000). The effects of 24-hr aqueous exposures to two different concentrations of DNP or vehicle control on mortality, U_{crit} , swim motion, and whole body triglyceride levels were measured.

2.2 Materials and Methods

2.2.1 Chemicals, Fish Maintenance and Exposure

All chemicals used in this study were reagent grade. Ethanol was obtained from VWR Canada (Mississauga, ON). All other chemicals were obtained from Sigma-Aldrich Canada (Oakville, ON) unless otherwise stated. All fish housing and experimental procedures were

approved by the Animal Research Ethics Board at the University of Saskatchewan in accordance with guidelines of the Canadian Council on Animal Care. Adult zebrafish (*Danio rerio*) were purchased from a local pet store and acclimated for three weeks prior to commencement of experiments in 30 liter holding tanks with particulate and charcoal filters, as well as ammonia biofilters. Dechlorinated municipal water was maintained at 28°C, partially changed every two days and regularly tested for water quality. Fish were fed twice daily with brine shrimp flake (Argent Chemical Laboratories, Redmond, WA) and San Francisco Bay Brand *Chironomus tentans* larvae (Newark, CA) and kept on a 14 h light:10 h dark photoperiod. Fish were fed immediately prior to 24 hour static exposures, but not during exposures or subsequent swim testing before euthanasia.

Adult zebrafish (3 replicate trials performed on different days with 1 fish per tank; n= 25 total fish per treatment) were aqueously exposed in static aerated 1 L glass tanks to either vehicle control (0.01% ethanol), 6 mg/L DNP, or 12 mg/L DNP without renewal for the 24 h exposure. Ammonia levels were measured by an aquarium ammonia water test following exposure since ammonia can affect U_{crit} (Tudorache *et al.*, 2008) but were found to be unchanged by the end of the exposure. Individual fish were exposed in a staggered manner so that fish could be rapidly transferred to the swim tunnel for swim testing when exactly 24 h of exposure had elapsed.

2.2.2 Swim Tunnel Design, Critical Swimming Speed and Swim Motion Analyses

A 100-mL Loligo Systems (Denmark) swim tunnel was used for swim testing. The swim tunnel was submerged in a 20 L buffer tank supplied with 28°C aerated, dechlorinated municipal water from a 20 L recirculating water bath (VWR International, Mississauga, ON). Water

velocities were calibrated using constant temperature anemometry according to manufacturer's instructions (Loligo Systems, Denmark). A Fastec Troubleshooter (High Speed Imaging, Winnipeg, MB) high speed camera (250 frames/sec) was mounted over the swim tunnel for swim motion analysis. For U_{crit} determination, a fish was placed in the swim tunnel and acclimated at zero water velocity for 30 minutes. After acclimation, the fish was forced to swim against a current of 1 cm/sec for 5 min, then water velocity was increased by a prescribed amount (U_{ii} - 2.5 cm/sec) at the end of a prescribed time interval (T_{ii} - 5 min). This process continued until the fish fatigued and was no longer able to swim against the water current. At that point, U_{crit} was calculated (Brett, 1964 and 1967):

(Equation 1)
$$U_{crit} = U_i + [U_{ii}(T_i/T_{ii})]$$

where U_i was the highest velocity maintained for an entire time interval and T_i was the time that elapsed at the fatigue velocity. Since fish cross sectional area was <10% of working section area of swim tunnel, solid blocking correction was not applied to the U_{crit} values (Bell and Terhune, 1970). U_{crit} values were converted from cm/s to body lengths/second (BL/s) in order to accommodate slight variations in fish size.

A Fastec Troubleshooter high speed camera and Midas Pro 4.0 capture software (Sacramento, CA) were used to acquire a 5 sec video at the beginning of every speed increment or at the first point where the fish was positioned in the middle of the swim tunnel in view of the camera. Frames were analyzed at U_i speed (the last speed maintained for an entire time interval prior to the fatigue speed). Videos of low quality were not included in analysis. Single digital images were extracted from the camera files using Adobe Premiere Elements 2.0 (San Jose, CA) and analyzed using Image Pro Express 6.0 (Bethesda, MD). The swim motion endpoints measured were compression ratio (ratio of compressed body length to total body length), left and

right tail beat amplitudes (distance relative to midline at the point of greatest compression, normalized to body length) and left or right tail beat angles (angle relative to midline at the point of greatest compression) (see Figure 2.1). Three different frames were analyzed for each endpoint and the average value for each individual was used for statistics.

2.2.3 Triglyceride Analysis

Following U_{crit} determination, fish were euthanized using tricaine methane sulphonate (MS-222), quickly weighed, measured with a vernier caliper, visually inspected for secondary sex characteristics in order to assign a sex, and frozen at -80°C until analyzed. Condition factor was determined by dividing the weight by the length³. Triglyceride levels were determined in whole body homogenates using lipase/glycerol kinase based reagents obtained from Sigma-Aldrich (Oakville, ON) according to previously described methods from this laboratory (Bennett *et al.*, 2007).

2.2.4 Data and Statistics

In initial data analyses, no significant effect of sex was found on all end-points (sex and treatment as variables) in a two-way analysis of variance (ANOVA). Therefore data from both sexes were combined and analyzed using a one-way ANOVA followed by Fisher's least significant difference (LSD) post hoc tests where appropriate with $p < 0.05$ considered to be

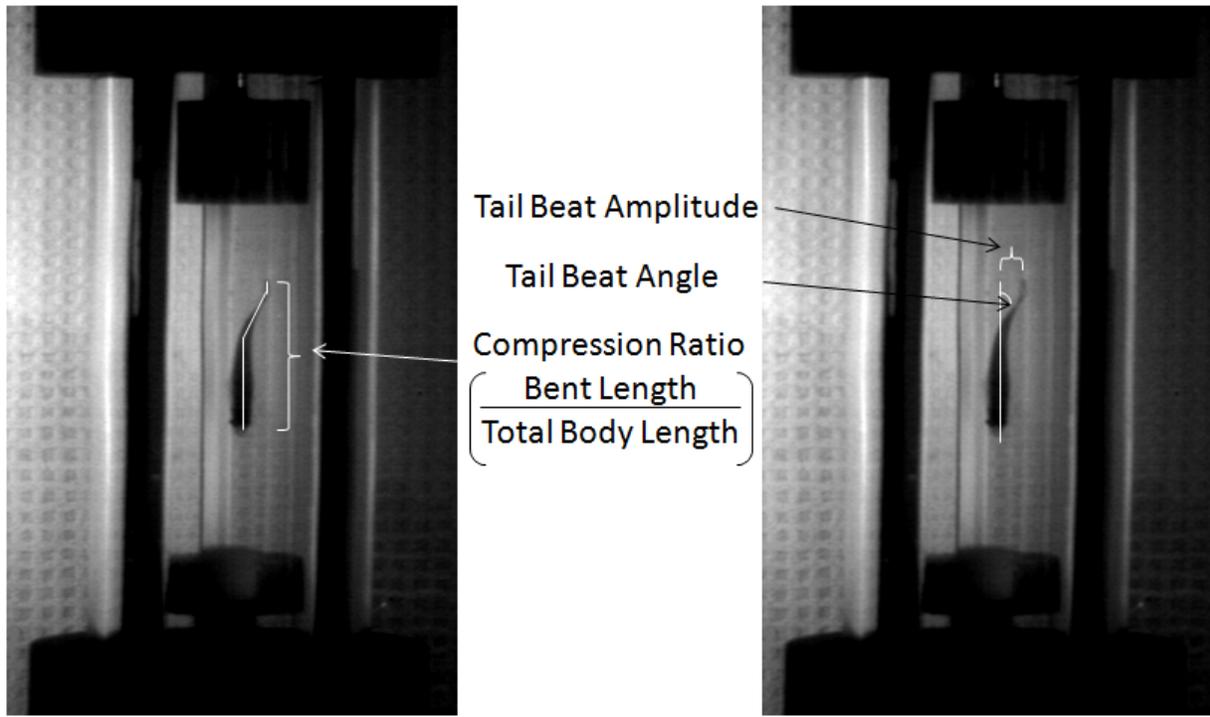


Figure 2.1. Swim motion image analysis endpoints. High speed camera pictures of an adult zebrafish swimming in the swim tunnel are shown to demonstrate the different measurements performed. The left picture demonstrates the body compression ratio which is the ratio of the bent length (denoted by the bracket) to the total body length (tracing). The right picture demonstrates how the tail beat amplitude (length from midline to the end of the fin while the fish is compressed during swimming) and the tail beat angle (the angle of the caudal peduncle relative to midline while the fish is compressed during swimming) were calculated.

significantly different. For tail beat amplitude measurements, data were analyzed using a one-way analysis of covariance (ANCOVA) followed by Fisher's LSD test where appropriate using body length as a covariate. Pearson's correlation analysis was used to assess significant linear relationships between swimming speed and tail beat frequency. Results are presented as mean \pm standard error of mean (SEM).

2.3 Results

2.3.1 Mortalities and Biometrics

After 24 h of exposure and following swim testing, there were no statistically significant differences in body weight or body length among the different exposure groups (Table 2.1). In addition, no difference was observed in the condition factor of the fish between the different exposure groups. However, exposure to 12 mg/L DNP resulted in a statistically significant increase in mortalities at 24 hr (before swim testing) compared to both the 6 mg/L DNP and vehicle control exposure groups (Table 2.1).

2.3.2 Effects of DNP on Swim Performance and Energy Stores

After 24 h of exposure, DNP significantly decreased critical swimming speeds of adult fish in a concentration-dependent manner compared to control (Figure 2.2). Tail beat frequency was also decreased in a concentration dependent manner by 24 h DNP exposure compared to control (Figure 2.3). No other significant changes were observed in the swim motion endpoints,

namely in right or left tail beat amplitude, tail beat angle or body compression ratio (Figure 2.4). Correlation analysis revealed that U_{crit} was highly correlated to tail beat frequency for all groups (Figure 2.5). The regression lines were superimposed for both DNP exposure groups but were both different from control. In general, the regressions of both DNP treated groups had lower y-intercepts and lower slopes than that of control (Figure 2.5). After 24-h exposure to DNP, whole body triglyceride concentrations in zebrafish were significantly elevated in a concentration-dependent manner compared to vehicle-exposed fish (Figure 2.6).

2.4 Discussion

The most significant finding of this study was that environmentally-relevant 2,4-dinitrophenol exposure for 24 h decreased the swim endurance of adult zebrafish. This decreased swim endurance was largely due to decreased tail beat frequency and not alterations in other aspects of swim motion. Paradoxically, after swimming to exhaustion, zebrafish exposed to DNP showed concentration-dependent increases in whole body triglyceride stores.

Environmental spill sites have reported DNP water concentrations ranging between 3-30 mg/L (Harris and Cocoran, 1995) which encompasses the nominal concentrations used in the current study (6-12 mg/L). The environmentally relevant concentration of 12 mg/L used in the current study caused significant mortality in adult zebrafish after only 24 h aqueous exposure. This agrees reasonably well with a previous study which reported a 96 h LC_{50} of 1.34 mg/L DNP in *Notopterus notopterus*, a tropical fish found in similar regions as zebrafish (Verma *et al.*, 1981).

Table 2.1. Mortalities and biometrics of adult zebrafish acutely exposed to 2,4-dinitrophenol. Adult zebrafish were aqueously exposed for 24 h to 2,4-dinitrophenol (DNP) or 0.1% ethanol vehicle in a static medium, with weight and length measured after swim testing at the end of the 24-h exposure. Data is expressed as mean \pm SEM. For weight, length and condition factor, n=25 fish per group. Percent mortality was calculated as the percent per day with n=3 independent exposure trials conducted on separate days. *p<0.05 compared to vehicle control, #p<0.05 compared to 6 mg/L DNP in Fisher's LSD post hoc test after one-way ANOVA.

	Body Weight (mg)	Body Length (cm)	Condition Factor	Mortalities (%)
Vehicle	439 \pm 17	3.09 \pm 0.04	1.48 \pm 0.06	0 \pm 0
6 mg/L DNP	433 \pm 18	3.07 \pm 0.04	1.63 \pm 0.08	6.7 \pm 7.2
12 mg/L DNP	480 \pm 22	3.11 \pm 0.05	1.50 \pm 0.06	54.1 \pm 6.2 ^{*#}

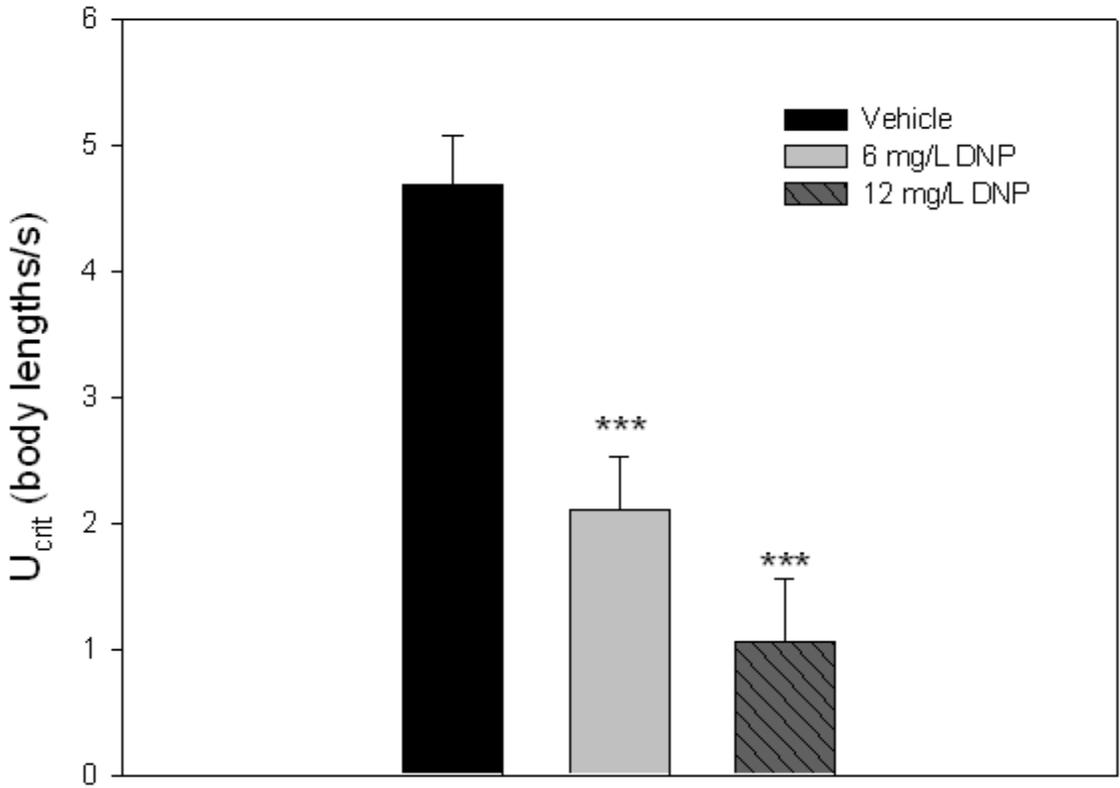


Figure 2.2. Critical swimming speeds of adult zebrafish acutely exposed to 2,4-dinitrophenol. Adult zebrafish were exposed for 24 hours to 2,4-dinitrophenol (DNP) or ethanol vehicle in a static medium and critical swimming speed (U_{crit}) measured in clean water immediately after the 24-h exposure. Sample sizes were $n=24$ for vehicle, $n=22$ for 6 mg/L DNP and $n=14$ for 12 mg/L DNP exposure groups. Data is expressed as mean \pm SEM. *** $p<0.001$ compared to vehicle control group in Fisher's LSD post hoc test after one-way ANOVA.

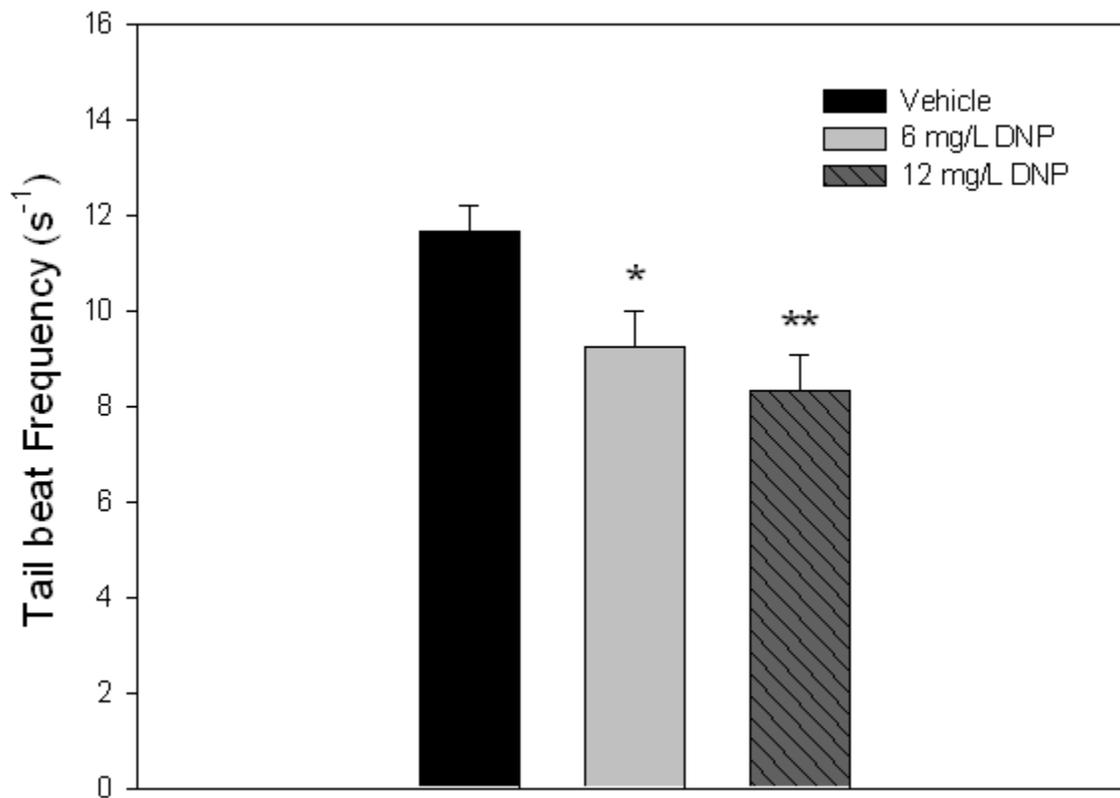


Figure 2.3. Tailbeat frequencies of adult zebrafish acutely exposed to 2,4-dinitrophenol. Adult zebrafish were exposed for 24 hours to 2,4-dinitrophenol (DNP) or ethanol vehicle in a static medium, then swim motion assessed in clean water immediately after the 24-h exposure. Tail beat frequencies were measured during the U_i speed (highest completed water velocity during critical swimming test). Sample size was $n=10$ for the vehicle, $n=6$ for 6 mg/L DNP, and $n=6$ for 12 mg/L DNP exposure groups. Data is expressed as mean \pm SEM. * $p<0.05$, ** $p<0.01$ compared to vehicle control in Fisher's LSD test after one-way ANOVA.

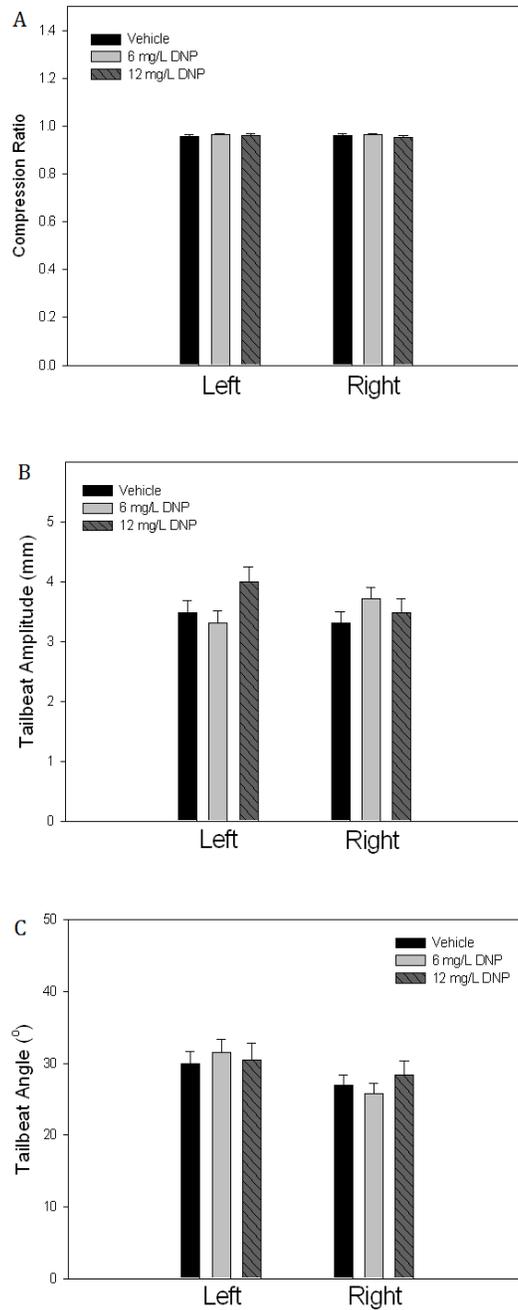


Figure 2.4. Swim motion parameters in adult zebrafish acutely exposed to 2,4-dinitrophenol. Adult zebrafish were exposed for 24 hours to 2,4-dinitrophenol (DNP) or ethanol vehicle in a static medium, swim motion was assessed in clean water immediately after the 24-h exposure. Compression ratios (A), tailbeat amplitudes (B), and tailbeat angles (C) were measured during the U_i speed (highest completed water velocity during critical swim test) for both the left and right directions. Data is expressed as mean \pm SEM. Sample size was $n=22$ for the vehicle control, $n=21$ for 6 mg/L DNP, and $n=12$ for the 12 mg/L DNP exposure groups. No significant differences were detected in separate one-way ANOVAs (compression ratio and angle) or ANCOVA with length as a covariate (amplitude) for each of these end-points.

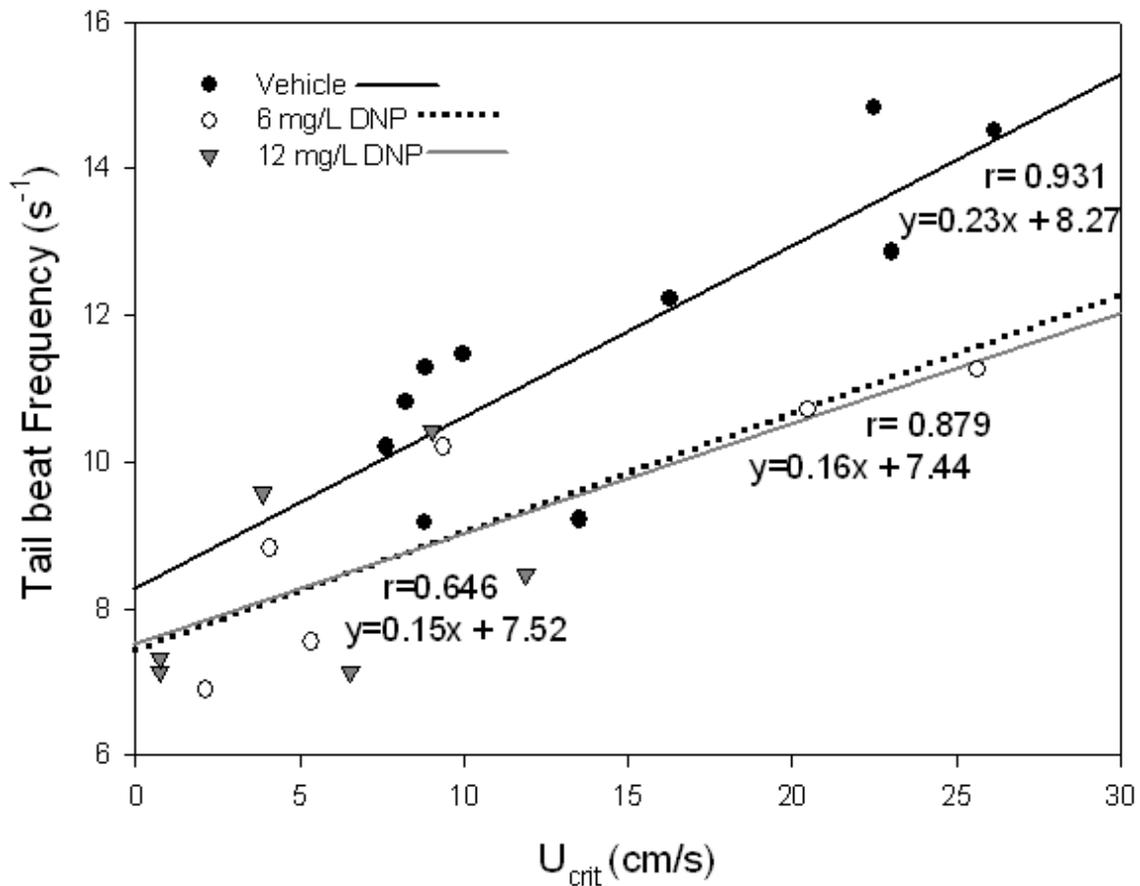


Figure 2.5. Correlation of critical swimming speed and tail beat frequency. Adult zebrafish were exposed for 24 hours to 2,4-dinitrophenol (DNP) or ethanol vehicle in a static medium, swim motion was assessed in clean water immediately after the 24-h exposure. Individual results from the critical swimming speed (U_{crit}) evaluations were plotted against their corresponding tail beat frequencies at U_i speed (highest completed water velocity during critical swim test). The slopes of regression lines for all three treatments were significantly ($p < 0.01$) different from zero. Sample sizes were $n=10$ for the vehicle control, $n=5$ for 6 mg/L DNP, and $n=6$ for 12 mg/L DNP exposure groups.

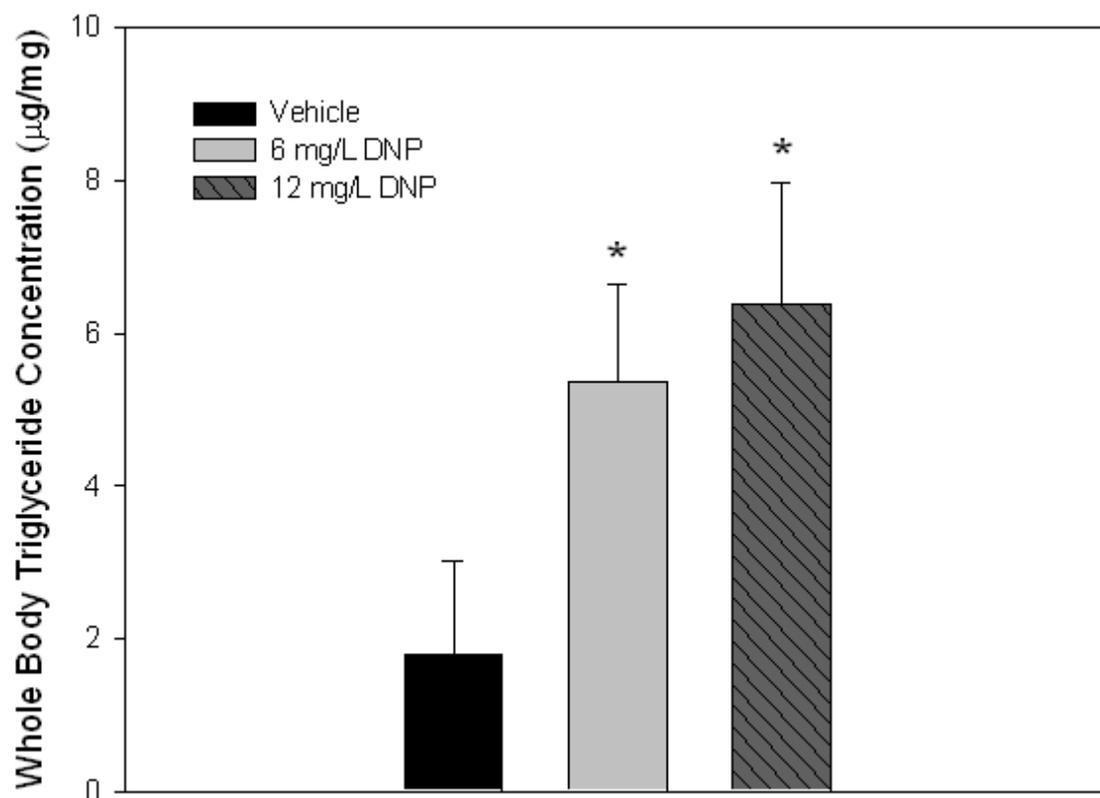


Figure 2.6. Whole body triglyceride concentrations in adult zebrafish exposed to 2,4-dinitrophenol. Adult zebrafish were exposed for 24 hours to 2,4-dinitrophenol (DNP) in a static medium and triglycerides measured in fish euthanized immediately after swim testing. Sample size was n=24 for the vehicle control, n=22 for 6 mg/L DNP, and n=14 for 12 mg/L DNP exposure groups. Data is expressed as mean \pm SEM. *p<0.05 compared to vehicle control in Fisher's LSD test after one-way ANOVA.

The shorter exposure time in the current study (24 h) than this previous study is the likely explanation for the slightly higher DNP concentration needed to produce 50% lethality.

However, exposure to lower, sublethal concentrations of DNP have the potential to exert profound population-level impacts if the ability of fish to swim is insufficient to avoid predation, capture prey or successfully migrate for spawning. The results of the current study agree with previous studies showing that swimming is impaired by the mitochondrial electron transport chain inhibitor, rotenone (Cheng and Farrell, 2007). With both DNP and rotenone, the results are consistent with the fact that ATP levels would decrease after exposure, leading to less energy for muscle activity during swimming. In the current study, the short exposure (24 h) also means that there is potential for this swimming impairment to be reversible as has been previously shown after acute exposure to pulp mill effluents (Howard, 1975), but this was not examined in the current study. Importantly, the decreased swim endurance is clearly explained by decreased tail beat frequency and not changes in any other aspects of swim motion, agreeing with the relationship described previously in normal fish (Bainbridge, 1963). The y-intercept of the U_{crit} versus tail beat frequency was higher for the vehicle control group than for the DNP exposure groups, highlighting the impaired ability of DNP exposed fish to beat their tails with sufficient frequency to sustain higher swimming speeds. The decreased slope of the DNP exposure groups paradoxically suggests that when DNP-exposed fish were able to attain a certain swimming speed, they had a lower tail beat frequency compared to vehicle control. At first this seems counter-intuitive, but may be explained by the fact that fish often swim in bursts when exposed to stressors (Hammer, 1995). If this is true, even though burst swimming was not quantified in this study, then the DNP exposed fish may have swam in a non-continuous manner: periods of

swimming followed by periods of coasting. If swim motion analysis favored clips of coasting for analysis, then this could explain the decreased tail beat frequency at a given U_{crit} when compared to vehicle-exposed fish. More detailed swim behavior analyses are warranted in future toxicology studies.

Contrary to the original hypothesis, higher whole body triglycerides were observed after 24 h of DNP exposure compared to vehicle control. In mammalian studies, DNP was originally used as a dieting aid for humans because it increases β -oxidation of fatty acids and thus leads to decreased lipid levels (Harris and Cocoran, 1995). However, it appears that this is not the case in zebrafish, at least for the acute exposure to DNP used in the current study. Triglycerides serve as the main source of energy for fish muscle during locomotion (Lemly, 1993; Weber *et al.*, 2003; Chatelier *et al.*, 2006; Magnoni and Weber, 2007; Bennett *et al.*, 2007). Several previous studies have reported that upon toxicant exposure, triglyceride levels decrease since energy is diverted to deal with detoxification processes and cellular repair (Calow, 1991; Benton *et al.*, 1994; Munkittrick and Dixon, 1998; Adams, 1999; Levesque *et al.*, 2002; Campbell *et al.*, 2003). In contrast, several recent studies have reported increased energy stores in fish exposed to uranium mining effluents compared to corresponding control fish (Kelly and Janz, 2008; Driedger *et al.*, 2009). Also, polychlorinated biphenyl-exposed eels (*Anguilla anguilla* L.) lost less weight compared to unexposed eels after an 800-km migration (Ginneken *et al.*, 2009).

The reason for the increase in triglyceride level observed after DNP exposure is not known, but a possible explanation is that interference with ATP production secondarily blocks the ability of fish to use their triglyceride stores. Lipid oxidation in red muscle is considered the main energy source during sustained exercise in fish (Richards *et al.*, 2002). DNP-mediated inhibition of oxidative metabolism in zebrafish may have forced an early switch from lipid

catabolism to anaerobic glycolytic energy production. If this switch to anaerobic metabolism also occurred with a greater reliance on white muscle, it would explain decreased triglyceride usage, early fatigue, and decreased U_{crit} in the DNP-exposed fish.

Another possible explanation may be related to the reported behavioral adaptation of some fish to decrease their spontaneous locomotor activity in response to toxicant exposure (Kienle *et al.*, 2009) which would conserve energy stores. The decreased swim endurance would be consistent with this decreased locomotion behavioral adaptation, but further energy conservation may have also occurred during the 24 h of exposure preceding the swim testing. In other words, higher metabolic costs are compensated by decreasing swimming activity. This compensation can cause problems since swimming activity is linked to such vital processes as food acquisition and predator evasion. Regardless of the reason, clearly the hypothesis that toxicants always cause depletion of energy stores needs to be revisited with further experimentation using a wider variety of toxicants with a large range of concentrations in order to clarify these disparate results.

In conclusion, the current study has demonstrated the ease and effectiveness of using zebrafish and swimming behavior in acute toxicity studies. Exposure of adult zebrafish to 6 or 12 mg/L DNP for 24 hr caused concentration-dependent impairment in swim endurance that was largely attributed to decreased tail beat frequency. At the same time, after 24-hr DNP exposure and swim testing, there was a concentration-dependent increase in triglyceride stores remaining in the zebrafish. This suggests either that DNP-exposed fish were unable to mobilize their triglyceride stores or they adapted their behavior by decreasing locomotor activity to conserve their energy stores. Future studies should be directed at examining the reversibility of the acute

and more chronic DNP-induced changes in swim performance and energy stores as well as examining potentially more sensitive, developmental life stages.

3.0 Persistent effects on adult swim performance and energetic in zebrafish developmentally exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

3.1 Introduction

Examination of developmental toxicity can serve as an important determination of the ability of pollutants to cause sublethal toxicity in fish since early life stages are often more sensitive to lower, more environmentally relevant concentrations of toxicants (McBee *et al.*, 1987; Westernhagen, 1988; Kendall *et al.*, 1990; McMurry *et al.*, 1995; Tanguay *et al.*, 2003). This is due to a number of reasons including undeveloped detoxification processes and the fact that rapidly dividing cells tend to be more sensitive since the energy is being devoted to cell growth and division rather than for protection against the toxicant (Westernhagen, 1988; Rogers and Kavlock, 2001). Lethal toxicity tests are more commonly used for regulation because it can be difficult to detect immediate sublethal effects during development. Thus, if exposed to a sublethal amount of toxicant, actual effects may not manifest until later in life and such a lag makes establishing cause-effect relationships difficult. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent and persistent organic pollutant with high chemical stability and lipophilicity (Rappe, 1984; Shlu *et al.*, 1988). TCDD and its congeners are not used in any industry but are unwanted byproducts in several chemical processes including smelting, chlorine bleaching of paper pulp, the manufacturing of some herbicides and pesticides, and are present in car exhaust (Hites, 1990). High biological and chemical stability enables TCDD to persist in the environment and in organic tissue for long periods of time.

Zebrafish (*Danio rerio*) serve as an effective model for examining TCDD-mediated developmental cardiovascular toxicity (for review see Carney *et al.*, 2006b). Exposure to lethal

doses causes blue sac disease-like symptoms including pericardial and yolk sac edema as well as craniofacial and spinal deformities (Elonen *et al.*, 1998). One of the first measurable deformity-induced effects is the cessation of blood flow to peripheral vessels followed by both pericardial and yolk sac edema, then finally to ventricular standstill and death (Hornung *et al.*, 1999). The critical period for TCDD to cause these developmental deformities in zebrafish appears to be within the first 96 hours after fertilization (Goldtone and Stegeman, 2006).

The molecular mechanism of TCDD toxicity appears to be dependent upon activation of the aryl hydrocarbon receptor (AHR), which then acts as a transcription factor, upregulating a host of genes including cytochrome P450 1A (CYP1A), a detoxification enzyme (Denison and Nagy, 2003; Prasch *et al.*, 2003). The role that CYP1A plays in mediating the effects of TCDD has been somewhat controversial. Some studies have reported that CYP1A is required to mediate TCDD toxicity (Teraoka *et al.*, 2003; Antkiewicz *et al.*, 2006), while other studies say it is not (Carney *et al.*, 2004). A previous study found that CYP1A activity was not increased above control in the first filial generation of zebrafish produced by adults developmentally exposed to TCDD even though characteristic deformities were present (King-Heiden *et al.*, 2009). However, other studies indicate that increases in CYP1A by AHR agonists were associated with a deformed cardiac phenotype (Bugiak and Weber, 2010) and decreased blood flow in the mid-brain (Teraoka *et al.*, 2009). More studies are required to clarify these discrepancies.

Carney *et al.* (2006) suggested that sublethal effects of TCDD exposure during development need to be studied. Swim performance tests may serve as an effective method for examining sublethal toxicity in fish. Swim performance has been shown to be linked to food acquisition, predator evasion, over winter survivability, and migratory fitness, all of which are

essential for fish survival and overall population health (Hammer, 1995; Plaut, 2001). Swimming performance has been shown to be affected by several factors including temperature, food availability, and toxicant exposure (for review see Hammer, 1995; see Chapter 2 of this thesis). This may account for the decrease in pink salmon (*Oncorhynchus gorbuscha*) experimentally-exposed to crude oil returning to native spawning grounds (Heintz *et al.*, 2000) or for the lower than expected numbers of returning sockeye salmon (*Oncorhynchus nerka*) to the Fraser River in British Columbia (Hanson *et al.*, 2008; Veldhoen *et al.*, 2009). The most common method for examining swim performance in fish is by examining the critical swimming speed (U_{crit}), an exhaustive exercise test (Brett, 1964). U_{crit} is both a measure of the fish speed and endurance. However, defects in swim performance may not be limited to decreases in swimming speed and endurance (U_{crit}) as toxicant exposure may also affect the way the fish swims, especially during a developmental study where exposure may cause deformities that persist later in life. Bainbridge (1963) suggests that simple measurements of tail beat amplitude and frequency may be an effective way of examining swim motion. These swim motion endpoints are often related to the swimming speed of the fish and may serve as an effective way of comparing how swimming motion and speed are related once exposed to toxicant.

The impacts of sublethal exposure to TCDD on fish energetics have not been characterized even though it has been reported to cause a similar wasting syndrome to that seen in mammals (Kleeman *et al.*, 1988). Locomotion accounts for a large portion of the expended energy budget in fish (Boisclair and Sirios, 1993) and lipids serve as the main source of energy for fish muscle during locomotion (Chatelier *et al.*, 2006; Magnoni and Weber, 2007). During toxicant exposure in fish, lipid levels have been reported to decrease in order to compensate for the energy demands involved in detoxification and cellular and tissue repair processes

(Munkittrick and Dixon, 1988; Calow, 1991; Benton *et al.*, 1994; Adams, 1999; Levesque *et al.*, 2002; Campbell *et al.*, 2003). In TCDD exposed mammals, decreased fat stores are not only due to decreased feeding behavior (Potter *et al.*, 1986), but also to direct interference with energy pathways by TCDD and AHR (Gorski *et al.*, 1990; Weber *et al.*, 1991; Croutch *et al.*, 2005). However, the effect of TCDD on energy metabolism at sublethal doses is unclear in mammals and unknown in fish.

Sublethal exposures to TCDD have been shown to cause persistent reproductive effects in fish (Geisy *et al.*, 2002; King-Heiden *et al.*, 2005; King-Heiden *et al.*, 2009), but no study so far has examined persistence of sublethal TCDD effects on swim performance, energetics, and cardiovascular structure. Zebrafish are a common model fish species used to examine developmental toxicity due to their large spawning numbers, transparent embryos, and rapid maturation, but their robust swimming speeds (Plaut, 2000) also make them excellent for use in studies examining toxic effects that could persist to adulthood. It is hypothesized that exposure to TCDD during the critical period of cardiovascular development (2-4 days post fertilization or dpf) will cause decreased swim performance, decreased triglyceride stores and increased cardiovascular deformities that will persist in zebrafish until adulthood. Furthermore, it is also hypothesized that increased CYP1A expression during this critical period of development will be required to mediate these toxic effects. To test these hypotheses, zebrafish larvae were exposed from 2-4 dpf to increasing concentrations of TCDD, then reared in clean water until 90 dpf. Mortalities, deformities and CYP1A activity were measured at 5 dpf. This was then compared to mortalities, deformities, triglyceride stores, swim performance, and cardiovascular structure at 90 dpf to examine persistence of TCDD effects.

3.2 Materials and Methods

3.2.1 Chemicals

TCDD (10 µg/mL), dimethyl sulfoxide (DMSO), toluene, tricaine methane sulphonate (MS-222), and ethoxyresorufin were purchased from Sigma-Aldrich (Oakville, ON). TCDD was originally dissolved in toluene. The toluene was evaporated under nitrogen and a 2 µg/mL TCDD stock was reconstituted in DMSO. Other TCDD concentrations were made from serial dilution of the 2 µg/mL stock in DMSO.

3.2.2 Fish Maintenance, Exposure, and Rearing

All fish housing and experimental procedures were approved by the Animal Research Ethics Board at the University of Saskatchewan in accordance with guidelines of the Canadian Council on Animal Care. Adult zebrafish were purchased from a local pet store and acclimated for at least four weeks in a static 30 liter holding tank with particulate and charcoal filters as well as ammonia biofilters. Fish were maintained in 28°C dechlorinated municipal water and kept on a 14 h light:10 h dark photoperiod. Fish were fed twice daily with brine shrimp flake (Argent Chemical Laboratories, Redmond, WA) and San Francisco Bay Brand *Chironomus tentans* larvae (Newark, CA). Water in the static tank was monitored with a partial change every two days to maintain quality. Twelve adult fish (four male, eight female) were placed into a static breeding tank overnight. Eggs were collected the following morning, washed with system water containing 0.002% w/v methylene blue (Sigma-Aldrich) and incubated in Petri dishes (50 eggs/10 mL) for 48 hours with daily renewal of the methylene blue solution. After 48 hours, 6

eggs were placed per well of a 12-well Falcon cell culture plate containing 3 mL of fresh dechlorinated municipal water.

Eggs were statically exposed for 48 hours total (from 48 to 96 hours post fertilization (hpf; i.e. 2-4 days post fertilization) to DMSO control (0.05% v/v) or TCDD at nominal concentrations of 0.01 ng/L, 0.1 ng/L, 1 ng/L and 100 ng/L (the latter considered a lethal, positive control group) with daily renewal. After 96 hpf, larvae were maintained in clean dechlorinated municipal water with daily renewal. Larvae were raised in 12-well tissue culture plates until 10 dpf, with daily feeding of *Paramecia multimicronucleatum* beginning at 5 dpf. At 10 dpf, 50 larvae were transferred to separate mesh holding nets (10 cm x 10 cm x 15 cm) for each treatment group in a 30 L static exposure tanks and raised until 40 dpf in clean water. Three rounds of breeding were carried out in a staggered manner and thus, 150 fish were raised passed 10 dpf per exposure group. Larvae were fed twice daily with food that was gradually transitioned from paramecium to brine shrimp flake and *C. tentans* larvae. From 40 dpf till 90 dpf, each replicate for each treatment group fish was maintained in clean water in separate 30 L static tanks. At 5 and 10 dpf, subsets of the larvae from each treatment were euthanized with an overdose of MS-222 and digital photographs taken under an Olympus dissecting microscope at 40x magnification. The incidence of deformities (craniofacial abnormalities, pericardial or yolk sac edema, spinal deformities) and mortalities were recorded at 5, 10, and 90 dpf.

3.2.3 *In Vivo* Ethoxyresorufin-o-deethylase (EROD) Activity

Ethoxyresorufin is converted by CYP1A into resorufin via its ethoxyresorufin-*O*-deethylase (EROD) activity (Nacci *et al.*, 1998; Noury *et al.*, 2006). Resorufin is a fluorescent product that accumulates in the gall bladder of fish and can be measured via densitometric

analysis (Nacci *et al.*, 1998; Noury *et al.*, 2006) using image analysis software. Fish from the different groups were incubated with 21 $\mu\text{g}/\text{mL}$ ethoxyresorufin dissolved in DMSO (0.5% v/v). Incubation was for 2 hours when larvae were at 5 dpf, after which fish were transferred to clean medium and examined under a Zeiss AxioVert Z1 fluorescent microscope (excitation/emission=530 nm/590 nm). The background intensities were subtracted from the measured gall bladder intensities. The relative intensity was calculated by dividing the corrected intensity by the average DMSO control intensity and expressed as fold-change compared to control.

3.2.4 Swim Tunnel Set-up, Critical Swimming Speed, and Swim Motion Analyses

A 100 mL Loligo Systems (Denmark) swim tunnel was used for swim testing. The swim tunnel was submerged in a 20 L buffer tank supplied with 28°C aerated, dechlorinated municipal water from a 20 L recirculating water bath (VWR International, Mississauga, ON). Water velocities were calibrated using constant temperature anemometry according to manufacturer's instructions (Loligo Systems, Denmark). A Fastec Troubleshooter (High Speed Imaging, Winnipeg, MB) high speed camera (250 frames/sec) was mounted over the swim tunnel for swim motion analysis. Access to the room was limited while experiments were in progress to minimize disturbance to fish.

Fish at 90 dpf were placed in the swim tunnel and acclimated for 30 minutes at zero water velocity. After acclimating, fish was forced to swim against a current of 1 cm/s. Water velocity was then increased by a prescribed amount (U_{ii} - 2.5 cm/s) at the end of a prescribed time interval (T_{ii} - 3 min). This process was repeated until the fish fatigued. At that point, U_{crit} was calculated (Brett, 1964):

(Equation 1)

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

where U_i is the highest velocity maintained for an entire time interval and T_i is the time that elapsed at the fatigue velocity. Since fish cross sectional area was <10% of working section area of swim tunnel, the solid blocking correction was not applied to the U_{crit} values (Bell and Terhune, 1970).

A Fastec Troubleshooter high speed camera and Midas 4.0 capture software (Sacramento, CA) were used to acquire a 5 sec video at the beginning of every speed increment or at the first point where the fish was positioned in the middle of the swim tunnel in view of the camera. Frames were analyzed at the U_i speed (the last speed maintained for an entire time interval prior to the fatigue speed). Poor quality videos were eliminated from the study. Single digital images were extracted from the camera files using Adobe Premiere Elements 2.0 (San Jose, CA) and analyzed using Image Pro Express 6.0 (Bethesda, MD). The swim motion endpoints measurements were similar to that used in a previous study from this laboratory (see Chapter 2 of this thesis): compression ratio (the ratio of the bent length to the total body length), left and right tail beat amplitudes, left and right tail beat angles, and tail beat frequency. The tail beat amplitudes and angles were calculated relative to midline at the point of greatest compression. Three different frames were analyzed for each endpoint and the average value was used in the statistical analysis.

3.2.5 Oxygen Consumption

Mean oxygen consumption rate (MO_2) was determined using a Fibox 3 fiber optic oxygen sensor and AutoResp 1 software (Loligo Systems, Denmark). The probe was calibrated

according to manufacturer's instructions and the closed respirometry method, whereby the swim tunnel remains closed to oxygen replenishment, was used in determining MO_2 :

(Equation 2)
$$MO_2 = ([O_2]_{t0} - [O_2]_{t1}) \times V/t \times BW$$

where $[O_2]_{t0}$ and $[O_2]_{t1}$ are the initial and final oxygen concentrations, V is the respirator volume minus the volume of the fish, t is the elapsed time, and BW is the body weight of the fish.

Two MO_2 measurements were determined at each speed increment, with the average value then used in statistical analysis. The aerobic scope of each fish was determined by subtracting the resting MO_2 from the $U_i MO_2$.

3.2.5 Triglyceride Analysis

Following U_{crit} determination, fish were euthanized using MS-222, weighed, measured with a vernier caliper, and frozen at -80°C until analyzed. The condition factor was calculated by dividing the weight by the length³. Whole body triglyceride levels were determined using lipase/glycerol kinase based reagents obtained from Sigma-Aldrich (Oakville, ON). Fish homogenates were prepared using the method described by Bennett *et al.* (2007).

3.2.6 Histological Analysis

A total of n=3-5 zebrafish at 90 dpf were used for histological determination of heart and vessel dimensions. Following determination of U_{crit} , fish were euthanized with an overdose of MS-222 before being transferred into neutral buffered formalin. After 36 hours, fish were transferred to 70% ethanol and stored until histological analysis could be performed. Whole fish

were paraffin-embedded and cross-sectioned (5 μm) in series beginning at the anterior end of the eyes and ending at the posterior of the abdominal cavity, then stained with hematoxylin/eosin and mounted. Digital micrographs were obtained with a Zeiss AxioVert Z1 light microscope. Photographs were taken at 200x magnification. Three serial sections each of the largest part of the ventricle, atrium, and bulbus arteriosis were averaged and the mean cross sectional area for each chamber was used in statistical analysis. The diameters of the dorsal aorta and posterior cardinal vein were determined in sections obtained immediately posterior to the posterior end of the gut cavity. Three serial sections were averaged for each blood vessel and the mean value was used in statistical analysis. All diameters were measured using AxioVision software.

3.2.7 Data and Statistics

Differences among treatment groups were analyzed using a one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc tests where appropriate with $p < 0.05$ considered to be significantly different. Tail beat amplitudes and U_{crit} were analyzed using a one-way analysis of covariance (ANCOVA) followed by Fisher's LSD post hoc test where appropriate using body length as a covariate. Results are presented as the mean \pm the standard error of the mean (SEM).

3.3 Results

3.3.1 Effect of TCDD on Zebrafish EROD Activity

At 5 dpf, larvae from all treatment groups accumulated fluorescent resorufin product in the gallbladder (Figure 3.1A). There was no significant increase in CYP1A activity in zebrafish exposed 0.01, 0.1, and 1 ng/L TCDD compared to control (Figure 3.1B). However, the group exposed to the lethal dose of TCDD (100 ng/L) had a 30-fold increase CYP1A activity compared to control (Figure 3.1B).

3.3.2 Deformities, Mortalities, Biometrics, and Energetics of Zebrafish Developmentally Exposed to TCDD

At 5 dpf, most DMSO-exposed larvae were normal (Figure 3.2A). Although not significantly different from control, TCDD-exposed larvae exhibited some incidence of the classic TCDD-associated deformities, namely spinal curvature, pericardial edema, yolk sac edema and craniofacial malformations (Figure 3.2A). However, by 90 dpf, the majority of fish in all treatments were grossly normal-looking, with deformities limited to a bend in the caudal peduncle in some fish (Figure 3B). At both 5 and 10 dpf there were no significant differences in mortalities or deformities in larvae exposed to sublethal amounts of TCDD (0.01, 0.1, 1 ng/L) compared to control (Table 3.1). In contrast, the positive control exposure group (100 ng/L TCDD) had a significant increase in both mortalities and deformities at 5 dpf when compared to control (Table 3.1). By 10 dpf, all the larvae exposed to 100 ng/L TCDD had died and thus deformity rates could not be obtained. At 90 dpf, there were no significant differences in

mortalities or deformities amongst the surviving treatment groups compared to control (Table 3.1).

At 90 dpf, body length was significantly decreased in the 0.01 and 0.1 ng/L TCDD groups, but not in the 1 ng/L TCDD group compared to control (Table 3.2). Neither body weight nor condition factor was different in TCDD-exposed groups compared to control (Table 3.2). Also at 90 dpf, whole body triglyceride concentrations following swim testing were elevated in the 0.1 and 1 ng/L, but not 0.01 ng/L, TCDD exposure groups compared to control (Figure 3.3).

3.3.3 Effects of TCDD on Swim Performance and Aerobic Scope

At 90 dpf, absolute critical swimming speed (expressed as cm/s) was decreased in the 0.1 and 1 ng/L TCDD exposure groups when compared to control (Figure 3.4A). Relative critical swimming speed (body lengths/s) was decreased in the 0.1 ng/L TCDD exposure group only, but with a trend toward a decrease in the 1 ng/L TCDD exposure group (Figure 3.4B). There were no differences in the swim motion parameters of the TCDD-exposed zebrafish (Figure 3.5) except for a significant decrease in the right tail beat angle of the 0.01 ng/L TCDD exposure group compared to control (Figure 3.5). There was also no significant difference in tail beat frequency among the exposure groups (Figure 3.5). In contrast, there was a significant increase in both resting MO_2 (measured at zero water velocity in swim tunnel) and MO_2 at U_i (measured at highest completed water velocity segment) in zebrafish exposed to 0.1 ng/L TCDD compared to control, but this did not translate to a change in aerobic scope (Figure 3.6).

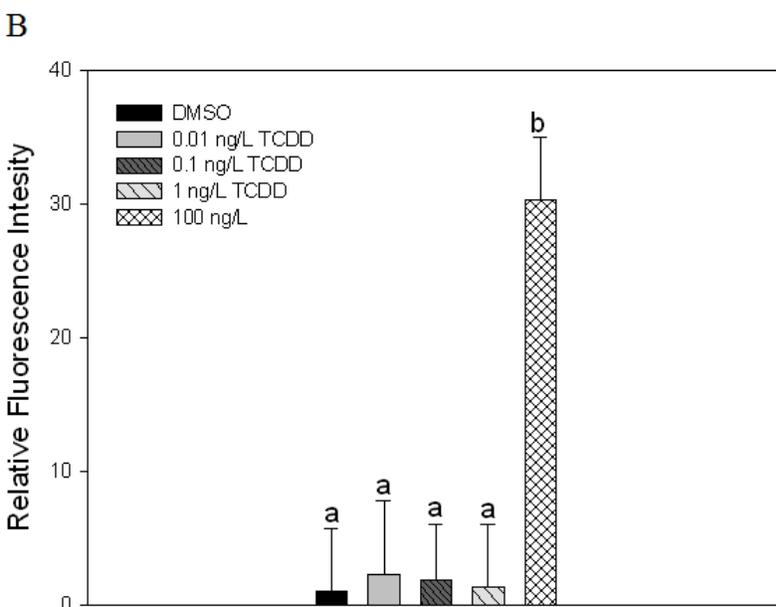
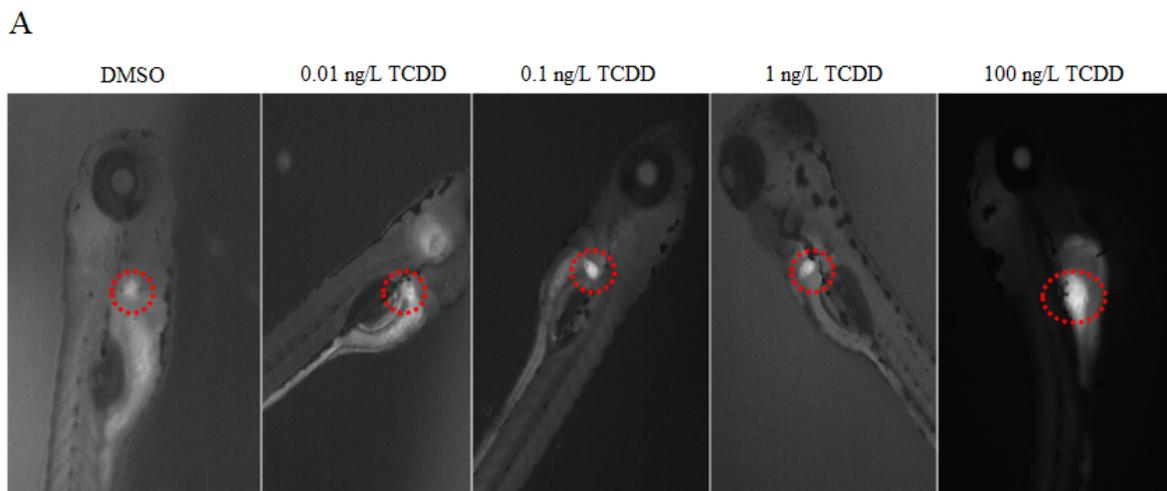


Figure 3.1. CYP1A activity in 5 dpf zebrafish exposed to TCDD. Zebrafish were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin between 2-4 days post fertilization (dpf). At 5 dpf, fish were exposed to ethoxyresorufin, a specific CYP1A substrate, for 2 hours to allow for resorufin formation and accumulation in the gall bladder which is circled in red (A). Excitation/emission was measured at 530 nm/590 nm and results were expressed as fold-increase in fluorescence intensity relative to DMSO control (B). Data is expressed as mean \pm SEM. Differences in letters denotes significant differences ($p < 0.01$) in Fisher's LSD post hoc test after one-way ANOVA. Sample sizes were $n=7$ for DMSO control, $n=5$ for 0.01 ng/L TCDD, $n=9$ for 0.1 ng/L TCDD, $n=7$ for 1 ng/L TCDD, and $n=7$ for 100 ng/L TCDD.

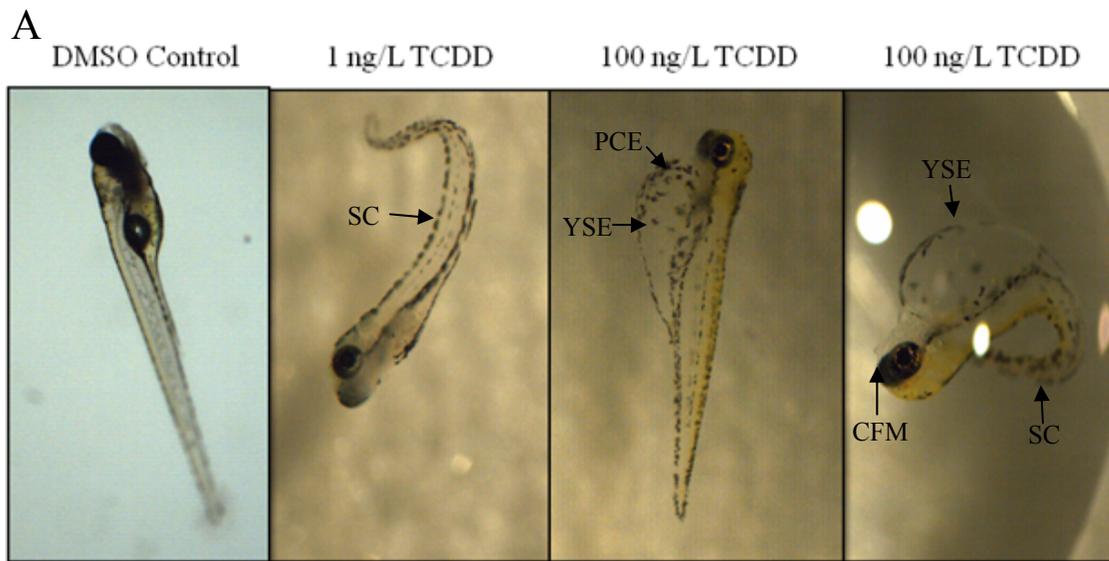


Figure 3.2. Characteristic deformities in zebrafish exposed to TCDD. Zebrafish larvae were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) between 2-4 days post fertilization (dpf). At 5 dpf (A) and 90 dpf (B), characteristic deformities were recorded. Images were taken at 40x magnification for the 5 dpf larvae or with the high speed camera for 90 dpf fish. Arrow in B denotes a spinal deformity at the caudal peduncle. SC- spinal curvature, PCE- pericardial edema, YSE- yolk sac edema, CFM- craniofacial malformation.

Table 3.1. Deformities and mortalities of zebrafish developmentally exposed to TCDD.

Zebrafish were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin between 2-4 days post fertilization (dpf) and deformities and mortalities were assessed at 5, 10, and 90 dpf. For 5 and 10 dpf determinations, 6 larvae per well were used for each n number. For the 90 dpf determination, 50 fish per breeding round were used for each n number. Data is represented as mean \pm SEM. * p < 0.001 versus vehicle control in Fisher's LSD post hoc test after one-way ANOVA. Sample sizes were $n=11$ for 5 dpf, $n=7$ for 10 dpf, and $n=3$ for 90 dpf time points. NS- no survivors.

	<u>Deformities (%)</u>			<u>Mortalities (%)</u>		
	5 dpf	10 dpf	90 dpf	5 dpf	10 dpf	90 dpf
DMSO	4.8 \pm 5.1	8.3 \pm 7.1	4.8 \pm 4.7	3.0 \pm 2.9	4.2 \pm 5.8	65.2 \pm 9.1
0.01 ng/L TCDD	11.9 \pm 5.1	16.7 \pm 7.1	8.3 \pm 8.3	4.5 \pm 2.9	4.8 \pm 4.4	74.0 \pm 9.1
0.1 ng/L TCDD	11.9 \pm 5.1	29.2 \pm 7.1	16.1 \pm 9.1	4.5 \pm 2.9	2.4 \pm 4.4	72.0 \pm 9.1
1 ng/L TCDD	9.5 \pm 5.1	25.0 \pm 7.1	10.0 \pm 6.5	3.0 \pm 2.9	7.1 \pm 4.4	77.8 \pm 9.1
100 ng/L TCDD	80.6 \pm 5.5*	NS	NS	22.2 \pm 3.9*	100 \pm 4.8*	NS

Table 3.2. Biometrics of 90 dpf zebrafish developmentally exposed to TCDD. Zebrafish were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin between 2-4 days post fertilization (dpf) and raised to 90 dpf. Data is presented as mean \pm SEM. * p <0.02 versus DMSO control in Fisher's LSD post hoc test after one-way ANOVA. Sample sizes were: $n=25$ for DMSO control, $n=36$ for 0.01 ng/L TCDD, $n=34$ for 0.1 ng/L TCDD, and $n=29$ for 1 ng/L TCDD exposure groups.

	Body Length (mm)	Body weight (mg)	Condition Factor
DMSO	19.0 \pm 0.5	90.9 \pm 9.6	1.3 \pm 0.2
0.01 ng/L TCDD	17.5 \pm 0.4*	73.8 \pm 7.4	1.5 \pm 0.1
0.1 ng/L TCDD	17.4 \pm 0.4*	69.4 \pm 7.6	1.4 \pm 0.1
1 ng/L TCDD	18.1 \pm 0.4	86.5 \pm 7.8	1.5 \pm 0.1

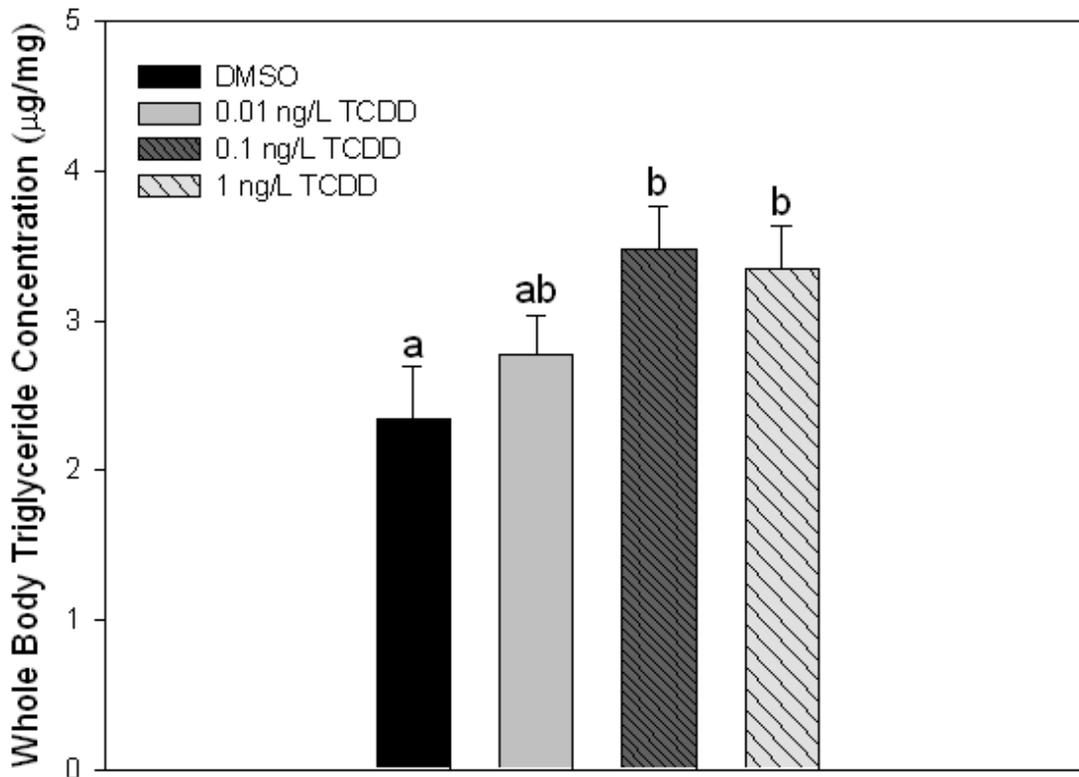


Figure 3.3. Triglyceride levels in 90 dpf zebrafish developmentally exposed to TCDD.

Zebrafish were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) between 2-4 days post fertilization (dpf) and raised to 90 dpf. Triglycerides were measured in fish euthanized immediately after swim testing. Data is presented as mean \pm SEM. Differences in letters denote significant differences ($p < 0.05$) in Fisher's LSD test after one-way ANOVA. Sample sizes were $n=16$ for DMSO control, $n=27$ for 0.01 ng/L TCDD, $n=25$ for 0.1 ng/L TCDD, and $n=23$ for 1 ng/L TCDD exposure groups.

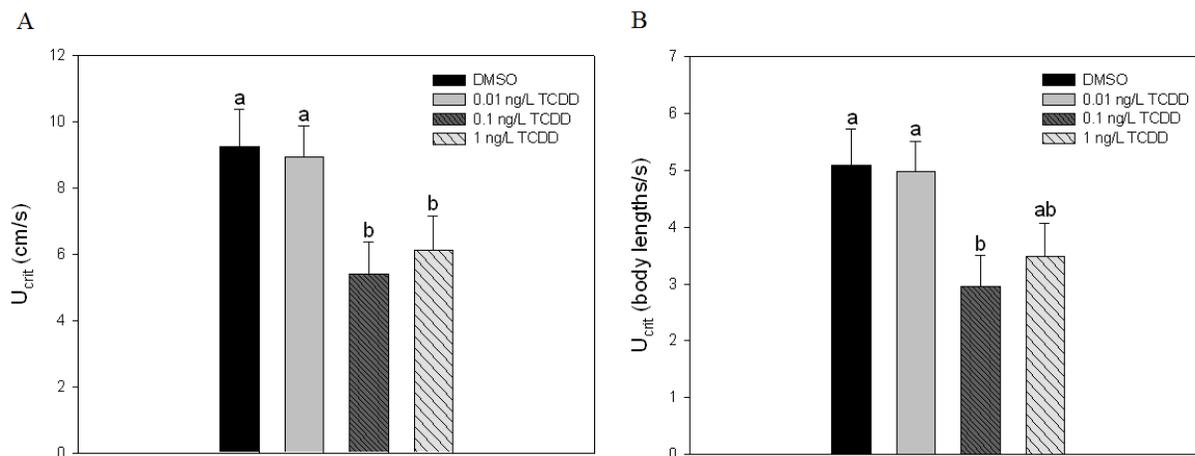


Figure 3.4. Critical swimming speeds of 90 dpf zebrafish developmentally exposed to TCDD. Zebrafish were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) between 2-4 days post fertilization (dpf) and raised to 90 dpf. Critical swimming speed (U_{crit}) is presented as absolute critical swimming speed (A) and relative critical swimming speed (B). Data is presented as mean \pm SEM. Differences in letters denote significant difference ($p < 0.05$) in Fisher's LSD post hoc test after one-way ANCOVA using body length as a covariate (A; cm/s) or after one-way ANOVA (B; body lengths/sec). Sample sizes were: $n=25$ for DMSO control, $n=36$ for 0.01 ng/L TCDD, $n=34$ for 0.1 ng/L TCDD, and $n=29$ for 1 ng/L TCDD exposure groups.

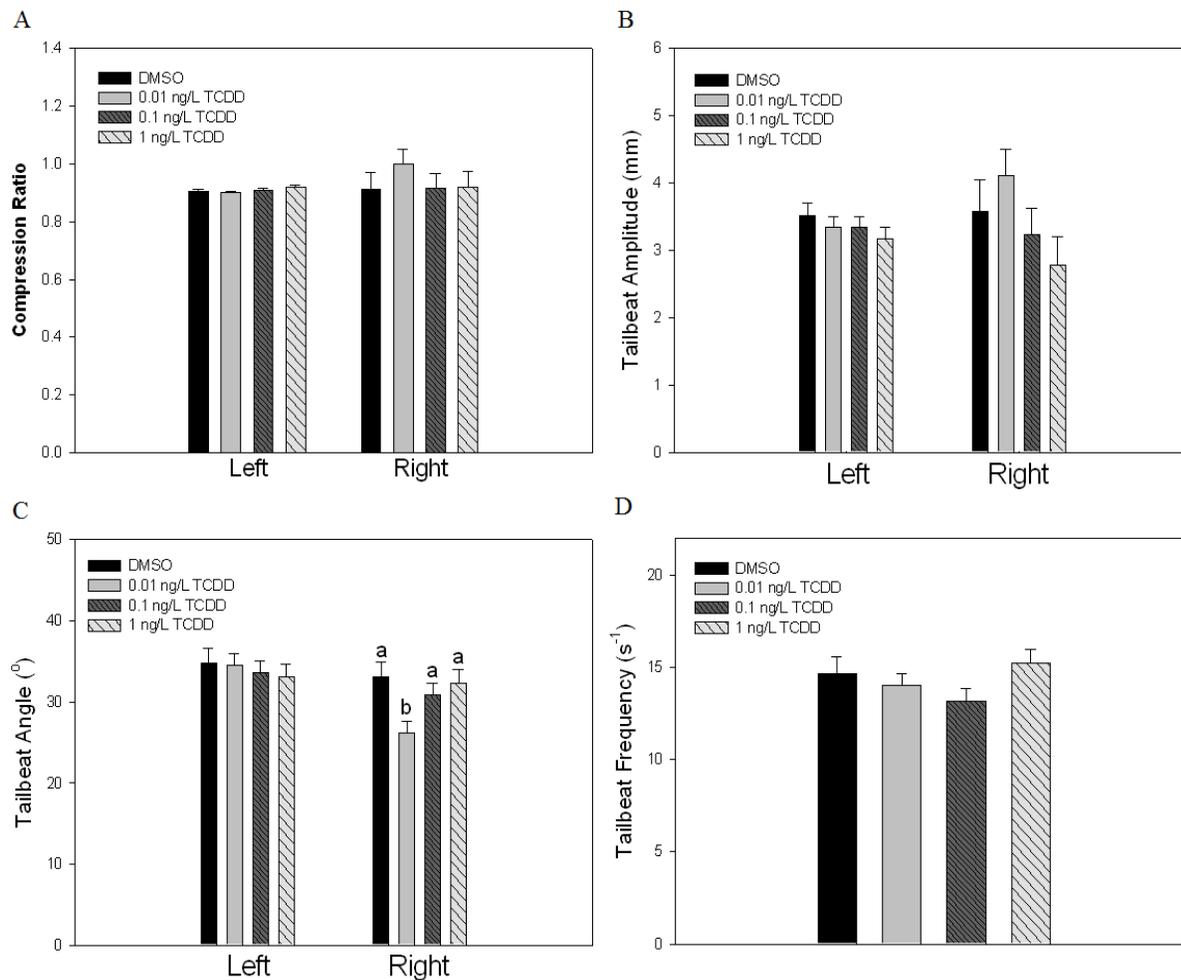


Figure 3.5. Swimming motion parameters of 90 dpf zebrafish developmentally exposed to TCDD. Zebrafish were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) between 2-4 days post fertilization (dpf) and raised to 90 dpf. Compression ratio (A), tailbeat amplitude (B), tailbeat angle (C), and tailbeat frequency (D) were the assessed during U_i speed of critical swimming speed determination. Data is presented as mean \pm SEM. Difference in letters denotes significant difference ($p < 0.05$) in Fisher's LSD post hoc test after one-way ANOVA. Sample sizes were $n=25$ for DMSO control, $n=36$ for 0.01 ng/L TCDD, $n=34$ for 0.1 ng/L TCDD, and $n=29$ for 1 ng/L TCDD exposure groups.

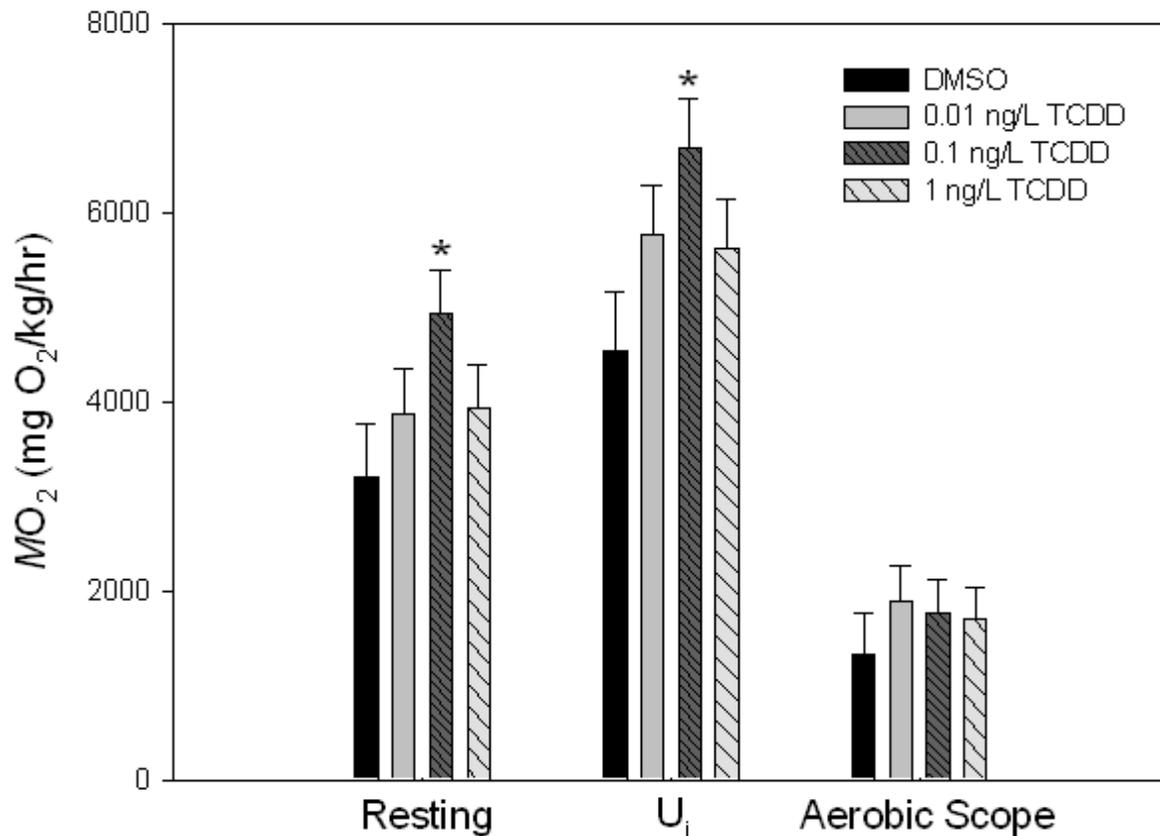


Figure 3.6. Oxygen consumption in 90 dpf zebrafish developmentally exposed to TCDD. Zebrafish were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) between 2-4 days post fertilization (dpf) and raised to 90 dpf. Oxygen consumption rate (MO_2) was measured after acclimation in the swim tunnel during rest (zero water velocity) and during U_i (highest completed water velocity segment). Aerobic scope was calculated by subtracting the resting MO_2 from the U_i MO_2 . Data is presented as mean \pm SEM. * $p < 0.05$ compared to DMSO control in Fisher's LSD post hoc test after one-way ANOVA. Sample sizes were $n=10$ for DMSO control, $n=14$ for 0.01 ng/L TCDD, $n=15$ for 0.1 ng/L TCDD, and $n=15$ for 1 ng/L TCDD exposure groups.

A



B

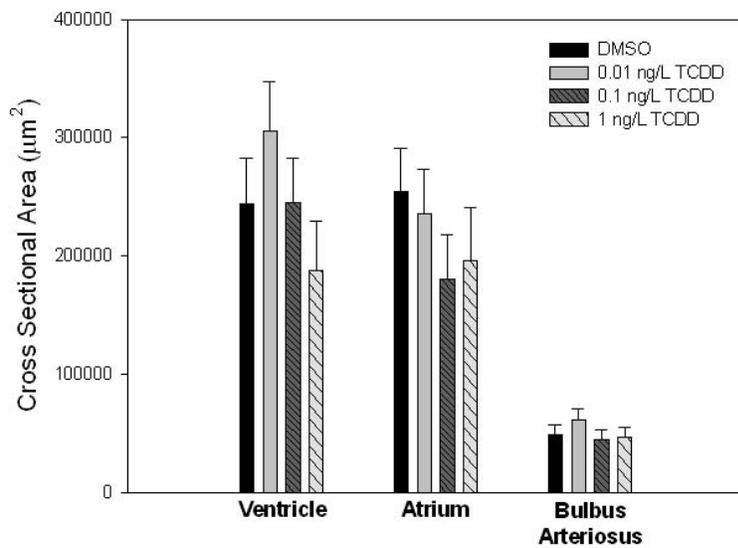


Figure 3.7. Cross sectional areas of heart chambers in 90 dpf zebrafish developmentally exposed to TCDD. Zebrafish were developmentally exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin between 2-4 days post fertilization (dpf) and were raised to 90 dpf. Cross sectional areas of the ventricle, atrium, and bulbus arteriosus were determined using digital image analyses (B), each at their point of maximum size (A: left panel for atrium, middle panel for ventricle and right panel for bulbus arteriosus). Results are presented as mean \pm SEM. Sample sizes were: n=6 for DMSO control, n=5 for 0.01 ng/L TCDD, n=6 for 0.1 ng/L TCDD, and n=5 for 1 ng/L TCDD exposure groups. V- ventricle; A- atrium; BA- bulbus arteriosus.

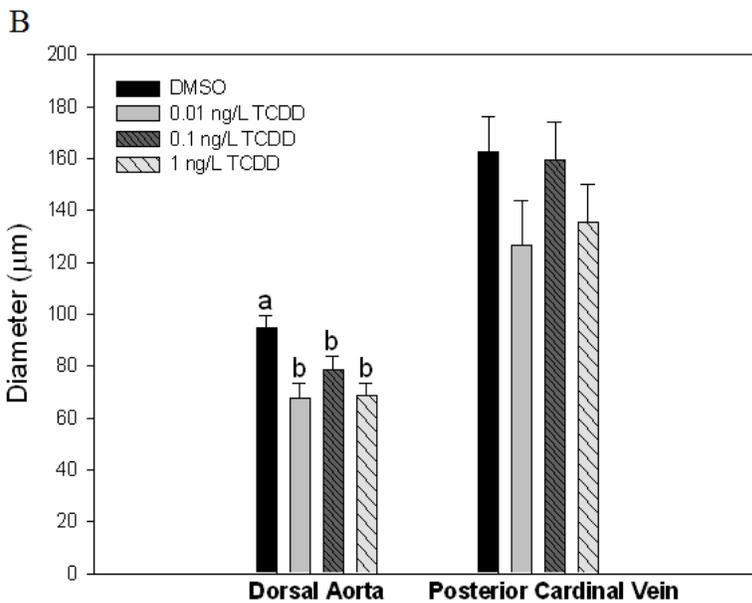
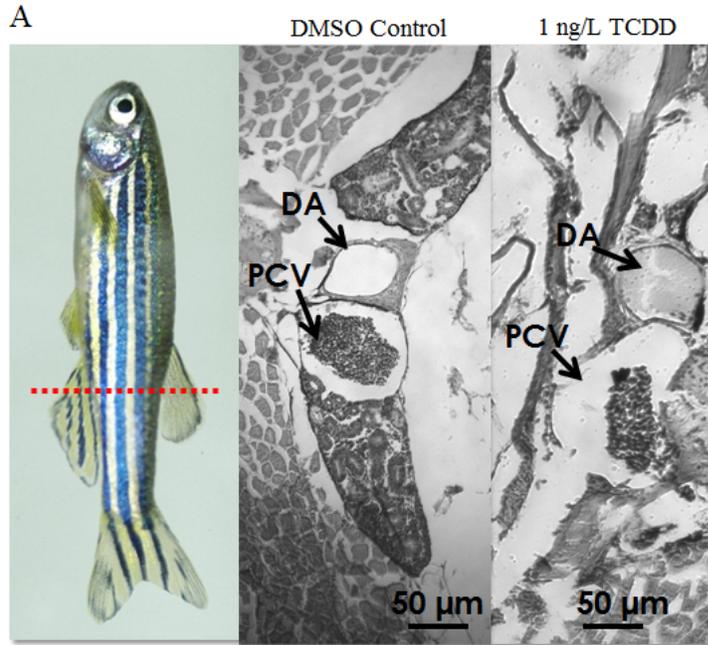


Figure 3.8. Blood vessel diameters in 90 dpf zebrafish developmentally exposed to TCDD. Zebrafish were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) between 2-4 days post fertilization (dpf) and raised to 90 dpf. Vascular cross sections were obtained at the point indicated by the dotted red line (A; immediately posterior to the posterior end of the gut cavity) and diameters were determined using digital image analysis (B). Data presented as mean \pm SEM. Differences in letters denote significant difference from DMSO control ($p < 0.05$) in Fisher's LSD post hoc test after one-way ANOVA. Sample sizes were: $n=5$ for DMSO control, $n=3$ for 0.01 ng/L TCDD, $n=4$ for 0.1 ng/L TCDD, and $n=5$ for 1ng/L TCDD exposure groups. DA- dorsal aorta; PCV- posterior cardinal vein.

3.3.4 Cardiovascular Changes in Adult Fish Exposed Developmentally to TCDD

At 90 dpf, all hearts in all treatments were qualitatively histologically normal (Figure 3.7A). There were also no differences in cross-sectional areas of zebrafish heart chambers (atrium, ventricle or bulbus arteriosus) among the different exposure groups (Figure 3.7B). In contrast, peripheral vascular diameters at 90 dpf were significantly changed, with a decrease in dorsal aorta diameter but not posterior cardinal vein diameter in all the TCDD-exposed groups compared to control (Figure 3.8B). However, at 90 dpf there were no histopathological changes in either blood vessel regardless of treatment (Figure 3.8A).

3.4 Discussion

The most significant finding of this study was that sublethal, developmental exposure (from 2-4 dpf) to TCDD caused a decrease in swim endurance at 90 dpf, an effect that persisted long after the period of TCDD exposure. The threshold for this effect was 0.1 ng/L TCDD. Furthermore, it is important to note the TCDD-exposed groups exhibiting this impaired swim endurance did not show any significant increase in gross deformities. Acute toxicant exposure during adulthood has been previously shown to decrease U_{crit} in fish (Webb and Brett, 1973; Howard, 1975; Kovacs and Leduc, 1982; Cripe *et al.*, 1984; Cheng and Ferrell, 2007; Tudorache *et al.*, 2008), but this is the first time that this effect has been reported after developmental TCDD exposure. While the exposure period was between 2-4 dpf, the persistent nature and chemical stability of TCDD may have resulted in residues persisting within the tissue reservoirs (Belair *et al.*, 2001) until adulthood. However, because of growth dilution and the short, low level TCDD exposure in the current study, this seems an unlikely explanation. When the

decreased swim endurance observed in the current study is considered in combination with reproductive toxicity reported previously (King-Heiden *et al.*, 2009), developmental exposures to sublethal levels of TCDD have the potential to cause population level effects.

In the current study, the highest TCDD exposure (100 ng/L) caused a high incidence and severity of deformities typical of developmental exposure to AHR agonists. Most of these larvae died in the period between 5 and 10 dpf, agreeing with a previous study from this laboratory (Bugiak and Weber, 2010). On the other hand, exposure to lower, sublethal TCDD levels (0.01, 0.1, and 1 ng/L) did not show any increases in deformities, yet the cumulative mortality to 90 dpf was quite high (~70%). In fact, this cumulative mortality did not differ compared to the control exposure group. Although not specifically quantified between 10 and 90 dpf, most mortalities in control and all surviving TCDD-exposed groups occurred shortly after 10 dpf, a period of naturally high mortality in all fish as they transition from yolk to exogenous feeding (Belanger *et al.*, 2010).

While the length of the fish was decreased by exposure to TCDD, the condition factor and weight were unaltered among the exposure groups at 90 dpf. Using a traditional toxicological approach, this would be taken to indicate that the general health of the zebrafish was largely unaffected by TCDD exposure. Both length and condition factor have been shown to be positively correlated with U_{crit} (Beamish, 1978; Nicoletto, 1991; Plaut, 2000; Lapointe *et al.*, 2006). This may explain why the decrease in absolute U_{crit} did not appear to be affected by length, because the condition factor was unaltered. However, relative U_{crit} (i.e. U_{crit} represented as BL/s) was also significantly decreased in the 0.1 ng/L TCDD exposure group with a similar trend to decrease, albeit non-significant, at the at 1 ng/L exposure. Since the goal of this study was to examine more environmentally relevant and sensitive effects of TCDD exposure, the

absolute swimming speed (cm/s) may serve as a more appropriate measure than relative swimming speed (BL/s). In other words, smaller fish will have a harder time swimming a given distance than larger fish, but must still try to do this in order to compete and survive.

There was a decrease in right tail beat angle in the 0.01 ng/L TCDD exposure group, but this was not seen in any of the other exposure groups. This alteration in swimming motion did not correspond to a decreased U_{crit} in this same group, suggesting swim motion was unrelated to swim endurance. If TCDD-exposed zebrafish with more severe deformities had survived to 90 dpf, perhaps it would have resulted in alterations in swimming motion. Also, the survivability of more severely deformed fish may have been low for reasons unrelated to swimming, such as an impaired ability to feed due to craniofacial malformations.

The elevated resting and U_i oxygen consumption in the 0.1 ng/L exposure group in the current study disagrees with a previous study where PCBs (also AHR agonists) decreased oxygen consumption compared to unexposed adult eels during a prolonged swim (Ginneken *et al.*, 2009). However, several methodological differences are likely explanations for this difference. Ginneken *et al.* (2009) did not perform exhaustive exercise tests while the current study did. Also, the developmental effects of TCDD in the current study are likely to be quite different than that produced by PCB exposure during adulthood. More importantly, in the current study the aerobic scope was unchanged by TCDD exposure, indicating that TCDD exposed fish can still mount a similar increase in metabolic rate in response to the demands of exhaustive exercise. Thus, although some differences in metabolic rate were noted in the current study, they do not provide an explanation for the decreased swim endurance observed at 0.1 and 1 ng/L TCDD..

In the current experiment where U_{crit} was decreased (0.1 and 1 ng/L TCDD), these groups also had elevated whole body triglyceride concentrations. Increased triglycerides after TCDD exposure is contrary to our original hypothesis and disagrees with previous studies using a variety of toxicants (Munkittrick and Dixon, 1988; Calow, 1991; Benton *et al.*, 1994; Adams, 1999; Levesque *et al.*, 2002; Campbell *et al.*, 2003). Even though TCDD was reported to cause chronic wasting in fish, the doses used were very high and eventually lethal (Kleeman *et al.*, 1988). Furthermore, although TCDD is known to disrupt lipid metabolism in mammals (Potter *et al.*, 1986; Alexander *et al.*, 1998; Croutch *et al.*, 2005; Sato *et al.*, 2008), the sublethal effects of TCDD on fish energetics are not well understood. In fact, a few previous studies do support the results of the current study with similar reports of increased triglyceride stores in fish collected from environments affected by industrial effluents (Kelly and Janz, 2008; Driedger *et al.*, 2009; Sancho *et al.*, 2009; Sancho *et al.*, 2010). Also, when examining simulated migration in PCB-exposed eels, it was found that exposed eels lost less weight compared to control eels following 800 km of swimming (Ginneken *et al.*, 2009). However, swim performance was not quantified in this study so it is not known whether the eels were altering their swimming to conserve energy or whether the PCBs had a direct effect on energy storage pathways. .

While the mechanism for the increased triglyceride levels in the exposure groups observed in this study are not known, it may be the result of behavioral adaptation of the fish. Other studies have suggested that the higher metabolic costs of dealing with stress are compensated by decreasing spontaneous swimming activity (Campbell *et al.*, 2002; Taylor *et al.*, 2004; Kienle *et al.*, 2009). Thus, the lower critical swimming speeds and higher triglyceride levels may be the result of the fish changing behavior to conserve energy to deal with toxicant exposure, leading to interference with vital processes such as food acquisition and predator

evasion (Hammer, 1995; Plaut, 2001). However, the low level TCDD exposure at such an early stage of development (2-4 dpf) in the current study is unlikely to have resulted in persistence of any significant TCDD body burden once growth dilution is taken into account. Alternatively, the sublethal, developmental TCDD exposure may have exerted persistent effects on the ability of fish to mobilize lipid stores via an epigenetic alteration of lipid metabolism pathways.

Epigenetic mechanisms are now considered to be a factor in TCDD-induced alterations in certain gene expression profiles (Wirgin and Waldman, 2004; Cui *et al.*, 2009; Beedanagari *et al.*, 2010). Regardless of the mechanism, if the fish are not able to effectively mobilize stores for energy for muscle contraction, this could be a contributing factor to the decrease in U_{crit} that was seen in this study.

A novel finding in the current study is that dorsal aorta, but not posterior cardinal vein, diameter decreased in all TCDD exposure groups compared to control. This contrasts with a previous study from this laboratory which found an increase in dorsal aorta diameter upon exposure to AHR agonists in 10 dpf zebrafish (Bugiak and Weber, 2010). The differences in findings are likely due to different locations studied between the current (posterior end of abdomen) and the previous study (anterior end of abdomen). Also, this may be due to a developmental difference since the previous study examined larval while the current study examined adult arteries. Regardless, the current study suggests that sublethal exposure to TCDD during development does have cardiovascular consequences that persist into adulthood. In relation to swim performance, a decreased dorsal aorta diameter would result in decreased blood perfusion to the periphery and, consequently, decreased peripheral oxygen levels which could account for the observed decrease in U_{crit} . Inconsistent with this idea, a decrease in dorsal aorta diameter was seen in the 0.01 ng/L TCDD group, a group that did not have decreased U_{crit} .

Furthermore, other vascular structures were not examined and may have exhibited important changes. Future studies need to more comprehensively examine vascular bed structure and function in order to better establish the relationship between vascular changes and swimming in fish.

In the current experiment, none of the sublethal TCDD exposure groups had an increase in CYP1A activity compared to control at 5 dpf, suggesting that AHR was not stimulated. This disagrees with a previous study from this laboratory which saw increased *cyp1a* mRNA levels in 5 and 10 dpf zebrafish larvae after exposure to a similar concentration (1 ng/L TCDD) as the current study (Bugiak and Weber, 2010). This discrepancy likely reflects differences in sensitivity between quantitative PCR techniques and *in vivo* EROD used in the current study. Supporting this, a previous study examining the sublethal effects of TCDD on reproduction similarly found that developmental deformities and reproductive impairment persisted into the first filial generation of zebrafish despite an absence of detectable CYP1A activity (King-Heiden *et al.*, 2009). Thus, although the results of the current study suggest that CYP1A induction may not be required to mediate TCDD toxicity at a sublethal level and that it is not involved in TCDD mediated decreases in critical swimming speed, the possibility of involvement cannot be completely eliminated until confirmation with more sensitive measures of CYP1A are used.

In conclusion, the current study has demonstrated that sublethal, developmental exposure (from 2-4 dpf) to sublethal concentrations of TCDD decreased swim endurance in 90 dpf zebrafish despite rearing in clean water for the entire time after exposure. The cause of this persistent decrease in swim endurance is not known, but may be related to behavioral adaptations limiting swimming capabilities, failure to mobilize triglyceride stores, cardiovascular deformities limiting blood flow to the periphery, or a combination of these factors. In addition, toxicities

related to sublethal, developmental TCDD exposure and swimming capabilities do not appear to be dependent on increased CYP1A activity.

4.0 Overall Discussion and Conclusions

One of the major goals of this thesis was to examine similarities and differences between acute exposure during adulthood and developmental exposure during the larval stage with respect to performance and energy usage during swimming in zebrafish. While the two toxicants used in this thesis were different both in their chemistry and modes of action, they are both known to cause energetic effects in fish. Thus, despite their differences, some comparisons can be made between these two toxicants.

4.1 DNP Versus TCDD

DNP and TCDD differ markedly in their toxicities. DNP is a rapidly metabolized protonophore that acutely effects energy utilization by interfering with the mitochondrial electron transport chain (Cutting *et al.*, 1933; Blaikie *et al.*, 2006). The other toxicological consequences, including increased β -oxidation, heart rate and ventilation rate are seen as secondary to the metabolic effects it exerts (Cutting *et al.*, 1933; Harper *et al.*, 2001; Blaikie *et al.*, 2006; McKim *et al.*, 2009). While accumulation of DNP has been observed in some fish species in a few heavily polluted sites, it is generally thought to be metabolized and eliminated quickly from the body once removed from the toxic environment (Harris and Cocoran, 1995). Even though DNP is found in a number of polluted sites, few studies had examined its sublethal effects.

Unlike DNP, acute effects of TCDD are thought to be minor unless at exceptionally high exposure levels. Instead, the long biological residency of dioxins creates a high capacity for biological accumulation and tends to produce important chronic or developmental toxicities (for

review see Goldstone and Stegeman, 2006). These toxic effects are wide ranging, and are not completely understood, with developmental, immunological, cardiovascular, reproductive, and neurological consequences (for review see Carney *et al.*, 2006b). While there is extensive literature examining the developmental effects of TCDD toxicity, only a few have looked at the sublethal impacts (Giesy *et al.*, 2002; King-Heiden *et al.*, 2005; King-Heiden *et al.*, 2009) and fewer still have looked at sublethal developmental impacts that persist to adulthood (King-Heiden *et al.*, 2009). All of these studies that examined sublethal impacts of TCDD focused only on reproductive success. Thus, this thesis was the first to examine sublethal toxicity of both DNP and TCDD by examining how these toxicants affect energy stores and swim performance.

4.2 Acute Versus Developmental Effects

4.2.1 Energy Utilization

DNP and TCDD are both known to have energetic effects. DNP is known to increase β -oxidation of fatty acids at sublethal doses in humans in order to mobilize the fatty acids to generate more ATP (Cutting *et al.*, 1933). TCDD is also known to decrease fat stores by causing chronic wasting through various molecular and behavior mechanisms including altered lipid biosynthesis (Alexander *et al.*, 1998) and decreased feeding behavior (Potter *et al.*, 1994). The sublethal effects of TCDD and DNP on triglyceride stores in fish have not been previously examined and cannot be assumed to produce the same energetic effects as lethal exposure. In fact, contrary to predictions based on these previous experiments using lethal exposures, the results in this thesis showed that triglyceride levels were elevated in zebrafish exposed to TCDD

or DNP compared to control fish. However, it should be noted that in both cases, triglycerides were determined following U_{crit} measurements and thus it could be argued that the apparent elevation is an artifact of toxicant effects on swimming and the resulting post-swimming condition, not a direct effect on energy metabolism. Alternatively, both DNP and TCDD may have direct effects on metabolism, impairing energy mobilization and resulting in larger energy stores in exposed fish. In support of this, developmentally TCDD-exposed adult zebrafish that were not used for swim testing exhibited similar trends for increased triglyceride levels compared to control fish, especially in the 1 ng/L exposure group (Figure 4.1). However, samples sizes for these unswum fish were small and were from a separate subgroup of fish that, qualitatively, did not appear as healthy. Thus, more detailed experiments are required to confirm whether the increased triglycerides do indeed arise before fish are forced to swim.

Alternatively, recent studies also suggest that behavioral adaptations often sacrifice locomotion in order to compensate for toxicant exposure and protect energy stores for other purposes (Campbell *et al.*, 2002; Taylor *et al.*, 2004; Kienle *et al.*, 2009). Although the amount of spontaneous activity was not quantified in the zebrafish in this thesis, this behavioral adaptation could explain why two very different toxicants were able to cause the same effect in triglyceride levels following U_{crit} determination. By decreasing their spontaneous activity or the amount of forced swimming during a swim test, the zebrafish in the current thesis may potentially have conserved their lipid stores in response to toxicant exposure. Whatever the reason, it is clear that the traditional notion that lipid stores decrease in response to toxicant exposure needs to be re-evaluated.

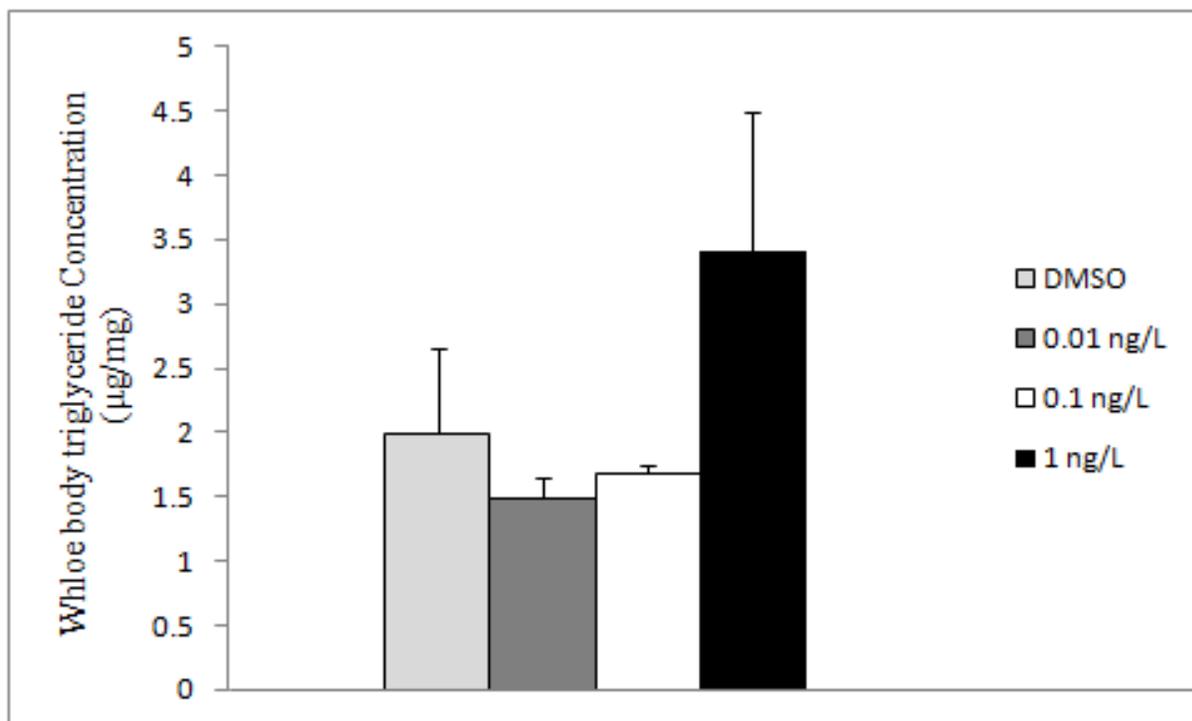


Figure 4.1. Triglyceride levels of unswum 90 dpf zebrafish developmentally exposed to TCDD. Zebrafish (n=3) were developmentally exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) between 2-4 days post fertilization (dpf) and raised to 90 dpf. Triglycerides were measured in fish euthanized without swim testing. Data is presented as mean \pm SEM.

4.2.2 Swimming

The swimming speeds of the control fish in the DNP study did not compare to the high swimming speeds obtained by Plaut and Gordon (1994; 14 BL/s), but did compare with earlier work done by Fuiman and Webb (1988; 4-6 BL/s). The cause of the difference is unknown but may be related to differences in fish housing, diet and swim tunnel set up. The TCDD study controls had similar relative swimming speeds as the DNP study (5 BL/s), but had considerably lower absolute swimming speeds (9 cm/s versus 14 cm/s). This was expected given the smaller size of the TCDD study fish (~1.7-1.9 cm long) compared to the DNP study fish (~3 cm long).

Both toxicants, DNP and TCDD, caused decreases in the critical swimming speed of zebrafish. The acute exposure to DNP appeared to result in a dose-response relationship whereas the developmental TCDD exposure appeared to be more threshold dependent, with the two higher exposure groups showing U_{crit} decreased to a similar degree. Of the swim motion parameters (tail beat angle and amplitude, compression ratio) examined, only tail beat frequency after exposure to DNP was altered in a manner consistent with U_{crit} changes. Because the DNP experiment used an acute exposure during adulthood, differences in swim motion parameters other than tail beat frequency were not expected since deformities were not produced by this DNP exposure regime. In contrast, the experiment with developmental TCDD exposures was predicted to change the manner in which the fish could swim due to alterations in skeletal form. In support of this prediction, right tail beat amplitude was altered in one of the TCDD exposure groups (0.01 ng/L) when compared to control. However, this did not affect swim endurance and the two higher TCDD exposure groups that did have decreased swim endurance showed no change in swim motion. Therefore, the sensitivity of the swim motion analyses may have been insufficient to detect changes in swim motion in the current thesis. Alternatively, TCDD and

DNP may have effects on other swimming behavior patterns that were not examined in this study. For example, zebrafish showed a burst and coast pattern of swimming. If swim motion tended to be analyzed during the only one type of swim pattern and if the proportion of bursting versus coasting were altered by toxicant treatment, this could provide an explanation for the decreased swim endurance without a change in swim motion. Future studies should use video cameras along with high speed photography in order to more effectively monitor qualitative aspects of swimming behavior.

Aside from changes in swim behavior, the cause of the decreased U_{crit} in the acute DNP experiment is likely due to its mitochondrial uncoupling effect. With less ATP being generated for muscle usage, it is expected that lower swim endurance would be observed. In contrast, the cause of the decreased U_{crit} in the developmental TCDD experiment is not clear. TCDD likely exerted effects in multiple tissue types in many different ways. Certain biometric, cardiovascular, and energetic endpoints were examined in this study and the decrease in U_{crit} may be primarily attributed to adverse effects of any of these including: decreases in body length, triglyceride usage, and dorsal aorta diameter. Decreased body length of TCDD-exposed zebrafish means that these fish must swim harder to cover the same distance as a larger fish, but this should translate into greater tail beat frequencies and/or increased triglyceride usage, both of which were not apparent in this thesis. If peripheral blood flow was impaired due to a smaller dorsal aorta diameter, then this may have caused a failure of triglycerides to be moved from storage sites to working muscle which in turn may explain decreased swim endurance. The exact linkages between energy, cardiovascular structure and swimming need to be clarified with further experiments.

From the studies presented in this thesis, it is clear that swimming performance, and mainly U_{crit} determination, is an effective method for determining sublethal toxicity that may lead to population level effects. U_{crit} is effective for determining both acute, adult toxicity as well as developmental toxicity. This is especially evident in the sublethal TCDD study, in which U_{crit} decreased despite no significant increases in mortalities or deformities, underscoring its apparently greater sensitivity than these more traditional toxicology end-points.

4.3 Deficiencies of Current Experiments and Future Experiments

It is difficult to make comparisons between the two exposures since one was a developmental exposure and one was an acute, adult exposure. The developmental exposure has broader environmental relevance since acute toxicity usually only occurs in heavily contaminated or spill sites. Future studies should examine developmental and chronic DNP exposure in order to better characterize how swim performance and energy stores are affected by this toxicant. Chronic exposure to DNP has been shown to decrease circulating red blood cells in fish (Verma *et al.*, 1981b), which, in combination with mitochondrial uncoupling, may have profound impacts on swimming capabilities. Although at the time of the current study, an O_2 sensing probe was not available, addition of oxygen consumption rate (MO_2) measurements in future DNP studies would be extremely beneficial. Also, tissue-specific changes in triglyceride levels (e.g. muscle versus liver) as opposed to changes in whole body triglyceride levels should be characterized. Another important energy storage molecule, glycogen, should also be examined. Finally, examination of lactate levels would also serve as a measure of the extent of anaerobic metabolism taking place in the fish. In summary, both the DNP and the TCDD study would benefit from a more thorough examination of energy usage following toxicant exposure.

The TCDD study also suggests that induction of CYP1A during early development was not required for TCDD-mediated decreases in U_{crit} . While previous studies from this lab suggest that CYP1A expression is required for the manifestation of certain toxic phenotypes during early development (Bugiak and Weber, 2010), this study supports other work that found a dissociation between CYP1A activity and deformities following sublethal TCDD exposures (King-Heiden *et al.*, 2009). However, more conclusive molecular studies are warranted. Given the persistent nature of TCDD, examination of adult gene expression profiles in arteries and gills following developmental exposure are warranted. Bugiak and Weber (2009) found that intraperitoneal injection of adult zebrafish with TCDD resulted in elevated CYP1A mRNA expression in both the mesenteric artery and liver. However, CYP1B1, CYP1C1, and CYP1C2 mRNA levels were elevated to a greater extent in the artery when compared to liver. It would be interesting to see if these results would be mimicked in adult zebrafish exposed developmentally to TCDD.

The availability of antisense morpholinos enables researchers to knockdown CYP1A mRNA translation during early development (Nasevicius and Ekker, 2000). Concomitant exposure to TCDD with CYP1A knockdown, followed by examination of swim performance later in life would be a more conclusive determinant of the role CYP1A plays in TCDD-mediated decreases in U_{crit} . This technique could be applied to a host of genes that are thought to be responsible for mediating lethal developmental deformities in zebrafish. It may also serve as a method for examining whether these genes play an endogenous role in swimming behavior in fish. Transgenic zebrafish lines that express green fluorescent protein in their vasculature would also be useful for examining subtle, sublethal developmental cardiovascular toxicity in zebrafish larvae (Bagatto and Burggren, 2006) and to more clearly examine of how persistent TCDD induced cardiovascular impairments can affect swimming capabilities.

5.0 References

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