The mechanism of retene toxicity in the early life stages of fish

by

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Abstract

Alkylphenanthrenes such as retene (7-isopropyl-1-methylphenanthrene) are aquatic contaminants commonly found in anthropogenically-, industrially-, and petroleum-contaminated environments, and have been implicated in crude oil toxicity. In the early life stages (ELS) of fish, exposures to alkylphenanthrenes produce signs of toxicity typical of those observed in exposures to halogenated aromatic hydrocarbons, particularly to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD, the most toxic congener, serves as the basis of the current mechanism-based risk assessment model. The model assumes that congeners that produce TCDD-like toxicity share a common mode of action and act additively. The mechanism of TCDD-like toxicity is assumed to be mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor involved in the xenobiotic response (e.g., induction of cytochrome P450 1A enzymes; CYP1A) and in normal development. CYP1A enzymes are not involved in the mechanism of TCDD toxicity.

Alkylphenanthrenes toxic to the ELS of fish are AhR ligands, but in contrast to TCDD, are readily metabolized by CYP1A enzymes. The byproducts of CYP1A metabolism have been implicated in retene toxicity. However, the target tissue of retene and the direct roles of AhR and CYP1A in retene toxicity are unknown, but are expected to be similar to those of TCDD.

The results presented in this thesis suggest that in the ELS of fish: (1) the primary target of retene is the cardiovascular system (Chapters 2 & 5); (2) retene toxicity is stage-specific (Chapter 2); (3) the mechanism of retene toxicity is mediated by AhR2, and is independent of CYP1A enzymes (Chapter 5); (4) multiple CYP1A-independent toxicities can result from exposures to different mixtures of CYP1A inducing (retene) and CYP1A inhibiting (α-naphthoflavone or 2-aminoanthracene) PAHs (Chapters 3 & 4); and (5) multiple concentration-dependent mechanisms of toxicity (i.e., synergism and response addition) can occur in co-
exposures of a CYP1A inducer (retene) with a range of CYP1A inhibitor (α-naphthoflavone) concentrations (Chapter 3). Thus, retene toxicity is mechanistically similar to that of TCDD toxicity, suggesting alkylphenanthrenes can be included in the current risk assessment model. However, the observed variable mixture toxicities and species differences in retene toxicity raise questions about the effectiveness of this model.
Co-Authorship

Chapters 2-5 were co-authored by Dr. Peter V. Hodson (my supervisor). Dr. Hodson contributed to the experimental design and data interpretation, and edited all chapters.

Chapter 2 (Stage-specific retene cardiovascular toxicity in Japanese medaka (*Oryzias latipes*) embryos) and 5 (AhR2-dependent, CYP1A-independent cardiovascular toxicity in zebrafish (*Danio rerio*) embryos exposed to retene) are co-authored by Kathleen Pelkki and Sally Shepardson (Saginaw Valley State University, University Center, MI). Kathleen Pelkki prepared sectioning tools, sectioned and stained half of the specimens examined by light and transmission electron microscopy, and digitalized the micrographs. Sally Shepardson contributed to the experimental design and success of the microscopy by providing expertise and training on the microtome and transmission electron microscope. Their efforts contributed to Figures 2-1 and 5-4, and provided valuable insights into the structural and ultrastructural effects of retene exposures. In addition, both individuals edited the manuscripts.

Chapter 4 (Embryotoxicity of retene in co-treatment with 2-aminoanthracene, a CYP1A inhibitor, in rainbow trout (*Oncorhynchus mykiss*) is co-authored by Meagan Ross and Ben Lemire. Both individuals assisted with the trout exposures, EROD and Western blot analyses, and synchronous scanning spectrofluorometry of water samples, which were the foundation of the experiment.

Chapter 5 is also co-authored by Dr. John P. Incardona (Northwest Fisheries Science Center, National Atmospheric and Oceanic Administration, Seattle, WA), who provided expertise and training on microinjection and microscopy techniques. Dr. Incardona contributed to the experimental design, performed the confocal microscopy and some stereomicroscopy which contributed to Figures 5-1, 5-2, and 5-6.
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List of abbreviations

2AA  2-aminoanthracene
AhR  aryl hydrocarbon receptor
ANF  α-naphthoflavone
Arnt aryl hydrocarbon receptor nuclear translocator
BNF  β-naphthoflavone
BSD  blue sac disease
CYP1A cytochrome P450 1A
DMSO dimethyl sulfoxide
EC50 median effective concentration
EROD ethoxyresorufin-O-dethylase
HAH halogenated aromatic hydrocarbon
HIF hypoxia inducible factor
IC50 median inhibitory concentration
LC50 median lethal concentration
LD50 median lethal dose
MeOH methanol
MO morpholino
PAH polycyclic aromatic hydrocarbon
PE pericardial edema
ROS reactive oxygen species
SI severity index
TCDD 2,3,7,8-tetrachlorodibenzo-p-dioxin
TEF toxic equivalency factor
TEQ toxic equivalency quantity
QSAR quantitative structure-activity relationship
XRE xenobiotic responsive elements
YE yolk sac edema
Chapter 1
General Introduction and Literature Review

1.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous environmental contaminants that contain 2-7 fused aromatic rings and exist in both unalkylated and alkylated forms (Fig. 1-1). PAHs are lipophilic, chemically stable, and have low water solubilities, which facilitates their accumulation and persistence in the environment. They readily undergo photodegradation, biodegradation, and metabolism (Douben, 2003).

PAHs are derived from petroleum, incomplete combustion of petroleum products and organic material, and are biotransformation products of biogenic precursors, such as plant resin acids (e.g., retene; Garcia et al., 1993). PAHs are invariably found in complex mixtures, which vary in PAH congener composition based on the source. In general, PAH mixtures derived from pyrogenic sources consist primarily of unalkylated PAHs; however, the amount of alkylated PAHs increases as combustion temperatures decrease. Petroleum sources (e.g., fuel, crude oil) are predominantly alkylated PAHs (Latimer and Zheng, 2003).

The environmental inputs of PAH are via: (1) natural sources, including oil seeps and forest fires; and (2) anthropogenic sources, including combustion of fossil fuels and petroleum spills. Due to their widespread distribution, increasing contamination, and toxicity, PAHs have been listed 8th on the Comprehensive Environmental Response, Compensation, and Liability Act’s priority list of hazardous substances (ATSDR, 2007).

In general, PAH are predicted to be type 1 narcotics (i.e., produce narcosis via a non-specific mode of action driven solely by lipophilicity; Verhaar et al., 1992), and some are known
carcinogens (see Di Giulio et al., 1995). Based on their carcinogenic and mutagenic potential, the U.S. Environmental Protection Agency has listed 16 PAHs as priority contaminants.

1.1.1 PAHs in the aquatic environment

In contrast to the decreasing levels of chlorinated aromatic hydrocarbons (e.g., polychlorinated biphenyls, furans, dioxins), the level of PAH contamination in the aquatic environment is increasing. A comprehensive analysis of PAH contamination in 38 lakes distributed throughout the U.S. has indicated that only 5% of the lakes showed a decrease in PAH contamination (Van Metre and Mahler, 2005). There was an increase in total PAH in 42% of the lakes, which coincided with an increase in anthropogenic activity (Van Metre and Mahler, 2005).
Van Metre and Mahler (2005) propose that the PAHs could surpass the hazardous threat of halogenated aromatic hydrocarbons (HAHs), which has serious implications given the known effects of HAHs on fish populations; HAHs have been implicated in the decline and extirpation of the lake trout (*Salvelinus namaycush*) population in Lake Ontario in the 1960s (Cook *et al*., 2003).

The major sources of PAH contamination in the aquatic environment include urban run-off, industrial and wastewater effluents, atmospheric deposition, natural oil seeps, and petroleum spills (Latimer and Zheng, 2003). Alkylated PAHs are commonly a large proportion of contaminants in these contaminated environments, with 2- to 5-ring alkylated PAHs as the predominant forms (Hellou *et al*., 1999; Wang *et al*., 1999; Brandt *et al*., 2002; Zakaria *et al*., 2002).

Atmospheric deposition presents a potentially significant input of alkylated PAHs. Diesel fuel contains a higher proportion of alkylated PAHs than unalkylated PAHs, which can be unaltered during combustion and released in gaseous phase or bound to particulate matter (Dobbins *et al*., 2005). Considering the overall use of diesel fuel, approximately 168-630 million U.S. gallons/day consumed for transportation alone (Kiuru, 2002), there is a considerable risk of alkylated PAH exposure to both terrestrial and aquatic organisms. Notably, the predominant forms of PAHs in diesel fuel are alkylanthracenes and alkylphenanthrenes, which are toxic to the early life stages of fish (discussed below; Kiparissis *et al*., 2001; Turcotte, 2008).

Oil spills are a notable source of alkylated PAHs and represent a considerable threat to aquatic environments due to the increasing worldwide demand and transport of crude oil. It was estimated that over 10 trillion U.S. gallons of oil was transported worldwide between 1972 and 1999 (Anderson and LaBelle, 2000). In that 22 year span, there were 278 crude oil spills greater
than 42,000 U.S. gallons from tankers worldwide, 118 of which occurred in coastal areas (Anderson and LaBelle, 2000).

1.1.2 Crude oil toxicity

The risk of oil contamination to aquatic organisms, particularly to the early life stages of fish, was brought to the forefront in 1989 when the Exxon Valdez oil tanker spilled nearly 11 million U.S. gallons of Alaska North Slope crude oil (Anderson and LaBelle, 2000) in the intertidal and nearshore spawning grounds of pink salmon (*Oncorhynchus gorbuscha*) and Pacific herring (*Clupea pallasi*) in Prince William Sound, Alaska. In the following years, population declines were observed in both species (Pearson *et al*., 1999; Bue *et al*., 1998), likely resulting from a decreased survival of early life stages. Subsequent studies revealed that oil exposures produced embryo-larval toxicity, including pericardial and yolk sac edema, skeletal and craniofacial malformations, abnormal development of fin rays, and mortality (Carls *et al*., 1999). Notably, the reported signs of oil toxicity, collectively termed blue sac disease (BSD), were typical of those previously observed in HAH exposures, particularly of those of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

The primary constituents implicated in crude oil toxicity are PAHs, specifically alkylated PAHs (Page *et al*., 2002; Khan, 2008). PAHs represent roughly 1-6% of the total crude oil constituents, 80-90% of which are alkylated (Wang *et al*., 2003). Furthermore, as oil weathers, there is a progressive increase in the percentage of overall alkylated PAHs, particularly in the relative abundance of alkylphenanthrenes. The shift in alkylated PAH composition is likely due to the rate of PAH degradation during weathering, which decreases with increasing ring size and degree of alkylation (Douglas *et al*., 1996). In general, there is a greater amount of alkylated
PAHs in tissues of aquatic organisms (e.g., oysters) than unalkylated PAHs in oil-contaminated environments (Hellou et al., 1994; Norena-Barroso et al., 1999).

Oil fractionation and toxicity studies by Saravanabhavan (2008) and Khan (2008) have implicated 3- to 5-ring alkylated PAHs in crude oil toxicity in the early life stages of fish. Specifically, the oil fractions producing toxicity were rich in naphthobenzothiophene, chrysenes, and alkylphenanthrenes (Khan, 2008; Saravanabhavan, 2008). Single compound exposures have confirmed the oil-like toxicity of alkylphenanthrenes (Billiard et al., 1999; Kiparissis et al., 2001; Turcotte, 2008). Thus, 3- to 5-ring alkylated PAHs, particularly alkylphenanthrenes, are of significant environmental concern due to their toxicity and persistence in oil-contaminated sites.

1.1.3 Alkylated PAH toxicity

Quantitative structure-activity relationship (QSAR) studies of alkylanthracenes and alkylphenanthrenes have identified that the toxicity of alkylated PAH: (i) differs from that of their unalkylated PAH counterparts in both mechanism and severity; (ii) differs among alkylated congeners; and (iii) can be influenced by alkyl group position and/or size (Kiparissis et al., 2001; Turcotte, 2008). For example, only alkylphenanthrenes substituted in the 1, 2, and/or 7-positions caused BSD, an outcome unlike that of alkylanthracenes (narcosis; Kiparissis et al., 2001; Turcotte, 2008) and unsubstituted phenanthrene (atrio-ventricular conduction block; Incardona et al., 2004). Furthermore, the toxicity of these alkylphenanthrenes generally increased with an increase in lipophilicity (Turcotte, 2008). Similar to TCDD and other embryotoxic HAHs, these alkylphenanthrenes are ligands of the aryl hydrocarbon receptor (AhR; Basu et al., 2001; Kiparissis et al., 2001; Billiard et al., 2002), a transcription factor involved in the regulation of a battery of genes including those involved in xenobiotic metabolism. Thus, these findings provide
evidence of a toxicological and a potential mechanistic commonality among alkylphenanthrene, crude oil (see above), and TCDD toxicity to the early life stages of fish.

1.1.4 Retene as a model alkylphenanthrene

Retene (7-isopropyl-1-methylphenanthrene; Fig. 1-1b) is a combustion product of wood (Ramdahl, 1983), biotransformation product of a conifer resin (dehydroabietic) acid found in pulp and paper mill effluents (Garcia et al., 1993), and is likely a constituent in a variety of petroleum sources. It has been found in industrially-contaminated sediments at concentrations up to 3300 µg/g dry weight (Leppanen and Oikari, 1999). In addition, it has been found in forest soils, coal, deep-sea sediments, and in pyrolysates of algae and bacteria (Wen et al., 2000 and references therein). Interestingly, a relatively high abundance of retene has been found in ancient sediments predating the emergence of coniferous plants (Jiang et al., 1998), suggesting additional unknown inputs.

Importantly, sediment-bound retene can dissolve into the water column and become bioavailable to fish (Oikari et al., 2002). In the early life stages of fish, retene causes concentration-dependent increases in BSD and mortality. In zebrafish (Danio rerio), semi-static retene exposures resulted in estimated EC50s of 544 and 445 µg/L at 144 and 240 hours post-fertilization (hpf; Billiard et al., 1999). In Japanese medaka (Oryzias latipes), static retene exposures using a partition-controlled delivery system produced an estimated 17-day EC50 of 9 µg/L, which was the lowest EC50 of all alkylphenanthrenes tested (Turcotte, 2008). In addition, retene exposures (semi-static) caused exposure-dependent increases in BSD in rainbow trout (Oncorhynchus mykiss) larvae, with an LC50 of 178 µg/L and a 14-day post-hatch least observable effect level (LOEC) occurring between 100 and 180 µg/L (Billiard et al., 1999).
Furthermore, a lethal body burden (also referred to as critical body residue; CBR) occurred at a concentration less than $2.3 \times 10^{-3}$ mmol/kg in rainbow trout larvae (Tabash et al., 1999), which is roughly 3 orders of magnitude lower than the CBR needed to induce narcosis. The CBR for narcosis in fish has been estimated to be 2-10 mmol/kg (McCarty et al., 1992). Taken together, these findings suggest that retene toxicity acts via a specific, versus a non-specific (i.e., narcotic), mode of action.

Retene can also be photomodified by ultraviolet (UV)-B radiation, resulting in an increase in larval toxicity. Vahniäinen et al. (2002) report a significantly increased mortality after two daily 3 h UV-B irradiations (2.8 or 5.4 kJ/m2) to vendace (*Coregonus albula*) and whitefish (*Coregonus lavaretus*) larvae pre-exposed to a sublethal concentration of retene. The photoxicity of retene resulted in LC50s of 41 µg/L in vendace larvae, and 15-16 µg/L in whitefish larvae, depending on the UV-B dose (Vahniäinen et al., 2002).

The mechanism of retene toxicity appears to be dependent upon retene metabolism, presumably via the activity of the AhR-regulated cytochrome P450 1A (CYP1A) metabolic enzymes (Hawkins et al., 2002; Brinkworth et al., 2003; Bauder et al., 2005; Hodson et al., 2007). CYP1A metabolism is described in detail in Figure 1-2a. Retene has a relatively weak binding affinity to AhR (Billiard et al., 2002), but is a strong inducer of CYP1A enzymes (EC50 = 63 µg/L; Basu et al., 2001), primarily in the endothelium of kidneys, vasculature, and heart tissues (Brinkworth et al., 2003). It has a rapid uptake and depuration rate in rainbow trout larvae, as evidenced by the presence of retene metabolites in bile after 12 h of exposure (Fragoso et al., 1999). The half-life of retene in trout was estimated at 14 h (Fragoso et al., 1999).

In retene-exposed rainbow trout larvae, Brinkworth et al. (2003) observed a marked increase in the signs of BSD one week following CYP1A induction. The lag time between CYP1A
Figure 1-2. The (a) catalytic cycle and (b) consequences of CYP1A metabolism. SOD, superoxide dismutase; NADPH, nicotinamide adenine dinucleotide phosphate; NADH, nicotinamide adenine dinucleotide.
induction and the onset of toxicity may represent the time needed to deplete the larvae of their defense (i.e., antioxidants) against the byproducts of CYP1A metabolism (reactive metabolites and reactive oxygen species; ROS; Fig. 1-2b). In the early life stages of fish, there is a limited supply of maternally-derived antioxidants, making the susceptible to oxidative damage. Indeed, retene exposures caused a decrease in glutathione and Vitamin E concentrations, and toxicity decreased in retene-exposed larvae co-treated with Vitamin E, implicating ROS in retene toxicity (Bauder et al., 2005).

Reactive metabolites have also been implicated in retene toxicity. Hodson et al. (2007) observed variable mixture toxicity in rainbow trout larvae co-treated with retene and α-naphthoflavone, a CYP1A inhibitor, although the overall trend suggested that toxicity decreased with an inhibited metabolism. Hawkins et al. (2002) reported an increase in retene toxicity after CYP1A induction by β-naphthoflavone. Furthermore, retene toxicity increased correspondingly with the presence of non-hydroxylated, non-conjugated metabolites after inhibition of CYP1A with piperonyl butoxide (Hawkins et al. 2002), which not only identified potential toxic retene metabolites, but also suggested the possible involvement of other CYP enzymes in retene toxicity. At present, the specific roles of the AhR, CYP1A enzymes, and CYP1A-derived byproducts in retene toxicity are unclear.

Taken together, these findings suggest that retene is a suitable model compound to study the potential risk of alkylphenanthrene toxicity in industrially-, anthropogenically-, and crude oil-contaminated environments. The target tissue and mechanism of retene toxicity in the early life stages of fish remains largely uncertain; however, they are expected to share commonalities with those of TCDD.
1.2 TCDD and TCDD-like toxicity

HAHs have been widely used in a variety of commercial products and typically form during the combustion of organic material (e.g., wood; Bumb et al., 1980) and the synthesis of chlorophenols (previously used as herbicides, insecticides, fungicides, and disinfectants; Poland and Knutson, 1982). They enter the aquatic environment via urban-run off, industrial effluents, and atmospheric deposition, and have widespread distribution in industrially-contaminated lake sediments (Oliver et al., 1989). Although the use of HAHs has declined, their chemical stability and resistance to degradation facilitates their long-term persistence in soils and sediments. Unlike PAHs, which can be readily metabolized by CYP enzymes, the halogen substitutions largely prevent HAH metabolism, and their persistence results in a strong propensity to bioaccumulate in food webs. Of the 419 possible HAH congeners, ~20 have been identified for their potential to produce early life stage toxicity in fish (Van der Berg et al., 1998). TCDD is the most potent HAH congener, and serves as the reference compound in risk assessment of HAH and PAH mixture toxicity (discussed below).

The toxicity of TCDD in the early life stages of fish is generally considered to result from an impact on the developing cardiovascular system, which ultimately leads to circulatory failure and death. A reduction in circulation is often the primary observable sign of TCDD-induced BSD (Chen and Cooper, 1999; Hornung et al., 1999; Belair et al., 2001; Dong et al., 2002; Teraoka et al., 2002; Carney et al., 2006). However, there is a controversy over whether the reduction in blood flow is caused by a direct effect on the heart or vasculature.

The embryonic vasculature in a number of fish species has been implicated as the target tissue after TCDD exposures. Cantrell et al. (1996) reported DNA damage-induced apoptotic cell death in the yolk vasculature, which likely lead to the onset of BSD at 5 days post-fertilization (Wisk and Cooper, 1990). Apoptosis was also implicated in a reduction in blood flow in the
midbrain vasculature of TCDD-exposed zebrafish embryos (Dong et al., 2002). Furthermore, a reduced number of branch points in the yolk sac vitelline vasculature were observed in TCDD-exposed trout larvae prior to the onset of edema (Hornung et al., 1999). Lastly, Guiney et al. (2000) suggested that yolk sac edema in TCDD-exposed lake trout larvae resulted from an increase in vascular permeability caused by an increased separation in intercellular junctions.

However, the most compelling evidence, i.e., evidence based on measured effects prior to the onset of any overt toxicity, suggests that the heart is the primary target of TCDD. In TCDD-exposed zebrafish embryos, the earliest measured response was a decrease in the number of cardiomyocytes at 48 hpf, which likely resulted in a decreased cardiac output, and a decrease in heart size in later stages (Antkiewicz et al., 2005). A decrease in heart size has also been reported in the TCDD-exposed embryo-larval stages of rainbow trout (Hornung et al., 1999). In zebrafish embryos exposed to TCDD at 72 hpf, cardiac-specific transcriptional responses preceded a reduction in ventricular stroke volume (Carney et al., 2006). Within the first 1-4 h after TCDD exposure, there was an up-regulation of cardiac genes involved in xenobiotic metabolism (i.e., CYP enzymes), cellular signaling pathways, and transcriptional regulation, which was followed by an overall down-regulation in genes involved in cell division and proliferation at 12 h (Carney et al., 2006).

Whether the conflicting proposed targets result from ontological or physiological differences among the fish species is unclear. Nevertheless, cardiovascular toxicity preceded the other notable signs of BSD (i.e., edema and skeletal deformities). TCDD-induced disruptions in erythropoiesis (Belair et al., 2001) and regression of the common cardinal vein (Bello et al., 2004) have also been reported in zebrafish.

The mechanism of TCDD cardiovascular embryotoxicity is dependent on the activation of AhR2 (the functional isoform in zebrafish), and is independent of CYP1A enzymes (Prasch et
al., 2003; Carney et al., 2004; Dong et al., 2004; Antkiewicz et al., 2006). However, the specific role of AhR2 in mediating toxicity is unclear.

1.3 AhR-mediated toxicity

The AhR is a highly conserved transcription factor that can be activated by a range of structurally diverse endogenous and exogenous compounds, including all the embryotoxic HAH and PAH congeners (Safe, 1990; Denison and Heath-Pagliuso, 1998; Hahn, 1998). Ligand binding enhances and stimulates translocation into the nucleus, where the AhR heterodimerizes with the AhR nuclear translocator (Arnt; Fig. 1-3). The resulting ligand-AhR-Arnt complex recruits a set of co-activators and interacts with xenobiotic responsive elements (XREs) in the promoter region of AhR-regulated genes, facilitating their expression. In addition, the complex can recruit co-regulators (e.g., histone acetyltranferases) to interact with XREs if promoter regions exist in nucleosomal configurations (reviewed in Bock and Köhle, 2006).

The full extent of AhR-regulated genes remains to be elucidated; however, the AhR is known to be involved in the biotransformation of xenobiotics, and in cell proliferation and differentiation (Nebert et al., 2000; Bock and Köhle, 2006). The AhR-regulated genes involved in the adaptive response to xenobiotic exposures include both phase I (i.e., CYP enzymes) and phase II (e.g., glutathione transferase A1 and NAD(P)H:quinone oxidoreductase 1) metabolic enzymes (Nebert et al., 2000). In addition, there is growing evidence that the AhR has a functional role in normal development and homeostasis (Fernandez et al., 1997; Lund et al., 2003; Walisser et al., 2004).

Thus, AhR activation by TCDD or TCDD-like compounds may cause toxicity via: (1) a direct tissue insult as a consequence of the xenobiotic response (i.e., oxidative stress via the byproducts of metabolism); and/or (2) a deregulation of normal AhR-regulated physiological
processes. Understanding AhR-mediated toxicity in fish is further complicated by the existence of multiple AhR isoforms. Three AhR isoforms have been identified in both rainbow trout (AhR1, AhR2α and β; Abnet et al., 1999) and zebrafish (AhR1a and b, AhR2; Karchner et al., 2005). In addition, there are multiple Arnt isoforms, which can influence the AhR-mediated effects. For example, a knockdown of Arnt1 protected zebrafish embryos prevented against TCDD-induced toxicity; however, Arnt2 knockdown did not affect toxicity (Antkiewicz et al., 2006).
The involvement of AhR in TCDD and unalkylated PAH toxicity has been studied extensively (Incardona et al., 2004, 2006), but its role in alkylated PAH toxicity is unknown.

### 1.4 Risk assessment model for TCDD-like compounds

To assess the hazard or risk of TCDD-like compounds, toxic equivalency factors (TEFs) are calculated by determining TCDD equivalencies (TEF = LD50_{TCDD}/LD50_{congener}; Safe, 1990). Since TCDD-like compounds are invariably found in mixtures, toxic equivalency quotients (TEQs) were proposed (TEQ = \sum (TEF_{congener} \times \text{concentration}_{congener})). TEFs and TEQs were originally developed to assess the risk of HAHs to human and other mammals (Safe, 1990). However, the approach has since been validated for fish, but the model is unique, i.e., fish TEFs/TEQs cannot be used to predict the risk to mammals (Van der Berg et al., 1998).

Ideally, TEFs should be derived from overt toxicity (i.e., LD50s), but toxicity data do not exist for all TCDD-like compounds. Thus, Van der Berg et al. (1998) proposed a tiered approach for deriving TEFs. In order of decreasing weight: Tier 1 – toxicity data; Tier 2 – biochemical effects *in vivo*; Tier 3 – biochemical effects *in vitro*; Tier 4 – estimates from QSARs. The biochemical effects are generally measured as the relative potency to induce CYP1A enzymes. CYP1A models are based on the observed correspondence between TEFs for induction and TEFs for toxicity.

The model was developed with specific criteria that qualify TCDD-like compounds for assessment. It was proposed that the compounds must: (i) have structural similarities to TCDD; (ii) bind to AhR; (iii) illicit an AhR-mediated dose-response similar to TCDD (i.e., have a parallel dose-response curve); and (iv) be persistent and bioaccumulative (Safe, 1990; Van der Berg et al., 1998). Thus, the compounds are expected to share a common mode of action (via the AhR), and consequently, should act additively. Furthermore, since TCDD embryotoxicity is not mediated
by CYP1A in fish (Carney et al., 2004), an additional criterion (i.e., CYP1A-independence) may need to be included in this mechanism-based risk assessment model.

Since PAHs do not meet all the criteria (i.e., are readily metabolized and generally do not bioaccumulate), the use of TEF/TEQ risk assessment for these compounds has been questioned. However, even TCDD-like HAH congeners do not always meet the prerequisites of the model (e.g., non-parallel dose-response curves for CYP1A induction; Parrott et al., 1995). Thus, it is imperative to establish alkylphenanthrene embryotoxicity as an AhR-mediated effect to validate the use of this model for this class of compounds.

1.5 Thesis objectives

Our working hypothesis is that alkylphenanthrene toxicity is mediated by the AhR and CYP1A enzymes (Fig. 1-4). Using retene as a model compound, the target tissue and involvement of AhR2 and CYP1A in alkylphenanthrene toxicity in the early life stages of fish will be investigated using: (i) time-course observational and histological studies (Chapters 2 and 5), and (ii) chemical inhibition and/or morpholino oligonucleotide knockdown of AhR (Chapter 5) and CYP1A enzymes (Chapters 3, 4, and 5). The results of these studies will be used to understand the similarity of alkylphenanthrene toxicity to that of TCDD, which will provide insight into the appropriateness and/or effectiveness of including alkylphenanthrenes in the current risk assessment models for TCDD-like compounds.

The following null hypotheses will be tested: (1) the proximate signs of retene toxicity do not differ from those of TCDD (i.e., cardiovascular dysfunction, edema); (2) the primary target of retene toxicity is not the cardiovascular system; and (3) the mechanism of retene toxicity is not mediated by AhR2 or (4) CYP1A enzymes.
1.6 References


Hodson, PV, Qureshi, K, Noble, CAJ, Akhtar, P, Brown, RS. (2007). Inhibition of CYP1A enzymes by α-naphthoflavone causes both synergism and antagonism of retene toxicity to rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* 81:275-285.


Figure 1-4. Working model of alkylphenanthrene toxicity in the early life stages of fish. AhR, aryl hydrocarbon receptor; CYP, cytochrome P450 enzyme; Arnt, aryl hydrocarbon receptor nuclear translocator; HIFα, hypoxia inducible factor; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; AA, arachidonic acid; EETs, epoxyeicosatrienoic acids; HETEs, hydroxyeicosatetraenoic acids.
Chapter 2

Stage-specific retene cardiovascular toxicity in Japanese medaka

(Oryzias latipes) embryos

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Abstract

Alkylated polycyclic aromatic hydrocarbons (PAHs), such as retene (7-isopropyl-1-methylphenanthrene), constitute a high proportion of PAHs in crude oil and have been implicated in crude oil toxicity. Retene exposures produce signs of blue sac disease (BSD), including pericardial and yolk sac edema, cardiovascular dysfunction, and craniofacial and spinal deformities. The mechanism of retene toxicity is not well-understood; however, it is thought to be similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) due to their comparable end-stage toxicity (BSD) and ability to bind to the aryl hydrocarbon receptor (AhR) and induce metabolic cytochrome P450 1A (CYP1A) metabolic enzymes. To investigate retene toxicity and identify potential tissue targets, time-course observational and histological studies were conducted in the embryo-larval stages of Japanese medaka (Oryzias latipes). Retene caused concentration-dependent increases in toxicity, which was alleviated by delaying the onset of exposure by 3 and 5 days. Pericardial edema was the primary overt sign of toxicity, which occurred in the late stages of cardiac and embryonic development (7 days post-fertilization; dpf). Both light and transmission electron microscopy of cardiac tissue revealed no structural differences between control and retene-exposed embryos at 6.5 dpf, just prior to the onset of toxicity at 7 dpf. As BSD progressed, an increase in pericardial edema appeared to mechanically stretch the heart into the tube-like structure typically observed in exposures to TCDD-like compounds. In embryos exposed to 320 and 560 µg/L retene, BSD increased at a constant and similar rate from 7 dpf to swim-up (14 dpf); however, the intercepts of the time vs. effect regression lines were significantly different, which may suggest a body burden threshold. Our results suggest: (i) retene toxicity does not result from a direct impediment of embryonic or cardiac development; (ii) the primary target is the heart; (iii) either the molecular target is only available in the first 3 days of
development, or retene toxicity results from an instantaneous or continuous toxic effect after a
functional CYP1A system is active; and (iv) retene toxicity differs from that of TCDD in both
time of onset and primary target. Thus, the findings may question the effectiveness of the current
risk assessment model for TCDD-like compounds, which assume a common mode of action.
2.1 Introduction

Alkylated polycyclic aromatic hydrocarbons (alkyl-PAHs) are relevant aquatic contaminants as they comprise 80-90% of PAHs in crude oils (Wang et al., 2003). As oil weathers, there is an overall shift towards 3- to 5-ringed alkyl-PAHs, predominantly alkylphenanthrenes (Heintz et al., 1999). In the early life stages of fish, exposures to crude oil or to alkylphenanthrenes, such as retene (7-isopropyl-1-methylphenanthrene), are known to produce a suite of developmental abnormalities, collectively termed blue sac disease (BSD), typically associated with exposures to the notable persistent organic pollutant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Billiard et al., 1999; Kiparissis et al., 2001). The signs of BSD include pericardial and yolk-sac edema, cardiovascular dysfunction, craniofacial and spinal deformities, and fin rot. Retene is also a combustion product of wood (Ramdahl, 1983), and a biotransformation derivative of abietic acid, a component of pulp and paper mill effluents. Retene has been found at concentrations up to 3300 µg/g wet weight in contaminated sediments downstream of pulp and paper mills (Leppanen and Oikari, 1999).

The mechanism of TCDD-like toxicity is not fully-understood, although it is thought to be mediated by the aryl hydrocarbon receptor (AhR; Prasch et al., 2003) – a ligand activated transcription factor involved in an adaptive response to xenobiotic exposures. Upon ligand binding, the AhR translocates into the nucleus, heterodimerizes with AhR nuclear translocator protein, and binds to xenobiotic responsive elements to initiate gene expression (see Hahn, 1998). The AhR induces a battery of genes, including those encoding phase I and phase II metabolic enzymes, including cytochrome P450 1A (CYP1A) enzymes (reviewed in Korashy and El-Kadi, 2006); however, many AhR-regulated genes remain to be elucidated. There is also growing evidence that the AhR plays a functional role in normal development and homeostasis (Fernandez et al., 1997; Lund et al., 2003; Walisser et al., 2004).
Interestingly, the cardiovascular system has been implicated as the primary target of TCDD (reviewed in Goldstone and Stegeman, 2006) and TCDD-like compounds. A reduction in blood flow is often reported as the primary observable sign in TCDD-like toxicity (Stegeman et al., 1995; Chen and Cooper, 1999; Hornung et al., 1999; Belair et al., 2001; Dong et al., 2002; Incardona et al., 2004). However, there is a discrepancy over whether the reduced blood flow results as a consequence of toxic effects in the heart or peripheral vasculature. A decrease in blood flow and an increase in vascular permeability have been linked to TCDD-induced apoptosis in brain and yolk vasculature (Cantrell et al., 1996, 1998; Toomey et al., 2001; Dong et al., 2002, 2004). Conversely, there is evidence that BSD may result from a disruption in cardiac structure (Hornung et al. 1999; Antkiewicz et al., 2005; Mehta et al. 2008) and/or function (Incardona et al., 2004). Furthermore, Handley-Goldstone et al. (2005) reported a differential expression of 516 cDNAs from zebrafish cardiac tissue after TCDD exposure, including an up-regulation of sarcomeric genes. Disrupting the coordinated expression of sarcomeric genes has been implicated in cardiomyopathies in birds and humans (Barrans et al., 2002; Walker and Catron, 2000). In addition to the uncertainty of the target, whether toxicity results from an impediment of normal development (e.g. altered regulation of the endogenous function of AhR) or a direct tissue insult (e.g. oxidative stress from byproducts of xenobiotic metabolism) remains to be determined.

To define the nature of retene toxicity for comparison to TCDD and to identify a potential target tissue, a systematic time-course observational and histological study was conducted with the embryo-larval stages of Japanese medaka (Oryzias latipes). We also conducted delayed onset experiments to identify susceptible stages to retene toxicity in embryogenesis, which may provide insight into a mechanism of action.
2.2 Methods

2.2.1 Experimental design

To assess the embryo-larval toxicity of retene and identify susceptible times and/or targets in development, medaka embryos were chronically exposed to a range (32-560 µg/L) of retene concentrations with onsets of exposures at 0 (day of collection), 3, and 5 days post-fertilization (dpf). To assess potential effects of retene on cardiac development, observational and histological analyses of medaka hearts were performed using stereo-, light and transmission electron microscopy.

2.2.2 Chemicals

Retene (98% pure) was obtained from ICN Biomedical (Aurora, Ont.). Methanol (HPLC grade; Fisher Scientific, Nepean, Ont.) was used as a solvent carrier for retene. Tricaine methane sulfonate (MS-222; Sigma-Aldrich, Oakville, Ont.) was used as a fish anesthetic.

2.2.3 Egg collection and maintenance

Japanese medaka eggs were collected from a breeding colony and were pooled in a Petri dish containing embryo rearing solution (1 mL of 10% NaCl, 1 mL of 0.3% KCl, 1 mL of 0.4% CaCl₂.2H₂O, 1 mL of 1.63% MgSO₄.7H₂O, in 95 mL of distilled H₂O). The eggs were randomly placed (15 eggs/treatment) in 200 mL of test solution in glass mason jars. The test water used was from the City of Kingston municipal water supply, which was dechlorinated via charcoal filtration and addition of 1.2 g/L sodium bisulfite. The treatments were maintained in a 0:24 light:dark photoperiod, and test solutions were renewed on a daily basis (static renewal method).
2.2.4 Experiment #1 - Dose-response and susceptible period analyses

Medaka embryos were exposed to a range of 5 concentrations (32, 56, 100, 320, 560 µg/L) of retene from fertilization to hatch (12 days). Methanol (0.002%) and water controls were included to account for any toxicity associated with the solvent carrier and exposure water. The embryos were maintained at a temperature of 26.5 ±0.5°C in a walk-in incubator. Three exposures series (in triplicate) were used which differed in the time of onset, i.e., 0, 3, and 5 dpf. At 8 dpf (after onset of BSD, prior to hatch), embryos were scored for signs of BSD, as described by Billiard et al. (1999). The following signs were scored based on presence absence (0-1) or severity (0-3, 3 as highest severity): yolk sac edema (YE; 0-3), pericardial edema (PE; 0-3), and stretched hearts (SH; 0-1). These were the only signs that could be definitively scored in ovo.

The BSD Index, presented as a 0-1 scale, was calculated by dividing the observed severity by the maximum BSD score possible (7). For example, if an embryo is showing a severity of 3 for PE, 2 for YE, and has a stretched heart, the BSD index = (3 + 2 + 1) / 7 = 0.86. At 12 dpf, the larvae were anesthetized in MS-222 until death.

2.2.5 Experiment #2 – Time of onset and target identification

Medaka embryos were exposed to a range (32-560 µg/L) of retene concentrations from fertilization to 11 dpf (2 days post-hatch). The embryos were maintained at an average temperature of 23.3 ± 1.1°C. The embryos were scored daily for stage of development (0-38, as described by González-Doncel et al., 2005), stage of heart development (0-5), heart rate, and signs of BSD (see Appendix A). For a more comprehensive analysis of BSD, the signs of toxicity were scored either as absent or present (0 or 1; craniofacial deformity, spinal deformity, fin rot, ocular edema, hemorrhaging, arrhythmia, ventricular standstill, absence of swim bladder, and mechanicosensory test) or as a graded scale (PE (0-5), YE (0-3), stretched heart (0-3), and
blood flow (0-3)). The graded scales reflected a wider dynamic range of response and a possible role in the mechanism of toxicity. The mechanicosensory test was used as an indicator of a narcotic-like effect and was measured as a response (i.e., swimming away) or non-response to touch. Refer to Appendix A for a detailed description of the BSD scoring methods.

2.2.6 Calculating BSD and Severity Indices

The BSD index was calculated as described above. It was used to describe and compare only the signs of BSD among treatments, without mortality.

The severity index (SI) weighs both the severity of BSD with mortality for a better representation of overall toxicity as lethality is the most severe effect. Separate SIs (pre- and post-hatch) were calculated for embryos and larvae, as it is difficult to score all signs of BSD because some are not easily visualized (e.g., craniofacial and spinal deformities) and some only present in the larval stage (e.g., no swim bladder inflation). The SIs were calculated using a modified equation presented in Villalobos et al. (2000), using the following equations:

Pre-hatch (embryonic) SI:

\[
SI = \left[ \sum_{i=1}^{n} (PE \cdot E_i) + \sum_{j=1}^{n} (YE \cdot E_j) + \sum_{k=1}^{n} (RP \cdot E_k) + \sum_{l=1}^{n} (SH \cdot E_l) + \sum_{m=1}^{n} (VS \cdot E_m) \\
+ \sum_{o=1}^{n} (AR \cdot E_\theta) \right] \div \text{maximum SI score}
\]
Post-hatch (larval) SI:

\[
SI = \left[\sum_{i=1}^{n}(PE \cdot Ei) + \sum_{j=1}^{n}(YE \cdot Ej) + \sum_{k=1}^{n}(RBF \cdot Ek) + \sum_{l=1}^{n}(SH \cdot El) + \sum_{m=1}^{n}(VS \cdot Em) \right. \\
+ \left. \sum_{o=1}^{n}(AR \cdot Eo) + \sum_{p=1}^{n}(CF \cdot Ep) + \sum_{q=1}^{n}(EE \cdot Eq) + \sum_{r=1}^{n}(SD \cdot Er) + \sum_{s=1}^{n}(HE \cdot Es) \right. \\
+ \left. \sum_{t=1}^{n}(FR \cdot Et) + \sum_{u=1}^{n}(NSB \cdot Eu) + \sum_{v=1}^{n}(NMOV \cdot Ev) \right] \div \text{maximum SI score}
\]

where \(Ei, Ej, Ek, El\), equal the number of embryos displaying a particular severity of pericardial edema, yolk sac edema, reduced blood flow, stretched heart, respectively; and \(Em, Eo, Ep, Eq, Er, Es, Et, Eu, Ev\) equal the number of larvae displaying ventricular standstill, arrhythmias craniofacial deformities, eye edema, spinal deformity, hemorrhage, fin rot, no swim bladder inflation, and no movement, respectively. A value of 23.5 was assigned for embryos and larvae that died with signs of BSD (0.5 higher than the maximum value), assuming that BSD was the cause of death. The maximum SI score (100% mortality) is 352.5 (23.5 x 15 fish). In other words, the severity index is the sum of the number of fish displaying a severity of each sign divided by the maximum SI score possible, creating representative view of overall toxicity on a 0-1 scale. For example, if in a treatment of 15 embryos, there were three embryos displaying a severity of 3 for PE, three displaying a severity of 2 for YE, five displaying a severity of 2 for SH, and one that died, then SI = \([3 \cdot 3 + 3 \cdot 2 + (5 \cdot 2) + 23.5] \div 352.5 = 0.14\).

Medaka embryos were viewed using a Nikon SMZ-10A stereomicroscope and digital photographs were taken with an Olympus Stylus 720SW digital camera at 50X magnification.
2.2.7 Experiment #3 – Histological analyses of heart structure

Medaka embryos were exposed to 320 µg/L (concentration determined from Experiments #1 and #2) and MeOH (0.002%) for 6.5 dpf (stage 34), and were then dechorionated and processed for histology. The chorions were enzymatically dechorionated for 19.5 h using 0.3 mg/mL protease from *Streptomyces griseus* (Sigma-Aldrich, Oakville, Ont.) in 1.9 mg/mL glycine (Sigma-Aldrich, Oakville, Ont.) solution at 34°C until the chorions could be easily removed with forceps.

Specimens were initially fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hrs, with post fixation in 2% osmium tetroxide for 2 hrs in the same buffer. Both fixation steps were at room temperature and followed by 4 buffer washes. Dehydration was in a graded series of ethanol at 10 min intervals and at room temperature. Plastic embedding was in Epon-Araldite resin. Cross-sections of the embryonic hearts were sliced using an RMC MT6000 ultramicrotome. For light microscopy, 500 nm thick sections were thermally adhered to slides and stained with filtered 1% toluidine blue. For electron microscopy, 70 nm sections were stained with uranyl acetate and lead citrate and viewed with a JEOL JEM1400 transmission electron microscope.

The electron micrographs of medaka hearts were analyzed for evidence of morphological differences in chamber wall thickness, amount of cardiac jelly, and organizational patterns of Z-lines and myofilaments (see Fig. 2-1).

Digital pictures of the light microscopy sections were captured using a Nikon Optiphot microscopy and Evolution MP 5.0 camera with Image Pro Plus 5.2.1 software (MediaCybernetics, Inc., Bethesda, MD), and were analyzed for morphological abnormalities. Ventricle size was measured in pixels by outlining the outer boundary of cross-sectioned ventricles (n = 5/treatment) using Image J software (U.S. National Institute of Health, Bethesda, MD).
Figure 2-1. Ultrastructural components of cardiac tissue analyzed in retene- and MeOH (0.002%)-exposed embryos. Arrowhead(s): (a) Cross-section of bundles of myofilaments; (b) Z-lines of the myofibril; (c) cardiac jelly in atrium. Scale bar = 2 µm.
2.2.8 Statistics

For all statistical tests, toxicity data were compared using a two-way analysis of variance followed by Student-Newman-Keuls pairwise multiple comparisons (SigmaStat Ver. 1.0, Jandel Scientific, Chicago, IL, USA; p < 0.05). Sigmoidal concentration-response curves and linear regression analyses were performed using GraphPad Prism software (Ver. 4.02, GraphPad Software, Inc., San Diego, CA, USA), and LC50s calculated using Probit Analysis (LC50 BAS 2.0, 1986, US EPA, Washington, DC).

The constraints of the sigmoidal concentration-dependent curves differed between those calculated for BSD and Severity Indices. A 0-1 (lower limit-upper limit) constraint was placed on the sigmoidal concentration-response curves of the Severity Indices (SIs), as all measurements of toxicity fall within the 0-1 scale, i.e., the highest SI value (1, representing 100% mortality in a treatment) can be reached. Thus, the SI EC50s calculated are estimated responses occurring at an SI value of 0.5. Conversely, an upper constraint cannot be placed on BSD Indices because it cannot be assumed that all embryos or larvae will develop the highest severity of all signs of BSD before they die. In other words, embryos or larvae may die before the maximum BSD Index score (1) is reached. Thus, the EC50s were calculated without an upper constraint, and may not occur at a BSD Index of 0.5.

2.3 Results

2.3.1 Experiment #1 – Dose-response and susceptible period analyses

Chronic retene exposures beginning at 0 dpf produced a concentration-dependent increase in BSD (R² = 0.94), with an 8-day EC50 of 170 µg/L (Fig. 2-2a). Toxicity increased
significantly beyond that of controls at 320 µg/L and 560 µg/L (P < 0.05); however, signs of BSD were observed in some embryos exposed to 100 µg/L retene. Delaying the onset of retene exposure by 3 and 5 dpf significantly reduced the signs of BSD (P < 0.05). Toxicity at all concentrations of retene in the delayed onset exposures did not differ significantly from that of controls.

With the exception of the highest concentration (560 µg/L) of retene with a 0 dpf onset of exposure, there was no significant difference in cumulative mortality (at 12 dpf) among treatments and between treatments and solvent controls (Fig. 2-2b); however, the exposure produced an estimated 12-day LC50 of 443 µg/L retene (95% c.l. = 312-744 µg/L retene). Solvent controls showed little mortality (< 14%). The mortality in the 560 µg/L treatments with a 0 dpf onset of exposure were over 7-fold higher (62%) than controls (6.9%; P = 0.005). Delaying the onset of exposure 3 and 5 dpf reduced mortality nearly 3.5 and 7-fold, respectively. There was a significant amount of mortality in water controls due to fungal growth.

2.3.2 Experiment #2 – Time of onset and target identification

Similar to Experiment #1, retene produced a concentration-dependent increase in toxicity (R² = 0.96; Fig. 2-3), with EC50s of 320 µg/L (SD = 41 µg/L) and 322 µg/L (SD = 24 µg/L) for the BSD index at 11 dpf and the severity index at 14 dpf, respectively. The larger variation in the BSD index is likely influenced by the confounding effect of hatched larvae. Hatched larvae are not included in the embryonic BSD index. The total possible BSD score for unhatched embryos differs from the score for larvae because fewer signs can be observed, and the chorion may affect the occurrence of edema and the extent of exposure to retene (Brinkworth et al., 2003). The
Figure 2-2. Delayed onset of retene and MeOH (0.002% v/v) exposures in the embryo-larval stages of medaka. Data are presented as the mean ± SE of 3 replicates (15 fish/replicate).
difference in EC50 in Experiment #1 was likely influenced by differing temperatures and scoring scales.

At 14 dpf, the lower retene concentrations (32, 100 µg/L) did not produce significant toxicity beyond that of controls; however, a slight response was observed in the 100 µg/L-exposed fish (avg. SI = 0.062, avg. BSD Index = 0.006; Fig. 2-3a). The higher concentrations (320, 560 µg/L) of retene produced a significant increase in percent mortality, BSD index, and overall SI (P < 0.001; Fig. 2-3a,b,d). Retene exposures produced an estimated 14-day LC50 of 448 µg/L retene (95% c.l. = 358-613 µg/L retene). The 320 and 560 µg/L exposures produced 44% and 53% mortalities, respectively, and a 1.8-fold difference in average BSD index values (0.24 and 0.42, respectively). From the onset of toxicity (7 dpf) to 14 dpf, the severity of retene toxicity increased linearly in 320 µg/L- and 560 µg/L-exposed fish (r² = 0.88 and 0.95, respectively; Fig. 2-3c). The slopes of the linear increase did not differ between the treatments, but the intercepts were significantly different (i.e., the lines had similar but parallel slopes; P < 0.001). Signs of BSD were not observed in control fish.

Retene had no observable effect on development through 6 dpf (stage 34, according to González-Doncel et al., 2005), a point at which the embryo is in the final stages of development. The heart appeared to develop normally through the cardiac looping stage, where the atrium migrates to a position adjacent to the ventricle (see Fig. 2 in Appendix A). The first overt sign of BSD (at 7 dpf) was pericardial edema (PE; Fig. 2-4a). As the severity of PE increased, the distension of the pericardial cavity began to mechanically stretch the heart (Fig. 2-4c). The stretching of the heart appeared to influence blood flow by pinching the extraembryonic vessels (ducts of Cuvier, vitellocaudal vein) at the point at which they entered the pericardial sac. There was no evidence of reduced blood flow prior to the onset of PE. As PE increased, the hearts continued to stretch into a tube-like structures, with long, thin atriums, and ventricles that were
significantly reduced in size (Fig. 2-4d-f). The atriums continued to contract until death; however, when blood flow ceased, ventricular standstill was apparent. There were no other arrhythmias observed. In addition, there were no significant differences in heart rate between control and retene-exposed embryos prior to the onset of BSD (6dpf), or until embryos displayed a severe amount of pericardial edema (PE 4-5).
Figure 2-4. Progression of pericardial edema (PE) and cardiac stretching. (a, b) The first overt sign of retene toxicity, PE 1: a bubbling of the pericardial sac underneath the jaw (arrow); edematous fluid surround the heart (asterisks). (c) PE 2: edematous fluid extending under the head region (arrow), occupying approximately 1/4 of the yolk sac volume. (d) PE 3: the edema fills approximately 2/3 of the yolk sac volume; as PE increases, the anchored heart stretches the atrium (A) into a tube-like structure and the ventricle (V) is reduced in size; (e) PE 4 and (f) PE 5: edematous fluid occupies 1/2 and > 1/2 of the yolk sac volume, respectively. In all frames: arrowhead, point where extraembryonic vessels enter the pericardial cavity (where heart is anchored); V, ventricle; A, atrium; E, edema. Scale bar = 0.1 mm.

2.3.3 Experiment #3 – Histological analyses of heart structure

There were no observable differences in heart morphology or ventricle size between retene- and MeOH-exposed embryos at 6.5 dpf (stage 34; n = 5/treatment).
2.4 Discussion

In the embryo-larval stages of fish, there is uncertainty regarding the mechanism and target of TCDD-like compounds. This was the first attempt at identifying a target and mechanism of retene embryotoxicity in medaka by examining the order in which pathology occurred and the timing of toxicity with respect to days from fertilization. Retene toxicity has been described in the embryo-larval stages of rainbow trout (Oncorhynchus mykiss; Billiard et al., 1999; Hawkins et al., 2002; Brinkworth et al., 2003; Hodson et al., 2007) and zebrafish (Danio rerio; Billiard et al. 1999); however, the mechanism of toxicity is largely unknown. As with most compounds, retene toxicity has only been inferred to be TCDD-like because similar manifestations in the end-stage toxicity. Thus, we chose use a time-course observational and histological approach to investigate the mechanism and tissue targets of retene.

Retene exposures induced concentration-dependent increases in signs of BSD (Fig. 2-2,3). The exposures had no overt affect on development until the final stages (stages 34-36) of embryonic development. The primary observable sign of BSD was localized pericardial edema at 7 dpf (stage 35-36; Fig. 2-4a,b), suggesting the heart as the proximate target in retene exposures. The proposed target is not consistent with TCDD embryotoxicity in medaka. Cantrell et al. (1996) reported apoptotic cell death and leakage of blood cells in the embryonic vasculature prior to stage 36, likely resulting in the onset of BSD at 5 dpf (Wisk and Cooper, 1990). There were no apparent effects on vascular structure or development prior to onset of pericardial edema in retene-exposed embryos. Furthermore, there were no differences in heart rates and no arrhythmias were observed prior to the onset of BSD, suggesting that retene toxicity may differ from its unsubstituted counterpart, phenanthrene, which produced signs of atrio-ventricular conduction block in zebrafish embryos (Incardona et al., 2004). The resulting tube-like heart in retene-exposed medaka embryos appears to result from mechanical stretching of a looped heart as
edematous fluid accumulated in the pericardial cavity (see Appendix A for detailed description; Fig. 2-4). It is conceivable that the increasing pericardial edema may also impart a pressure-induced impediment of cardiac function (i.e., cardiac tamponade) that may facilitate the progression of the secondary signs of BSD.

Histological analyses of heart tissues prior to the onset of BSD (6.5 dpf) revealed no overt structural differences, including ventricle size, in retene-exposed medaka. If the heart is the primary target, this finding suggests that the primary observable sign of BSD (pericardial edema) does not result from abnormal cardiac development. Thus, toxicity may arise from a direct insult (e.g., oxidative damage) on cardiac tissue; however, more direct studies are needed to confirm this hypothesis. Notably, Antkiewicz et al. (2005) reported an increase in apoptosis in cardiomyocytes in TCDD-exposed zebrafish embryos, which were not secondary to edema. It is worth noting that a hemorrhage occurred in the heart region of a control embryo, producing a small amount of pericardial edema (PE 1), but did not result in the subsequent increase in edema and heart stretching observed in retene-exposed embryos. This random observation may provide further evidence that the structural integrity of cardiac tissues may be affected after retene exposures.

The evidence suggesting that retene does not affect normal development prior to the onset of BSD is seemingly contradictory to the finding that delaying the retene exposures to 3 and 5 dpf alleviates BSD, which suggests that the target is only available within the first 3 days of exposure. If the early developmental stages are sensitive to retene exposure, it would suggest that metabolic activation is not required for retene toxicity. This would suggest that parent (i.e. unmetabolized) retene is the toxic form, which is inconsistent with previous reports that implicated retene metabolites in toxicity (Hawkins et al., 2002; Hodson et al., 2007). Hodson et
al. (2007) identified a decrease in hydroxylated retene derivatives that corresponded with a
decrease in toxicity after CYP1A inhibition by α-naphthoflavone.

Conversely, the absence of BSD in delayed onset exposures may infer that retene
accumulates in the embryo until the detoxification pathways become activated. A functional
CYP1A system in medaka embryos has not been reported earlier than 5 dpf (Wisk and Cooper,
1992). If the retene accumulates within the first 5 days of development, it may reach a body
burden that exceeds the capacity of the embryos ability to combat the subsequent production of
high levels of metabolic byproducts (e.g., reactive oxygen species), leading to oxidative damage,
which has been implicated in retene toxicity (Bauder et al., 2005). Alternatively, the effect could
also be continuous and cumulative. For example, in the 2 dpf after CYP1A activation the
byproducts of metabolism could continually deplete antioxidant (e.g., Vitamin E) levels
effectively lowering the body burden threshold. The embryo-larval stages of fish are particularly
susceptible to these effects, as they have a finite supply of maternally-derived antioxidants in
their yolk. The fact that no toxicity was observed in retene-exposed embryos when the onset of
exposure was delayed until 5 dpf (i.e., when CYP1A is functional) lends more support to these
hypotheses. Furthermore, in 320 and 560 µg/L retene exposures, there were similar rates of BSD
progression; however, the intercepts of the time vs. effect regression lines were significantly
different (Fig. 2-3), which may also suggest a body burden threshold. The involvement of
CYP1A in retene (Hawkins et al., 2002; Brinkworth et al., 2003; Hodson et al., 2007) and TCDD
(reviewed in Goldstone and Stegeman, 2006) toxicity has been suggested. Retene exposures in
rainbow trout larvae suggest that CYP1A induction precedes the onset of BSD (Brinkworth et al.,
2003), and that BSD is mediated by the byproducts of CYP1A metabolism (Hawkins et al., 2002;
Bauder et al., 2005; Hodson et al., 2007).
In TCDD-exposed medaka embryos, delaying the onset of exposure 4 dpf does not affect toxicity (Wisk and Cooper, 1990). Toxicity was only alleviated in TCDD exposures if the onset was delayed until 6 dpf (Wisk and Cooper, 1990). Thus, taken together, our findings suggest that retene toxicity differs from that of TCDD. Current mechanism-based risk assessment models express risk by calculating TCDD equivalents (see Safe, 1992). Since this model assumes a common mode of action of TCDD-like compounds, the findings of the present study may question the effectiveness of the model. It is conceivable, however, that the different toxicities may arise via the same mechanism, but are a function of variations in pharmacokinetics, i.e., retene is less lipophilic (slower uptake rate, less AhR activation) and is far more readily metabolized (higher excretion rate) when compared to TCDD. Further, more direct comparative studies of retene and TCDD toxicities are needed to begin investigating the commonality in the mode of action.

In summary, our findings suggest that retene: (1) produces concentration dependent increases in toxicity in the embryo-larval stages of medaka; (2) does not overtly affect embryonic development until the final stages of development (stages 34-36); (3) targets the heart; (4) delaying the onset of exposure 3 and 5 dpf alleviates toxicity; and (5) toxicity differs from that of phenanthrene (unalkylated form) and TCDD.

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2.6 References


Chapter 3
Evidence for multiple mechanisms of toxicity in larval rainbow trout
(Oncorhynchus mykiss) co-treated with retene and α-naphthoflavone

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Abstract

Alkylated polycyclic aromatic hydrocarbons, such as retene (7-isopropyl-1-methylphenanthrene), induce cytochrome P450 1A (CYP1A) enzymes and produce dioxin-like toxicity in the embryo-larval stages of fish characterized by the signs of blue sac disease (BSD). The signs of toxicity are well-characterized; however, the mechanism is not well-understood. To elucidate the role of CYP1A in retene toxicity, larval rainbow trout (*Oncorhynchus mykiss*) were co-treated with a range of concentrations of α-naphthoflavone (ANF), a known CYP1A inhibitor. The co-treatment produced synergistic toxicity at 3.2-100 µg/L ANF, after which toxicity at 180 µg/L ANF dropped to levels typical of retene-only. At 320 µg/L ANF, toxicity increased with or without retene, indicating that ANF alone is capable of inducing BSD. In addition, the additive toxicity of retene-only and 320 µg/L ANF-only approximately equals that of the co-exposed larvae (100 µg/L retene + 320 µg/L ANF), indicating response addition. Thus, two mechanisms of action occurred in co-exposed larvae at different concentrations of ANF. In trout larvae, there was a correlation between toxicity and CYP1A protein concentrations, and in juvenile trout, ANF produced a concentration-dependent inhibition of ethoxyresorufin-O-deethylase (EROD) activity without a measurable drop in CYP1A protein. Taken together, the mechanism underlying the synergistic toxicity is EROD-independent and may be AhR-dependent. This study demonstrates that multiple, exposure-dependent mechanisms can occur in mixture toxicity, suggesting that current risk assessment models may drastically underestimate toxicity, particularly of mixtures containing both CYP1A inducers and inhibitors.

Keywords: retene, α-naphthoflavone, mechanisms, CYP1A, AhR, mixture toxicity
3.1 Introduction

Alkylated polycyclic aromatic hydrocarbons (PAHs), such as retene (7-isopropyl-1-methylphenanthrene), are dominant forms PAHs in crude oils (80-90% of total; Wang et al., 2003) and have been implicated as the primary agents of oil toxicity (Carls et al., 1999). In addition, alkylated PAHs have been implicated in recruitment failure of fish downstream of pulp and paper mills (Sandstrom et al., 1994). Chronic exposures to alkylated PAHs produce dioxin-like toxicity in the early life stages of fish, characterized by the signs of blue sac disease (BSD), which include: yolk sac and pericardial edema, hemorrhaging, skeletal deformities, fin rot, and cardiovascular dysfunction (Billiard et al., 1999). Retene, an alkyl-phenanthrene, is a biotransformation product of dehydroabietic acid, a component of pulp mill effluents (Ramdahl, 1983). It has been found in industrially-contaminated sediments at concentrations up to 3300 µg/g dry weight (Leppanen and Oikari, 1999), and causes exposure-dependent increases of BSD (Billiard et al., 1999; Hawkins et al., 2002).

The mechanism of alkyl-phenanthrene toxicity is not well-understood; however, it is thought to be aryl hydrocarbon receptor (AhR)- and cytochrome P450 1A (CYP1A)-mediated (Brinkworth et al., 2003; Hawkins et al., 2002). The AhR is a ligand-activated transcription factor of the bHLH Per/Arnt SIM family that is involved in the regulation of a number of genes. In the cytosol, an inactive AhR remains bound to two 90 kDa heat shock proteins and an AhR inhibitory protein (see Hahn, 1998). Upon ligand binding, the complex dissociates with the heat shock and inhibitory proteins, translocates into the nucleus, and heterodimerizes with an aryl hydrocarbon receptor nuclear translocator (ARNT) protein. The resulting ligand-AhR-ARNT complex binds to DNA recognition sequences (xenobiotic response elements), and initiates the up-regulation of a battery of genes, including the genetic transcripts for phase I (e.g., CYP1A)
and phase II enzymes (e.g., glutathione transferase A1, NAD(P)H:quinone oxidoreductase 1; reviewed in Korashy and El-Kadi, 2006).

Retene can bind to both AhR1 and AhR2 isoforms (Billiard et al., 2002) and increase expression of CYP1A (Fragoso et al., 1998). This increase in CYP1A and subsequent metabolism of retene may be part of the mode of action (Brinkworth et al., 2003; Hawkins et al., 2002). CYP1A metabolism is not 100% efficient and may produce reactive oxygen species (ROS) and reactive metabolites as by-products, resulting in oxidative damage and adduct formation (Halliwell and Gutteridge, 1999). In larval trout, retene exposure reduces tissue concentrations of glutathione and Vitamin E, suggesting that toxicity may be a result of oxidative stress (Bauder et al., 2005).

To investigate the role of CYP1A metabolism in retene toxicity, we co-treated larval trout with retene and α-naphthoflavone (ANF), a known CYP1A enzyme inhibitor and AhR antagonist. Gillner et al. (1985) suggested that ANF in its non-planar configuration is a poor fit for the AhR site and cannot promote the dissociation of the HSPs, thus preventing dimerization with ARNT (Wilhelmsson et al., 1994). Reversible inhibition of CYP1A activity may occur as a result of ANF binding directly to the active site or heme moiety (Miranda et al., 1998). Hence, if metabolism is involved in the mechanism of retene toxicity, a co-treatment with ANF should reduce toxicity. Previously, Hodson et al. (2007) identified a non-linear interaction between retene and ANF toxicity in co-treated rainbow trout larvae. At a high concentration of ANF, retene toxicity was antagonized when hydroxylation was virtually eliminated, suggesting that toxicity was caused by hydroxylated retene derivatives. Thus, our second objective was to identify thresholds associated with retene and ANF mixture toxicity for the identification of toxic retene metabolites.
The results of our study identified an interactive effect of retene and ANF, which did allow for the direct fulfillment of our objectives. An EROD-independent synergism and response addition was observed, which illustrates the potential for multiple, concentration-dependent modes of action in the mixture toxicity of both CYP1A inducers and inhibitors.

3.2 Methods

3.2.1 Experimental design

To confirm the CYP1A inhibitory effect of ANF, an ethoxyresorufin-O-deethylase (EROD) assay was conducted using the S9 of livers from co-exposed juvenile trout. The juvenile trout were exposed to a mixture of 100 µg/L of retene and 3.2, 10, 32, 100, or 320 µg/L ANF for 48 hrs. The same range of ANF concentrations was used in the absence of retene, along with methanol (0.0002% v/v), water, and retene controls. Combined with the EROD activity data, Western blots of the S9s were assessed to investigate the mode of ANF inhibition.

Larval rainbow trout were exposed by semi-static daily renewal of a range (3.2-320 µg/L) of ANF concentrations alone and in co-treatment with 100 µg/L retene. This concentration of retene is chronically sub-lethal, producing signs of BSD without a significant amount of mortality (Billiard et al., 1999). Methanol (0.0002% v/v) and water controls were used to account for any toxicity associated with the carrier solvent and exposure water. The chronic exposures began just prior to hatch and continued until swim-up (28 days), at which time the larvae were scored for signs of BSD. Chronic toxicity was assessed by analyzing mortality rates and severity of BSD, and CYP1A protein levels were measured by Western blot analysis.
3.2.2 Chemicals

Retene and ANF (both 98% pure) were obtained from ICN Biomedical (Aurora, ON). HPLC-grade methanol (Fisher Scientific, Nepean, ON) was used as a carrier for both retene and ANF. β-Naphthoflavone (BNF) was obtained from Acros Organics (Fair Lawn, NJ, USA) and was used as a positive control in EROD assays, as it is a potent CYP1A inducer and causes BSD in the early life stages of fish (Billiard et al., 2006). Tricaine methane sulfonate (MS222; Sigma-Aldrich Canada, Oakville, ON) was used as a fish anesthetic, as brief exposures do not alter EROD induction at the concentration used in this study (Hodson et al., 1996).

3.2.3 Protocol

The trout eggs and juveniles were received from Rainbow Springs Trout Hatchery (Thamesford, ON) and acclimated to 10 and 12°C, respectively, for 1 hour in their original container. The eggs were divided into 4 stainless steel bowls containing fresh water (with aeration), and left for an acclimation period of 4 days. The juveniles were placed in holding tanks and were acclimated for 1 week prior to the experiments to Lake Ontario water (alkalinity = 90 mg/L CaCO₃) from the City of Kingston municipal supply, which was dechlorinated by charcoal filtration and addition of 1.0 mg/L sodium bisulfite. For both the larval and juvenile exposures, the treatment water was aerated and maintained under a 0:24 h, and 16:8 light:dark photoperiod, respectively. Water chemistry was measured daily and conditions were within the optimal range for trout development (temperature = 8.92 ± 0.18 °C; dissolved oxygen = 114 ± 4.66 %; pH = 8.21 ± 0.08; conductivity = 312 ± 2.21 µS; total ammonia = 7.09 ± 4.36 µM).

For the CYP1A inhibition study, five 1-5 g juveniles were placed in 10 L of test solution in a 20 L bucket lined with food grade polyethylene bags for each treatment. The treatments were
run in duplicate and the water and chemicals were renewed at 24 h. At the end of the 48 h exposure, the juveniles were sacrificed and their livers were removed, homogenized, and centrifuged at 9000 x g to obtain an S9. The S9 fractions were flash-frozen and stored at -80°C until the EROD and Western blot assays were conducted.

For the chronic toxicity test, 25 larvae were placed in stainless steel bowls containing 2 L of test solution. The treatments were run in triplicate in two separate exposure times with two separate batches of eggs; two replicates were conducted in the first exposure, and the third replicate was conducted in the second exposure.

At swim-up, the larvae were anaesthetized with 100 µg/L MS222 until movement ceased, and were then scored for BSD as described by Billiard et al. (1999). The following signs were scored based on presence/absence (0-1) or severity (0-3, 3 as highest severity): pericardial edema (PE; 0-3), yolk sac edema (YE; 0-3), craniofacial deformity (CF; 0-1), spinal deformity (SD; 0-1), fin rot (FR; 0-1), and no movement (mechanicosensory test) (NMOV; 0-1). The signs with a severity scale (edemas) are ones suggested to be the proximate cause of death due to an impediment of cardiovascular function (e.g., cardiac tamponade), and can be scored visually as a range of fluid accumulation in their respective compartments.

After BSD scoring, the larvae were weighed prior to and following yolk sac removal, and placed in cryogenic vials (5 larvae/vial). The vials were flash-frozen in liquid nitrogen and stored at -80°C for Western blot analysis.
3.2.4 Severity and BSD indices

The severity index (SI) is the sum of the number of embryos displaying a severity of each sign divided by the maximum SI score, creating a representative view of overall toxicity on a 0-1 scale. It was calculated using a modified version of the Villalobos et al. (2000) equation:

$$SI = \left[ \sum_{i=1}^{n} (PE \cdot E_i) + \sum_{j=1}^{n} (YE \cdot E_j) + \sum_{k=1}^{n} (CF \cdot E_k) + \sum_{l=1}^{n} (SD \cdot E_l) + \sum_{m=1}^{n} (FR \cdot E_m) + \sum_{o=1}^{n} (NMOV \cdot E_o) \right] \div \text{maximum SI score}$$

where $E_i$, $E_j$ equal the number of embryos displaying a particular severity of pericardial edema, yolk sac edema, respectively; and $E_k$, $E_l$, $E_m$, $E_o$ equal the number of embryos displaying craniofacial deformities, spinal deformity, fin rot, and no movement, respectively. A value of 10.5 was assigned for lethality (0.5 higher than the maximum value). The maximum SI score (100% mortality) was 262.5 (10.5 x 25 fish). For example, if in a treatment of 25 larvae there were three larvae displaying a severity of 3 for PE, two displaying YE 2, five displaying CF, and one died, then $SI= [(3 \cdot 3) + (3 \cdot 2) + (5 \cdot 1) + 10.5] \div 262.5 = 0.12$.

The BSD index was calculated for surviving embryos (dead larvae were excluded) similar to the SI, where the severities of signs are summed and divided by the maximum score (maximum BSD score = 10). BSD indices were only calculated in treatments with less than 65% mortality, which allowed for enough data points to observe trends without being largely influenced by a reduced number of individual BSD scores.
3.2.5 EROD analysis

Juvenile livers were prepared for EROD analysis according to Hodson et al. (1996). Livers were homogenized in HEPES buffer (5.2 g/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; 11.184 g/L KCl, pH 7.5) and centrifuged for 20 min at 9000 x g to obtain a S9. Crude EROD activity was measured using a Spectramax Gemini Spectrofluorometer (Molecular Devices, Sunnyvale, CA; $\lambda_{\text{excitation}} = 530$ nm, $\lambda_{\text{emission}} = 586$ nm). Specific EROD activity (picomoles per minute per mg protein) was calculated by normalizing the crude activity to the total protein of the S9s, as measured by Biorad Reagent (Biorad, Hercules, CA, USA) standardized to bovine serum albumin. BNF (10 $\mu$g/L) was used as a positive control, as it is a potent CYP1A inducer and causes BSD in the early life stages of fish (Billiard et al., 2006).

3.2.6 Western Blot analysis

CYP1A protein blot was measured using an SDS-PAGE/chemiluminescent detection system as described by Sibani (2001). One juvenile trout liver or 5 larvae per treatment were homogenized in 1 mL of HEPES buffer and centrifuged to obtain an S9, as described above. A mouse CYP1A monoclonal antibody against rainbow trout CYP1A (C10-7; Cayman Chemical, Ann Arbor, MI, USA) was used as a primary antibody, and HRP (horseradish peroxidase)-goat anti-mouse IgG (H+L) conjugate (Zymed® Laboratories, South San Francisco, CA, USA) as a secondary antibody. The resulting band densities were analyzed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA) and standardized to the total protein in each well measured using Biorad Reagent. Four independent gels were run with CYP1A
controls, retene-exposed larvae, and larvae exposed to either a concentration series of ANF or to ANF and retene (co-exposure).

3.2.7 Measurement of waterborne retene

Water samples were taken every second day from treatments containing retene. A 1.5 mL water sample was taken and added to 1.5 mL of ethanol in a 7 mL scintillation vial. The vials were stored at 4°C until analysis. Waterborne retene concentrations were measured using synchronous scanning spectrofluorometry (Quanta-Master Fluorescence Spectrometer; PTI Ltd., London, ON, Canada) with an excitation range of 290-310 nm and an emission range of 340-360 nm against a standard curve of retene diluted in 50:50 ethanol:water.

3.2.8 Statistics

EROD activity, mortality, and severity index data were analyzed using a three-way analysis of variance followed by Student-Newman Keuls (SNK) pairwise multiple comparisons (SigmaStat for Windows V 3.0©; SPSS Corporation, Chicago, IL, USA; P < 0.05). A two-way analysis of variance was used to analyze within treatment differences of the above measurements, and BSD index data, and was followed by an SNK pairwise multiple comparison. Spearman correlation, linear and non-linear regression, and IC50 analyses were performed using GraphPad Prism software (Ver. 4.02, GraphPad Software, Inc., San Diego, CA, USA).
3.3 Results

3.3.1 Chemical exposure

Waterborne retene concentrations averaged 86% (SE=1.7%; N=140) of nominal concentrations when sampled immediately following addition (T0). Over a 24 hour period, retene concentrations dropped an average of 20% (N=22) from the actual T0 concentration. The drop in concentration reflects the partitioning of the chemical to the exposure container and into the trout larvae. Waterborne concentrations of ANF were not measured, as the concentrations used were below the detection limit of the fluorescence spectrometer.

3.3.2 CYP1A inhibition in juvenile trout

Retene-only exposed trout caused high CYP1A synthesis and high EROD activity relative to controls (P < 0.001; Fig. 3-1 and 3-2a). On its own, ANF was able to both induce CYP1A and inhibit EROD activity (Fig. 3-1). Relative to retene, EROD activity was significantly lower in juveniles exposed to all concentrations of ANF (P < 0.001; Fig. 3-2). An increase in EROD activity above control values was only measured in 32 and 100 µg/L ANF only-exposed fish (P < 0.001 and P = 0.045, respectively).

Juvenile trout co-treated with retene and ANF produced a concentration-dependent decrease in EROD activity ($R^2 = 0.98$), with an IC50 of 30 µg/L ANF (Fig. 3-2). Activity first reduced below retene controls at 32 µg/L ANF (P = 0.026) and decreased to background levels at 320 µg/L. EROD activity plotted against CYP1A protein blot values suggests that ANF may be inhibiting EROD activity at the active site of the enzyme (Fig. 3-1).
Figure 3-1. EROD activity versus CYP1A protein concentrations in livers of juvenile trout exposed to 100 µg/L retene (dark circles) or a range of ANF concentrations (open symbols) (a) in co-treatment with 100 µg/L retene, and (b) ANF alone. The data have been arranged to show: (1) retene only caused high CYP1A synthesis and high EROD activity; (2) all concentrations of ANF caused CYP1A synthesis, but only low EROD activity; and relative to retene-only, (3) retene + low concentrations of ANF (3.2-32 µg/L) caused higher CYP1A synthesis with similar EROD activity; (4) retene + high concentrations of ANF (100-320 µg/L) caused similar CYP1A protein concentrations with progressively lower EROD activities. The dotted lines outline the range of values from retene only-exposed juveniles. Shaded areas are indicative of inhibited EROD activity and not inhibited CYP1A synthesis (higher protein and similar or lower EROD activity, or similar protein levels with lower EROD activity). The data represent the mean relative EROD activity and Western blot band density of individual juvenile livers.
Figure 3-2. EROD activity of juvenile trout livers after 48 h exposure to BNF (10 µg/L), retene (100 µg/L), and a range of ANF concentrations, alone and in co-treatment with 100 µg/L retene. The data represent the mean EROD activity ± 95% confidence limits (N = 5), normalized to methanol controls.

BNF (positive control) caused a 7-fold higher EROD activity than retene-only exposed juveniles. The high EROD activity confirms that the EROD assay was sensitive enough to detect a known CYP1A inducer.

3.3.3 Mortality of larvae

There was a significant effect of treatment (P < 0.001) and concentration (P < 0.001) on the percent mortality of larvae exposed to ANF alone and in co-treatment with retene (Fig. 3-3). Mortality in both retene- and ANF-only exposures did not exceed that of controls. A synergistic toxicity occurred in co-treated larvae exposed to 5.6-100 µg/L concentrations of ANF (P < 0.05).
Figure 3-3. Cumulative percent mortality of trout larvae chronically exposed to water, methanol (MeOH; 0.0002% v/v), retene (100 µg/L), and a range of ANF concentrations, alone and in co-treatment with 100 µg/L retene. The data represent the mean percent mortality ± SE (N = 3 replicates, 25 larvae per replicate).

At the lowest ANF concentration (3.2 µg/L) with retene, mortality was higher but not statistically significantly different from that of controls or ANF only (P = 0.061). Mortality peaked at 32 and 56 µg/L ANF (100%) and then dropped off in a concentration-dependent manner to 180 µg/L ANF, where it returned to control levels. There was no significant difference in mortality among replicates when the effects of treatment and concentration were considered.
3.3.4 Chronic toxicity – Severity and BSD indices

As expected, retene controls produced a severity index higher than that of water and solvent controls (P < 0.02). In ANF-only exposed larvae, toxicity was only lower than retene exposures at 18 µg/L ANF, and increased significantly from water and solvent between 180 and 320 µg/L ANF (P = 0.035). In co-treated larvae, a synergistic toxicity (i.e., more than additive) was observed in the severity of BSD (P < 0.001; Fig. 3-4a). All treatment concentrations caused significantly higher toxicity than controls and their respective ANF-only concentrations (P < 0.03). Toxicity increased to a maximum between 18 and 56 µg/L ANF and decreased to levels near that of retene-exposed larvae at 180 µg/L ANF (P = 0.037).

The BSD index was influenced by the amount of mortality, but similar trends were observed (Fig. 3-4b). ANF alone did not produce significant signs of BSD in all but the highest concentration. At 320 µg/L, ANF produced signs of BSD with a higher severity than retene controls (P < 0.001), which corresponded to an increased severity of ANF and retene co-exposure. The sum of the BSD indices of larvae exposed to retene-only (100 µg/L) and 320 µg/L ANF-only were not statistically different from that of the larvae co-exposed to the same concentrations, indicating response addition. All co-treated larvae showed significantly more severe BSD when compared to water, solvent, and retene controls (P < 0.001). The co-exposure treatments with 18-56 µg/L ANF produced 100% mortality in two or more replicates, and therefore were not plotted.

3.3.5 CYP1A induction in trout larvae

ANF modulated CYP1A induction on its own and in co-treatment with retene (Fig. 3-5). ANF alone induced CYP1A in 10, 32, and 100 µg/L-exposed larvae. The 100 µg/L exposures
Figure 3-4. Severity index and BSD index of trout larvae chronically exposed to water, methanol (MeOH; 0.0002% v/v), retene (100 µg/L), and a range of ANF concentrations, alone and co-treatment with 100 µg/L retene. (a) Severity index includes both mortality and BSD, with the highest value designated to mortality. (b) BSD index represents a standardized score of the signs of BSD in surviving larvae. BSD indices were only presented in treatments with less than 65% mortality, and therefore values were not plotted for larvae exposed to retene and 18-56 µg/L ANF (hash marks). The data represent the respective mean index score ± SE (N = 3 replicates; Severity index, 25 larvae per replicate; BSD index, variable n per replicate).
Figure 3-5. Severity index versus CYP1A protein concentrations of trout larvae chronically exposed to retene (100 µg/L), ANF only (3.2-320 µg/L), and retene + ANF (100-320 µg/L). The data represent individual band densities with the respective severity index of the five pooled larvae per band.

consistently produced 3-fold higher CYP1A induction than 10 and 32 µg/L treatments. At 320 µg/L ANF, induction was at levels comparable to those of retene only exposed-larvae, suggesting that ANF was 1/3 as potent as retene (i.e., a concentration 3-times higher was required to cause the same CYP1A response).

In co-treated larvae, CYP1A induction was consistently at or above the levels induced by retene controls for 100-320 µg/L ANF concentrations (Fig. 3-5). Co-treatment with retene and
100 µg/L ANF did not induce CYP1A beyond that of retene alone. However, co-treatment with 180 and 320 µg/L ANF increased CYP1A levels an average of 1.5- and 1.6-fold, respectively.

The CYP1A protein measurements identified a significant correlation between CYP1A induction and toxicity ($r^2 = 0.72$; Spearman $r = 0.86$; Fig. 3-5), suggesting that toxicity is AhR- and/or CYP1A-mediated.

In all gels, there were no measurable levels of CYP1A in water and solvent control larvae.

### 3.4 Discussion

The results of this study illustrate the potential for synergism between a CYP1A inducer (retene) and CYP1A inhibitor (ANF), and a concentration-dependent interactive toxicity and mode of action of AhR ligands. The objective of this study was to investigate the role of CYP1A in retene toxicity; however, synergistic and additive toxicities were observed in larval trout co-treated with a range of ANF concentrations.

Synergistic toxicity was observed in larval trout co-treated with retene and low to intermediate concentrations (3.2-100 µg/L) of ANF (Fig. 3-4). Toxicity was enhanced in the intermediate (32-100 µg/L ANF) concentrations, despite a concentration-dependent decrease in EROD activity (Fig. 3-1). Thus, the synergistic toxicity cannot be explained by changes in EROD activity alone. Furthermore, the lowered EROD activity in juveniles exposed to ANF alone and in co-treatment with retene may infer that ANF is acting on the active site of the enzyme (Fig. 3-1), although more direct measurements are needed. EROD activity in this study was extrapolated from in vitro analyses and may not reflect activity in vivo. However, inhibition of CYP1A activity is consistent with Hodson et al. (2007), who reported an exposure-dependent
reduction in retene metabolites in tissue and bile metabolites of larval trout co-treated with ANF. Direct CYP1A inhibition has been observed in ANF-exposed rainbow trout hepatocytes (Aluru et al., 2005) and zebrafish embryos co-treated with BNF (Wassenberg and Di Guilio, 2004; Billiard et al., 2006; Timme-Laragy et al., 2007). In addition, ANF has been reported as a competitive inhibitor of human CYP1A1 and CYP1A2 (Chang et al., 1994), which has been attributed to the high affinity binding of ANF in the active site in a configuration with the phenyl group in close proximity to the heme iron (Sansen et al., 2007).

The trend of toxicity between 180 and 320 µg/L ANF in co-exposed larvae shifted to response addition (Fig. 3-4b), where the additive toxicity of retene-only and 320 µg/L ANF-only approximately equaled that of the co-exposed larvae (100 µg/L retene + 320 µg/L ANF). This suggests that the mechanism of retene and ANF at high ANF concentrations (> 180 µg/L) may be similar, and that the compounds when co-exposed are working independently. The lack of EROD activity measured after exposure to 320 µg/L ANF alone and in co-treatment with retene suggests an EROD-independent mechanism.

Furthermore, there was a significant correlation between CYP1A protein concentration and toxicity in the exposed larvae (Fig. 3-5), suggesting that toxicity is dependent on the induction of CYP1A. Therefore, if CYP1A expression is indicative of AhR activation, toxicity may be AhR-mediated. Further studies are needed to characterize the involvement of AhR in toxicity; however, our results do not refute this mode of action. Thus retene, ANF, and mixture toxicity may be CYP1A-independent and may be AhR-dependent. These results have been reported in BNF+ANF co-exposures (Billiard et al., 2006), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposures (Antkiewicz et al., 2006), and PAH (e.g. benz[a]anthracene; Incardona et al., 2006) exposures in zebrafish. In these exposures, CYP1A knockdown using antisense
morpholino oligonucleotides had no effect or potentiated toxicity; however, signs of BSD were absent in AhR2-morphants (Billiard et al., 2006; Incardona et al., 2005, 2006).

ANF alone was capable of inducing CYP1A expression at concentrations greater than 3.2 µg/L (Fig. 3-5). In the larvae, ANF induction potency appeared to be 1/3 of that of retene, as there was an apparent increase in CYP1A expression to retene (100 µg/L) control levels at 320 µg/L. This rise in CYP1A protein concentration at 320 µg/L ANF corresponded to a significant increase in signs of BSD. Wassenberg and Di Giulio (2004) observed a similar trend in zebrafish, and reported a dose-response increase in toxicity in embryos exposed to ANF concentrations greater than 100 µg/L. The relatively weak CYP1A induction potential of ANF is consistent with previous reports (Aluru et al., 2005; Timme-Laragy et al., 2007). In rainbow trout hepatocytes, ANF-induced CYP1A gene and protein expression was half that of β-naphthoflavone (BNF) at the same concentration (Aluru et al., 2005). Timme-Laragy et al. (2007) reported that cyp1a mRNA induction in ANF-exposed zebrafish larvae was about 8-fold lower than in BNF-exposed larvae.

3.4.1 Possible mechanisms

ANF is a complex compound that has pleiotropic effects. There have been many discrepancies with regards to its inhibitory effects and subsequent role in mixture toxicity. ANFs’ inhibitory effect on CYP1A induction has been attributed to suboptimal binding capacity for AhR due to its angular, non-planar configuration, thus preventing AhR-ARNT dimerization (Gillner et al., 1985). It has also been reported to bind competitively with TCDD for the AhR binding site (Wilhelmsson et al., 1994), despite a 40 times lower affinity (Blank et al., 1984). Conversely, ANF may inhibit CYP1A enzyme activity both competitively and non-competitively.
It is plausible that the variable ANF mixture toxicity with AhR ligands results from a combination of these characteristics, and likely changes with dose. These inconsistencies are further complicated by the finding that ANF is capable of inhibiting AhR-mediated effects without a reduction of CYP1A protein expression (Aluru and Vijayan, 2004), and that two isoforms of AhR2 (α and β) exist in rainbow trout and may regulate distinct sets of genes (Abnet et al., 1999).

ANF may also act by modulating the activity of critical CYP enzymes. In addition to CYP1A(1), ANF is known to inhibit a number of CYP enzymes, including CYP1A2, 2C8, 2C9, 2A6, 2B6 in human hepatocytes (Chang et al., 1994), and 2K1 and 3A27 in rainbow trout (Miranda et al., 1998). Altering CYP function may contribute to toxicity by inhibiting xenobiotic detoxification and/or other critical endogenous processes. For example, many CYP enzymes, including CYP1A and CYP2Cs, influence cardiovascular and renal function via arachidonic acid metabolism (reviewed in Roman, 2002). The cardiovascular system has been suggested as the target of dioxin-like compounds in the embryo-larval stages of fish (reviewed in Goldstone and Stegeman, 2006) and disruptions in cardiovascular and renal function are potential sources of edema (proximate sign of toxicity).

To our knowledge, the inhibitory effect of ANF has not been studied in fish CYP1B1 and 1C1, which synergistically increase in BNF+ANF co-exposures (Timme-Laragy et al., 2007). Expressions of these mRNAs were approximately 7- and 3-fold lower than cyp1a mRNA, respectively; however, they may be responsible, in part, for the shift in retene metabolites in previous CYP1A inhibition studies in rainbow trout larvae (Hawkins et al., 2002; Hodson et al., 2007). Piperonyl butoxide (PBO) inhibition of CYP1A produced a distinct set of bile metabolites of retene that were not hydroxylated or conjugated, and the abundance and persistence of these
metabolites may be responsible for toxicity (Hawkins et al., 2002). Co-exposure of trout to retene and ANF caused a progressive shift to less polar metabolites and an overall decrease in the number of retene metabolites with increasing ANF concentrations (Hodson et al., 2007). Toxicity was antagonized when retene metabolites were virtually absent, which implicates metabolites as a causative agent.

The interactive effect of the retene and ANF in co-treatment may also be, in part, due to a retene-induced enhancement of ANF metabolism. Andries et al. (1988) observed that TCDD not only created a 10-fold increase in ANF metabolism, but also a shift in metabolites. A rise in ANF metabolism could contribute to toxicity in three ways: (1) increased enzyme inhibition; (2) an increase in ROS; and (3) an increase in toxic ANF metabolites. A number of hydroxyl- and oxide-ANF metabolites have been described (Nesnow and Bergman, 1981; Andries et al., 1988); however, the 9-hydroxy metabolite of ANF is the most biologically active, increasing the potency of CYP inhibition 7- to 8-fold (Nesnow and Bergman, 1981). Thus, ANF metabolism can further decrease retene metabolism (and/or metabolism of an unidentified endogenous compounds), which in turn could increase and sustain AhR activation, as suggested by Hawkins et al. (2002). Secondly, since ANF is metabolized by CYP enzymes (Nesnow and Bergman, 1981), an increase in metabolism can contribute additional ROS, whose role has been suggested in retene toxicity (Bauder et al., 2005). If a 10-fold increase in ANF metabolism occurs, the resulting increase in ROS-induced oxidative stress may account for the synergistic toxicity observed in this study.

### 3.4.2 Summary

The results of this study suggest that the variable increase in toxicity observed in rainbow trout larvae co-treated with retene and a range of ANF concentrations is independent of EROD activity and may be AhR-dependent. Moreover, two distinct mechanisms of toxicity (synergism
and response addition) appear to be acting in co-exposed larvae depending on the concentration of ANF. The mechanism of enhanced toxicity of ANF in co-exposures with dioxin-like compounds remains uncertain; however, it is likely influenced by the pleiotropic effects of ANF.

These findings provide evidence that multiple, exposure-dependent mechanisms can occur in mixture toxicity, suggesting that current models risk assessment models of dioxin-like compounds, which assumes additivity and a common mechanism, may be oversimplified and may drastically underestimate mixture toxicity. The significance of these findings is amplified by the fact that PAHs are almost always found in complex mixtures, likely containing both inducers and inhibitors of CYP1A. Furthermore, the toxic mechanisms of alkylated PAHs are little known but essential to these mechanistic-based risk assessment models. To further elucidate the mechanism of retene toxicity, alone and in co-treatment with ANF, other methods of CYP1A inhibition and AhR antagonism (e.g., morpholino knockdown) are needed.

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3.6 References


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Chapter 4

Embryotoxicity of retene in co-treatment with 2-aminoanthracene, a CYP1A inhibitor, in rainbow trout (*Oncorhynchus mykiss*).
Abstract

Environmentally-relevant mixtures of polycyclic aromatic hydrocarbons (PAHs) (e.g., crude oils) are often rich in alkyl-PAHs, such as retene (7-isopropyl-1-methylphenanthrene), which produce dioxin-like toxicity in the embryonic stages of fish. The mechanism of alkyl-PAH toxicity is not well-understood, but was previously thought to be mediated by cytochrome P450 1A (CYP1A) enzymes. To understand the role of CYP1A in retene toxicity we co-exposed free-swimming rainbow trout (Oncorhynchus mykiss) embryos to 100 µg/L retene and to a range of 2-aminoanthracene (2AA; a known CYP1A inhibitor) concentrations. Ethoxyresorufin-O-deethylase (EROD) assays of juvenile trout co-exposed with 2AA and retene confirmed the CYP1A inhibitory capacity of 2AA (IC50 = 62 µg/L 2AA). In two independent experiments, 2AA alone and in co-treatment with retene produced a concentration-dependent increase in toxicity to embryonic trout. The toxicity observed in 2AA only-exposed embryos is the first reported for embryonic stages of fish, with LC50s of 19 and 125 µg/L 2AA and overall EC50s of 17 and 38 µg/L 2AA in the two experiments. Toxicity increased in embryos co-exposed to retene and 2AA, resulting in LC50s of 14 and 17 µg/L 2AA and overall EC50s of 7 and 3 µg/L 2AA. The exposure-response curves for 2AA alone and 2AA with retene were parallel, suggesting a common mode of action between the two treatment regimes, and may infer a common mechanism between retene and 2AA. Taken together with the juvenile EROD data, 2AA alone and in co-treatment with retene may be EROD (CYP1A)-independent. The mixture toxicity was not consistent with previous co-exposures of CYP1A inducer and inhibitors, suggesting that current risk assessment models may not be a good predictor of relevant PAH mixtures.

Keywords: polycyclic aromatic hydrocarbon; retene; 2-aminoanthracene; cytochrome P450 1A; mixture toxicity.
4.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants often found in complex mixtures derived from pyrogenic and petrogenic sources. In the embryolarval stages of fish, PAHs are known to cause dioxin-like toxicity (blue sac disease; BSD) in both single compound and mixture exposures [1-3]. PAHs have varying modes of action [4] which makes understanding mixture toxicity difficult and problematic.

Alkyl-PAHs are the predominant forms of PAHs in crude oils (80-90% of total) [5]. Furthermore, as oils weather there is a progressive shift towards 3- to 5-ringed alkyl-PAHs, primarily alkyl-phenanthrenes [6], which have been implicated in crude oil toxicity to embryonic fish [7]. Alkyl-phenanthrenes, such as retene (7-isopropyl-1-methylphenanthrene), are known to produce exposure-dependent increases in BSD [1,8]. Retene is also a product of wood combustion [9] and has also been found in contaminated sediments downstream of pulp and paper mills [10].

The mechanism of retene toxicity is has been generally considered to be mediated by the aryl hydrocarbon receptor (AhR) and cytochrome P450 1A (CYP1A) [11,12]. The AhR is a ligand activated transcription factor that is thought to contribute to dioxin-like toxicity through altered expression of AhR-responsive genes, including CYP genes [13,14]. CYP1A-mediated toxicity can result from oxidative stress and adduct formation via the production of reactive oxygen species and reactive metabolites during CYP1A oxygenation of PAH [15].

Alkyl-PAHs are unique in that the alkyl groups provide an additional site for hydroxylation, which can alter their toxicity when compared to their unsubstituted counterparts. Retene is primarily metabolized by CYP enzymes to benzylic alcohols, not phenols [16], which may account for the difference in pathology compared to phenanthrene. In zebrafish, phenanthrene toxicity has been attributed to atrio-ventricular conduction block [17], whereas
retene produced signs of BSD with no observable arrhythmias (Scott and Hodson, unpublished data).

Thus, the objectives of this study were to (1) investigate the role of CYP1A in retene toxicity, and concurrently, (2) study the interactive toxicity of a PAH mixture containing both a CYP1A inducer and inhibitor. To accomplish these objectives, we co-treated free-swimming rainbow trout (*Oncorhynchus mykiss*) embryos with retene and 2-aminoanthracene (2AA), a known CYP1A inhibitor in fish.

Aminoanthracenes have been found in industrially-contaminated sediments [18] and in waste products of coal-derived liquids [19]. 2-Aminoanthracene produces both *in vitro* and *in ovo* concentration-dependent inhibition of CYP1A in fish co-treated with β-naphthoflavone (BNF), a model CYP1A inducer [20,21]. The inhibitory mechanism of 2AA is due to a covalent association with the CYP1A enzyme, rendering it inactive. 2-Aminoanthracene is a known mutagen [22]; however, Wassenberg and Di Giulio [21] observed little toxicity in killifish (*Fundulus heteroclitus*) embryos exposed to a range of 2AA concentrations.

The results of our study revealed an exposure-dependent toxicity of both 2AA on its own and in co-treatment with retene, which may be independent of EROD activity and CYP1A induction. These findings are not consistent with those previously reported in co-exposures between CYP1A inducers and inhibitors, suggesting that the current risk assessment model may not be a good predictor of PAH mixture toxicity.
4.2 Materials and Methods

4.2.1 Chemicals

Retene (98% pure) was obtained from ICN Biomedical (Aurora, ON), and 2AA (96% pure) was from Sigma-Aldrich (Oakville, ON). Reagent grade dimethyl sulfoxide (DMSO; Fisher Scientific, Ottawa, ON) was used as a carrier solvent for retene and 2AA. Tricaine methane sulfonate (MS-222; Sigma, Oakville, ON) was used as a fish anesthetic, as it does not alter ethoxyresorufin-O-dethylase (EROD) induction at the concentration used in this study [23]. BNF was obtained from Acros Organics (Fair Lawn, NJ, USA) and was used as a positive control in EROD assays. All other EROD assay materials are described elsewhere [23,24], and materials used in SDS-PAGE Western blots and the chemiluminescent detection are described extensively in Sibani [25].

4.2.2 Fish care and maintenance

Trout eggs and juveniles were obtained from Rainbow Springs Trout Hatchery (Thamesford, ON) and were acclimated to 8 and 12°C, respectively, upon arrival. They were acclimated for 1 h in their shipping container and then placed into their respective holding tanks. The eggs were placed in stainless steel bowls and held until the first sign of hatching, and the juveniles were housed in a 100 gal holding tank. Aerated Kingston municipal Lake Ontario water (alkalinity = 90 mg/L CaCO₃) was used during the holding and exposure periods, which was dechlorinated by charcoal filtration and the addition of 1.0 mg/L sodium bisulfite.
4.2.3 CYP1A and EROD studies – juvenile trout

Juvenile trout were used to as a surrogate for testing the CYP1A inhibitory effect of 2AA in free-swimming embryos. Juvenile trout were chosen for the EROD and Western blot assays due to the difficulties of obtaining consistent and quantifiable measurements of EROD activity in free-swimming embryos. Although there may be relative differences in AhR/CYP1A protein concentrations and toxic responses between the two life stages, the biochemistry of these proteins does not change. Thus, the interactions with AhR and/or CYP1A are expected to be similar between juvenile trout and free-swimming embryos.

To confirm the CYP1A inhibitory effect of 2AA, juvenile trout were exposed to a range of five 2AA concentrations (10-1000 µg/L), alone and in co-treatment with 100 µg/L retene. The range of concentrations of 2AA were chosen based on previous reports that described CYP1A inhibition without signs of BSD [21], and 100 µg/L retene was chosen because it produces signs of BSD without a significant amount of mortality in the early life stages of rainbow trout [1]. The activity of the CYP1A enzyme was measured using an EROD assay of S9 fractions prepared from the liver tissue of the exposed juveniles. BNF (10 µg/L) was used as a positive control, as it is a known CYP1A inducer that is capable of producing BSD in embryo-larval stages of fish [26]. To further investigate the mode of inhibition, Western blot analyses were performed to compare CYP1A protein concentrations and CYP1A activity.

In the CYP1A inhibition study, five 1-3 g juvenile trout were placed in 10 L of test solutions in a 20 L bucket lined with food grade polyethylene bags. The exposure containers were maintained under a 16:8 h light:dark photoperiod and a 15°C room temperature. The chemical exposures were 48 hrs in duration, with a renewal of water and test compounds at 24 hrs. At 48 hours, the juveniles were sacrificed and their livers were removed and homogenized.
The homogenate was centrifuged at 9000 x g to obtain an S9 fraction for EROD and Western blot analyses. The S9 was flash-frozen in liquid nitrogen and kept at -80°C until further analysis.

4.2.4 Chronic toxicity studies – free-swimming trout embryos

To investigate the involvement of CYP1A metabolism in retene toxicity and the mixture toxicity of a mixture of a CYP1A inducer and inhibitor, rainbow trout embryos were chronically exposed to seven concentrations (10-1000 µg/L) of 2AA, alone and in co-treatment with 100 µg/L retene. The exposures followed a semi-static daily renewal protocol beginning just prior to hatch and continued until swim-up. At swim-up (25 days post-hatch), chronic toxicity was assessed by measuring the mortality and severity of BSD in the exposed embryos. The treatments were run in duplicate on two separate exposure occasions (4 total replicates) with two independent batches of eggs to reduce pseudoreplication. The two replicated exposure occasions are herein referred to as exposure periods.

In the chronic toxicity test, 25 eggs were placed in 2 L of test solution in randomly ordered stainless steel bowls. The exposures followed a semi-static daily renewal protocol. The room was maintained at 10 ± 1°C and a 0:24 h light:dark photoperiod. The eggs were exposed only to a minimal amount of low light intensity (~ 1 h) during the daily change-over of test solutions. Dead embryos were removed and recorded as necessary. At swim-up, the embryos were anesthetized in 100 µg/L MS222 until movement ceased, and they were scored for presence and severity of the signs of BSD. The following signs were scored based on presence/absence (0-1) or severity (0-3, 3 as highest severity): pericardial edema (PE; 0-3), yolk sac edema (YE; 0-3), ocular hemorrhaging (OH; 0-1) craniofacial deformity (CF; 0-1), spinal deformity (SD; 0-1), fin rot (FR; 0-1), and no movement (mechanicosensory test) (NMOV; 0-1). The signs with a 0-3
severity scale (pericardial and yolk sac edema) are ones that can be scored visually as a range of fluid accumulation in the respective compartments. Edema is likely the proximate cause of death due to an impediment of cardiovascular function. After being scored, the embryos (5 per sample) were flash-frozen and kept at -80°C until further analysis.

In both experiments, water quality measurements were taken daily and were within the optimal range for trout development (temperature = 8.50 ± 1.1°C (replicates 1 and 2), 8.2 ± 0.6°C (replicates 3 and 4), dissolved oxygen = 11.6 ± 1.0 mg/L, pH = 8.23 ± 0.53, total ammonia = 6.20 x 10^{-5} moles/L). In addition, water samples from one replicate (alternating) were obtained daily at 0 and 24 h to describe the chemistry of the test compounds in the exposure solutions.

In both the juvenile and embryonic exposures, DMSO (0.0001% v/v) and water controls were included to account baseline responses associated with the carrier solvent and exposure water.

4.2.5 Severity index calculation for overall toxicity

The severity index (SI) is the sum of the number of embryos displaying a severity of each sign divided by the maximum SI score, creating a representative view of overall toxicity on a 0-1 scale. It was calculated using a modified version of the Villalobos et al. [27] equation:

$$SI = \left[ \sum_{i=1}^{n}(PE \cdot E_i) + \sum_{j=1}^{n}(YE \cdot E_j) + \sum_{k=1}^{n}(OH \cdot E_k) + \sum_{l=1}^{n}(CF \cdot E_l) + \sum_{m=1}^{n}(SD \cdot E_m) + \sum_{o=1}^{n}(FR \cdot E_o) + \sum_{p=1}^{n}(NMOV \cdot E_p) \right] \div \text{maximum SI score}$$
where $E_i, E_j$ equal the number of embryos displaying a particular severity of pericardial edema, yolk sac edema, respectively; and $E_k, E_l, E_m, E_o, E_p$ equal the number of embryos displaying ocular hemorrhaging, craniofacial deformities, spinal deformity, fin rot, and no movement, respectively. A value of 11.5 was assigned for lethality (0.5 higher than the maximum value). The maximum SI score (100% mortality) was 287.5 ($11.5 \times 25$ fish). For example, if in a treatment there were four embryos displaying a severity of 3 for PE, two displaying a severity of 2 for YE, six displaying CF, and two died, then $SI = \left[ (4 \times 3) + (3 \times 2) + (6 \times 1) + (2 \times 11.5) \right] / 287.5 = 0.16$.

4.2.6 EROD analysis of juvenile trout livers

EROD activity was measured by a kinetic fluorescence method described elsewhere [23,24]. Crude EROD activity was estimated using a Spectramax Gemini Spectrofluorometer (Molecular Devices, Sunnyvale, CA; $\lambda_{\text{excitation}} = 530$ nm, $\lambda_{\text{emission}} = 586$ nm). Specific EROD activity (picomoles per minute per mg protein) was calculated by normalizing the crude activity to the total protein of the S9. Protein concentrations were measured using a Bio-Rad colorimetric assay (BioRad, Hercules, CA, USA) with a Spectra Max Plus microplate spectrophotometer (Molecular Devices) and were standardized to bovine serum albumin. Each sample was assayed in triplicate against a standard curve of resorufin.

4.2.7 Western blot analysis of juvenile trout livers

The S9 samples were run on a 10% SDS-PAGE gel as described in Sibani [25], standardized to total protein measured in the Bio-Rad colorimetric assay. A positive control sample was obtained from a stock of liver S9 of BNF-exposed juveniles, and was used in all gels.
to ensure comparable data. Gels were blotted to a nitrocellulose membrane and incubated in a primary (CYP1A (fish) monoclonal antibody, C10-7; Cayman Chemical, Ann Arbor, MI, USA) antibody and a horseradish peroxidase-goat anti-mouse IgG (H+L) conjugate secondary antibody (Zymed® Laboratories, South San Francisco, CA, USA). The resulting band densities were analyzed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA) and were standardized to the positive control.

4.2.8 Measurement of waterborne retene and 2AA

Water samples were taken every second day from treatments containing retene. A 1.5 mL water sample was taken and added to 1.5 mL of ethanol in a 7 mL scintillation vial. The vials were stored at 4°C until analysis. Waterborne retene and 2AA concentrations were measured using synchronous scanning spectrofluorometry (Quanta-Master Fluorescence Spectrometer; PTI Ltd., London, ON, Canada) and were analyzed using FELIX software (PTI Ltd., London, ON, Canada). Retene (λexcitation range = 290-310 nm, λemission range = 340-360 nm) and 2AA (λexcitation range = 358-438 nm, λemission range = 450-530 nm) samples were run against standard curves of retene or 2AA diluted in 50:50 ethanol:water.

4.2.9 Statistics

A 3-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls (SNK) pairwise multiple comparison was used to analyze EROD, percent mortality, and SI data. A 2-way ANOVA followed by an SNK pairwise multiple comparison was use to analyze within treatment differences, and measured waterborne concentrations of retene and 2AA. The ANOVAs were run using SigmaStat for Windows (V 3.0©; SPSS Corporation, Chicago, IL, USA;
P < 0.05). Non-linear regression (sigmoidal concentration-reponse curves) IC50, and EC50s were analyzed and calculated using GraphPad Prism software (Ver. 4.02, GraphPad Software, Inc., San Diego, CA, USA). LC50 calculations were performed using Probit Analysis (LC50 BAS 2.0, 1986, US EPA, Washington, DC).

4.3 Results

4.3.1 Chemical exposure in the chronic toxicity test

Immediately following addition (T0), waterborne concentrations of retene (100 µg/L) averaged 83% of the nominal concentration (SE = 2.0%, N = 41). The concentrations dropped to 24% of the nominal concentrations (SE = 2.3%, N = 26) after 24 h.

The range of waterborne 2AA concentrations averaged 82% of the nominal concentrations (SE = 5.7%, N = 125) and dropped 2.5-fold to 33% (SE = 3.0%, N = 51) of the nominal concentration after 24 h. At each concentration, there was no significant difference in measured 2AA between treatment regimes (2AA only and retene + 2AA); however, there was a significant difference in 2AA concentration between exposure periods (P < 0.001). In the first exposure period, the measured concentrations of 2AA were approximately 2-fold higher than in the second exposure period with respect to nominal concentrations > 32 µg/L 2AA. Thus, with the exception of the EROD inhibition study, the data herein have been analyzed and presented using measured concentrations of 2AA.
4.3.2 CYP1A inhibition in juvenile trout

As expected, retene exposure produced high levels of both CYP1A synthesis and EROD activity (Fig. 4-1A). The equally high BNF (10 µg/L)-induced EROD activity confirms the sensitivity of the assay (Fig. 4-2). Relative to retene only-exposed juveniles, 2AA alone caused similar CYP1A induction at all concentrations, but with about 1/3 less EROD activity (Fig. 4-1B). The EROD activity of all concentrations of 2AA only-exposed fish were significantly lower than retene-only exposed juveniles (P < 0.001), but were not higher than that of DMSO controls.

Juvenile trout co-treated with retene and 2AA produced a concentration-dependent inhibition of EROD activity (R² = 0.99; Fig. 4-2), with an IC50 of 62 µg/L 2AA. The EROD activity of co-exposed juveniles dropped significantly below retene-only levels at 100 µg/L 2AA and reached control (DMSO) levels at 320 µg/L 2AA. Similar to 2AA only-exposures, the EROD and Western blot data suggest that 2AA may be directly inhibiting CYP1A function (Fig. 4-1).

4.3.3 Chronic toxicity in trout embryos

The two exposure periods produced similar trends in toxicity (Fig. 4-3). Both treatment regimes (2AA and retene + 2AA) produced concentration-dependent increases in toxicity (R² > 0.90). There was a significant increase in mortality and overall toxicity (severity index, i.e., mortality + BSD) in embryos co-treated with retene and 2AA when compared embryos exposed to 2AA only (P < 0.001). All concentrations of 2AA caused significantly higher toxicity than controls in embryos exposed to 2AA alone and in co-treatment with retene (P < 0.001).
Figure 4-1. EROD activity versus relative CYP1A protein concentrations in livers of juvenile trout exposed to 100 µg/L retene (dark circles) and/or a range of 2AA concentrations (open symbols). Panel A: 2AA in co-treatment with 100 µg/L retene; Panel B: 2AA only. The data have been arranged to show: (1) retene only caused a moderate amount of CYP1A synthesis and relatively high EROD activity; and relative to retene-only, (2) 2AA caused variable and sometimes higher levels of CYP1A synthesis, but only low EROD activity, and (3) at 56 µg/L 2AA, retene + 2AA co-exposure caused an increase in CYP1A synthesis only, followed by a concentration-dependent decrease in both CYP1A protein levels and EROD activity. The dotted lines outline the range of values from retene only-exposed juveniles. Shaded areas are indicative
of inhibited EROD activity and not inhibited CYP1A synthesis (higher protein and similar or lower EROD activity, or similar protein levels with lower EROD activity). No CYP1A protein was detected in control fish and the average background (DMSO) EROD activity was 0.58 pmol/min/mg protein. The data represent the mean relative EROD activity and Western blot band density of individual juvenile livers.

Figure 4-2. Relative EROD activity in juvenile trout livers exposed to BNF (10 µg/L), retene (100 µg/L), and a concentration series of 2AA alone and co-treatment with 100 µg/L retene. The data represent the mean EROD activity ± 95% confidence limits (N = 5), normalized to DMSO (0.0001% v/v) controls.
For example, in the first exposure period, maximal mortality was observed in concentrations ≥ 41 µg/L 2AA for both treatment regimes (Fig. 4-3A). Overall toxicity was significantly higher than retene-only exposed embryos at concentrations > 24 µg/L 2AA in 2AA-only-exposed embryos (P = 0.015), and at all concentrations in co-exposed embryos (P < 0.001). The EC50 in the 2AA-only exposure was 2.4-fold higher than in co-exposed embryos (Fig. 4-3C).

2AA was less toxic in the second exposure period, which was likely influenced by the inherent differences between the batches of eggs. When compared to the first, the second exposure period produced an LC50 roughly 7- and 1.2-fold higher in embryos exposed to 2AA alone and in co-treatment with retene, respectively (Fig. 4-3). The overall EC50 was 3.5-fold lower in the 2AA-only exposure and 3-fold higher in the co-exposure in the second exposure period (Fig. 4-3).

The slopes of the linear portion of the exposure-response curves within each experiment (i.e., 2AA only vs. retene + 2AA) were not significantly different, indicating parallel curves. Thus, the nature of the exposure-response curve of co-exposed embryos did not change; however, toxicity increased in embryos co-treated with 2AA and retene, shifting the curve to lower concentrations.
Figure 4-3. (A,B) Cumulative percent mortality and (C,D) severity index (overall toxicity) observed in the two exposure periods of trout embryos to retene (100 µg/L), a concentration series of 2AA alone and co-treatment with 100 µg/L retene, water, and DMSO (0.0001% v/v). The data represent the mean percent mortality and mean severity index score ± SE (N = 4 replicates; 25 embryos per replicate) plotted against measured 2AA concentrations.

4.4 Discussion

The results of this study suggest that: (1) 2AA is capable of inducing CYP1A enzyme and inhibiting its function; (2) 2AA alone or in co-treatment with retene causes a concentration dependent increase in toxicity in trout embryos; and (3) the mechanism for both treatment regimes (2AA and retene + 2AA) is EROD-independent, which supports a protective role of CYP1A.
In embryos co-exposed to one concentration of retene (100 µg/L) and a range of 2AA (10-1000 µg/L) there was a concentration-dependent increase in toxicity (Fig. 4-3), despite an exposure-dependent decrease in both EROD activity and CYP1A protein levels (Fig. 4-1A and 2). It should be noted that relative CYP1A protein concentrations and EROD activity were measured in juvenile trout, which may not be representative of the induction and activity in free-swimming embryos; however, previous retene co-exposure studies with α-naphthoflavone suggest that the trends in CYP1A inhibition are similar in both life stages [28,29].

On its own, 2AA caused a concentration-dependent increase in toxicity (Fig. 4-3), which, to our knowledge, is the first reported toxicity of 2AA to embryonic fish. The increase in 2AA embryotoxicity was not correlated with an increase in CYP1A protein levels or EROD activity in juvenile trout (Fig. 4-1B). It induced the expression of CYP1A enzymes, producing similar protein levels at concentrations > 56 ug/L 2AA, but failed to increase EROD activity. Thus, 2AA is capable of both inducing CYP1A and inhibiting its function. The effect of 2AA on EROD activity is consistent with those reported in other fish species [20,21]. In channel catfish, 2AA reduced EROD activity in liver microsomes from BNF-induced fish, but did not induce EROD activity in microsomes from un-induced fish [20]. Thus, our data suggest an EROD-independent mechanism for both treatment regimes (2AA and retene + 2AA), and supports a protective role of CYP1A. Furthermore, if CYP1A protein concentrations are indicative of AhR activation, toxicity may also be AhR-independent, as there was a constant (2AA only) or concentration-dependent decrease (retene + 2AA) in CYP1A protein levels with increasing toxicity. Further, more direct studies are needed to confirm these inferences; however, our results do not refute these mechanisms. AhR-independent toxicity has been previously reported in mixtures rich in alkylated PAHs (weathered crude oil) [3], and CYP1A-independent toxicity has been observed in
unsubstituted PAH (e.g., benzo[a]anthracene) [30], TCDD [31], and BNF + α-naphthoflavone exposures [26].

Our results were in general agreement with a co-exposure of 2AA and BNF in killifish embryos [21]. At the same 2AA concentrations used in this study, 2AA-alone induced CYP1A and produced a concentration-dependent decrease in EROD activity when co-exposed with BNF. The co-exposure resulted in an exposure-dependent increase in toxicity but 2AA alone was only slightly toxic to killifish exposed at 500 µg/L [21]. The difference in 2AA toxicity between trout and killifish is unclear, but may be due to different life stage sensitivities (unhatched vs. free-swimming embryos).

The two experiments in this study produced similar trends in toxicity, adding weight to the observed effects (Fig. 4-3). Both experiments produced similar concentration-dependent curves, with a slightly reduced toxicity between experiments, which is likely due to biological variability between separate batches of eggs. In addition, the linear portion of the exposure-response curves of 2AA only and retene + 2AA were parallel, suggesting a similar mode of action, which may infer a common mechanism between retene and 2AA.

It is plausible that retene and 2AA share a common mechanism, as they both can be metabolized to highly reactive carbocations after sulfation. After hydroxylation by CYP enzymes, the intermediate metabolites can undergo phase II metabolism via various conjugations reactions (e.g., sulfation), creating more polar compounds and facilitating excretion [15]. Sulfation adds an electron-withdrawing sulfate group, which can heterolytically cleave producing a carbocation metabolite in compounds that can stabilize the cation via mesomerism (reviewed in [32]) – a characteristic shared by both benzylic alcohols (primary retene metabolite) [16] and aromatic hydroxylamines (primary 2AA metabolite) [33]. The resulting carbocations react transiently with nucleophiles, such as proteins and DNA [34]. It is noteworthy that the formation
of benzylic alcohol metabolites of alkyl-PAHs may be enhanced when CYP1A is inhibited [35-37]. In humans, CYP1A1 primarily metabolizes 5- and 6-methylchrysene via ring oxidation; however, CYP3A4, a human isoform of rainbow trout CYP3A27 [34], produced considerable amounts of methyl hydroxylations [36]. Similarly, CYP3A4 primarily hydroxylates the benzylic position of 1-methylpyrene [35]. Thus, inhibiting CYP1A may shift metabolism to other CYP enzymes that may preferentially hydroxylate alkylated PAHs in exocyclic positions, potentially producing reactive carbocation derivatives.

Previous co-exposure studies with CYP1A inducers and inhibitors have reported synergistic toxicity in the embryonic stages of fish [21,28,29]. In killifish embryos, concentration-dependent synergistic toxicities were observed in co-exposures of BNF with multiple CYP1A inhibitors (piperonyl butoxide, fluoranthene, and 2AA), and benzo[a]pyrene with α-naphthoflavone [21]. Previous retene co-exposures with α-naphthoflavone identified both synergistic and additive mixture toxicities in embryonic trout [28,29]. Taken together with the mixture toxicity of two environmentally-relevant contaminants presented in this paper, the variable responses suggest that the current risk assessment model of dioxin-like compounds is oversimplified, at least with regards to PAH mixture toxicity. The current model assumes that dioxin-like compounds have a common mode of action via the AhR, and consequently, will act additively in mixtures [38]. Furthermore, the model only assesses the risk of AhR ligands and does not consider the potentially strong influence that compounds with little to no AhR binding capacity (e.g., phenanthrene) [39] can have on mixture toxicity [11].

In conclusion, in rainbow trout embryos, 2AA alone, or in co-treatment with retene, produces concentration-dependent increases in toxicity which may be independent of EROD activity and CYP1A induction. Thus, toxicity may be CYP1A- and AhR-independent; however, more direct studies are needed. In addition, the linear portion of the exposure-response curves of
2AA and retene + 2AA were parallel, suggesting a similar mode of action and may infer a common mechanism between retene and 2AA. However, more direct studies are needed to confirm the involvement of AhR, CYP enzymes, and carbocations in the mechanism of 2AA and retene toxicity. In addition, our results provide further evidence that the current risk assessment model may not be a good predictor of PAH mixture toxicity.

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4.6 References


Chapter 5

AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene

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Abstract

In the embryo-larval stages of fish, alkylphenanthrenes such as retene (7-isopropyl-1-methylphenanthrene), cause a suite of developmental abnormalities typical of that of exposures to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The signs of TCDD-like toxicity include: pericardial and yolk sac edema, cardiovascular dysfunction, and skeletal deformities. The cardiovascular system has been suggested to be the primary target of TCDD-like compounds, and the mechanism is mediated via the aryl hydrocarbon receptor 2 (AhR2) and is independent of cytochrome P450 1A (CYP1A) enzymes. Current mechanism-based risk assessment models use TCDD as a reference compound; however, the mechanism of retene toxicity and its etiological commonality to TCDD toxicity remains largely uncertain. To investigate the mechanism of target tissue of retene toxicity, we used observational, histological, and genetic knockdown techniques in the embryonic stages of zebrafish (Danio rerio). Retene toxicity was first observed at 36 hours post-fertilization (hpf), with pericardial edema and reduced blood flow as the proximate signs of toxicity. At 52 hpf, there was a lack of parachordal vessels in retene-exposed embryos, suggesting a delay in angiogenesis. By 72 hpf, retene-exposed embryos exhibited a significant increase in pericardial edema, atrial edema, and girth, and an observable decrease in bulbus arteriosus and ventricle size, and blood flow. In addition, the presence of round erythrocytes typical of the primitive stage erythropoiesis suggested a disruption in definitive stage erythropoiesis. A knockdown of CYP1A by antisense morpholino oligonucleotides did not affect retene toxicity; however, a blockade of AhR during retene exposures prevented toxicity. Furthermore, after AhR2 knockdown, CYP1A proteins were still observed in the vasculature and eye tissue, providing evidence for an additional functional AhR isoform. Thus, the mechanism of retene cardiotoxicity is AhR2-dependent and CYP1A independent, similar to TCDD. However,
the onset and proximate signs of retene toxicity differs from that of TCDD, suggesting multiple
AhR-mediated effects.

Key words: alkyalted polycyclic aromatic hydrocarbons, retene, aryl hydrocarbon receptor,
cytochrome P450 1A, embryotoxicity.
5.1 Introduction

Alkylphenanthrenes such as retene (7-isopropyl-1-methylphenanthrene) are constituents of crude oil and have been implicated in the toxicity of crude oil to the early life stage of fish (Khan, 2008). Exposures to crude oil and alkylphenanthrenes produce exposure-dependent increases in embryo-larval toxicity, characterized by the signs of blue sac disease (BSD; Billiard et al., 1999; Kiparissis et al., 2001). The signs of BSD include pericardial and yolk sac edema, craniofacial and spinal deformities, and cardiovascular dysfunction, and resemble those caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD, the most potent congener, serves as the reference compound for assessing mixture toxicity of TCDD-like compounds. The current risk assessment model calculates risk by determining TCDD equivalents, and assumes that TCDD-like compounds act via the same mechanism as TCDD (see Safe, 1992).

The mechanism of TCDD toxicity to fish is mediated via the aryl hydrocarbon receptor 2 (AhR2, the functional AhR isoform; Prasch et al., 2003; Dong et al., 2004; Antkiewicz et al., 2006), a ligand-activated transcription factor involved in the expression of a battery of genes involved in xenobiotic metabolism (e.g., cytochrome P450 1A) and in normal development and homeostasis (Fernandez-Salguero et al., 1996; Hahn, 1998; Walliser et al., 2004). Upon activation, the AhR translocates into the nucleus and heterodimerizes with the AhR nuclear translocator. The resulting complex binds to xenobiotic responsive elements (XREs) in the promotor region of AhR-responsive genes, initiating transcription (Hahn, 1998). The AhR-mediated mechanism of TCDD-induced cardiotoxicity remains largely uncertain; however, it is independent of CYP1A activity (Carney et al., 2004). Xenobiotic metabolism by CYP1A enzymes can cause toxicity through the production of reactive oxygen species (ROS) and/or reactive metabolite intermediates (Di Giulio et al., 1995).
The involvement of AhR and CYP1A in alkyl-phenanthrene toxicity was suggested by structure-activity studies of alkyl-polycyclic aromatic hydrocarbons, which identified a possible link between toxicity of alkyl-phenanthrenes and their binding affinity to the AhR. AhR-binding alkylphenanthrenes produced BSD, which generally followed a rank order of potency in accordance to an increase of lipophilicity (Turcotte, 2008; Kiparissis et al. 2001). Furthermore, changes in the spectrum of byproducts of metabolism have implicated CYP1A activity in the mechanism of retene toxicity (Hawkins et al., 2002; Brinkworth et al., 2003; Bauder et al., 2005; Hodson et al., 2007). In retene-exposed rainbow trout (Oncorhynchus mykiss) larvae, CYP1A induction precedes the signs of BSD (Brinkworth et al., 2003), and toxicity decreased after chemical CYP1A inhibition (Hawkins et al., 2002; Hodson et al., 2007). In addition, Bauder et al. (2005) implicated ROS-induced oxidative stress in retene toxicity, as there was a decreased glutathione and Vitamin E concentrations in retene-exposed trout larvae, and reduced retene toxicity when the larvae were co-treated with Vitamin E.

Retene toxicity has been previously reported in the embryo-larval stages of zebrafish (Billiard et al. 1999); however, the toxicity reported was primarily late-stage larval toxicity, with little insight into the proximate signs of BSD during the embryonic stages of development. In the present study, we used a more comprehensive approach to study retene toxicity using observational, histological, and immunofluorometric analyses. Furthermore, we used antisense morpholino oligonucleotides to investigate the involvement of AhR and CYP1A in retene toxicity. The objective was to: (i) further investigate retene toxicity, and (ii) investigate the etiological and mechanistic commonality of retene and TCDD toxicity.

The results of our study suggest that retene produces an AhR2-dependent, CYP1A-independent cardiovascular toxicity in zebrafish embryos. Thus, the mechanism of retene toxicity
is similar to that of TCDD; however, there are early and late stage pathology differences that may suggest multiple AhR2-mediated effects.

5.2 Materials and Methods

5.2.1 Chemicals

Retene (98% pure) was obtained from ICN Biomedical (Aurora, ON). Dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) was used as a carrier solvent for retene. Stock solutions prepared at a concentration of 20 mg retene/mL DMSO.

5.2.2 Zebrafish exposures

Maintenance of wild-type AB, nacre, and Tg(fli1-EGFP) strains of zebrafish and egg collection protocols are described elsewhere (Incardona et al., 2004, Incardona et al., 2005). After collection and/or injection, the eggs were incubated at 28.5°C until 50% epiboly, at which time they were exposed to 12.5 μg/mL retene or DMSO (0.0006%, v/v). The exposures were carried out in glass Petri dishes (up to 50 embryos/dish) containing 40 mL of test solution. Retene exposures were run in triplicate, and all injected DMSO controls were run in duplicate. Due to time and egg number constraints, only 1 replicate of uninjected DMSO controls containing 45 eggs was conducted. We expect that this would not have an effect on our interpretation of our results, as all morpholino-injected DMSO controls showed no signs of toxicity. Furthermore, no toxicity has been observed in previous DMSO exposures at similar concentrations (data not shown).
5.2.3 Morpholino knockdown

Antisense morpholino oligonucleotides (Gene Tools, Philomath, OR) were used to sterically block the translation of AhR2 and CYP1A mRNAs, thus knocking down the gene products. The morpholino oligonucleotide sequences were as follows: AhR2 (ahr2-MO), 5’-TGTACCGATACCCGCCGACATGGTT-3’; CYP1A (cyp1a-MO), 5’-TGGATACTTTCCAGTTCTCAGCTCTCTC-3’; and generic standard control (std-MO), 5’-CCTCTTACCTCAGTTACAATTATA-3’. The eggs were injected as described by Incardona et al. (2006) and were maintained at 28.5°C until observational analysis.

5.2.4 Stereo- and confocal microscopy

Embryos were mounted in 3% methylcellulose and 24, 36, 48, and measured at 72 hpf. Digital stills and videos were obtained as previously described (Incardona et al., 2006). CYP1A immunofluorescence (at 72 hpf) and endothelial fluorescence of fli1-EGFP embryos (at 52 hpf) were imaged using a Zeiss LSM 5 confocal microscope.

5.2.5 Histology

At 72 hpf, zebrafish embryos were fixed in 4% paraformaldehyde, washed in PBT (phosphate buffered saline, 0.2-0.5% Triton X-100) and dehydrated in a graded series of ethanol concentrations at 10 min intervals at room temperature. The embryos were placed in propylene oxide (2 x 10 min), followed by an embedment in Epon-Araldite. The specimens were sliced at 500 nm using an RMC MT6000 ultramicrotome. The sections were thermally-adhered to a glass slide and stained with 1% filtered toluidine blue.
The sections were viewed with an Olympus BX51 microscope and photographs were digitally captured using an Olympus QColor 5 camera and QCapture Pro software (Ver. 5.1.1.14, Media Cybernetics, Inc., Bethesda, MD). The cross-sectional size of atrial walls, ventricles, and brain were measured in pixels by outlining the boundaries of the tissues using Image J software (U.S. National Institute of Health, Bethesda, MD). The atrial wall thickness was calculated by subtracting the lumen area from the area measured at the outer boundary of the atrium (i.e., myocardial layer).

5.2.6 Statistics

The prevalence and severity of pericardial edema were analyzed using a two-way analysis of variance, followed by a Student-Newman-Keuls multiple pairwise comparison. A t-test was used to analyze heart chamber and brain size measurements. All statistical analyses were performed using SigmaStat Ver. 1.0 (Jandel Scientific, Chicago, IL; P < 0.05).

5.3 Results

5.3.1 Retene toxicity

Retene embryotoxicity was first observed at 36 hpf, with pericardial edema, reduction in blood flow, and reduced erythrocyte number as the proximate signs of toxicity (Fig. 1) {Supplemental videos: embryos_36hpf.mov and bloodflow_36hpf.mov}. In some retene-exposed embryos, the ventricles appeared smaller {Supplemental video: ventricle_36hpf.mov}, and a pooling of blood cells occurred in the common cardinal vein at 36 hpf, which suggests the
Figure 5-1. Embryotoxicity at 36 hpf in zebrafish embryos exposed to retene and DMSO (0.006%, v/v). See also Supplemental video: embryos_36hpf.mov and bloodflow_36hpf.mov. Scale bar = 0.1 mm.

At 72 hpf, there was a significant difference in the prevalence and severity of pericardial edema in retene-exposed embryos (P < 0.001; Fig. 3). Retene-induced craniofacial deformities and intracranial hemorrhaging were also observed. In addition, retene exposures produced circumferential atrial edema, i.e., a significant increase in extracellular space between the atrial myocardium and endothelium (P = 0.003; Fig. 4a,b). There was no significant difference in the atrial lumen area between control (DMSO) and retene-exposed zebrafish embryos; however, there was roughly a two-fold distension in the myocardium in retene-exposed atriums, which was likely edema-induced. There was no measurable difference in the cross-sectional diameter of ventricles; however, in some embryos, retene exposures caused an apparent decrease in both ventricle and bulbus arteriosus size (Fig. 2c,d), which appeared to impair blood flow {Supplemental video: heart_72hpf.mov}. The timing and sequence of the chamber effects...
Figure 5-2. Cardiovascular effects of retene and DMSO (0.006%, v/v) exposures in zebrafish embryos. (a,b) 52 hpf, a delay in vascular development as evidenced by a lack of parachordal vessels (PAV) connecting the intersegmental vessels (Se) in retene-exposed embryos. (c,d) 72 hpf, a reduction in bulbus arteriosus and ventricular size in retene-exposed embryos. Images: (a), (b) tail segments of Tg(fli1-EGFP) embryos; (c), (d), ventral view of heart region of nacre embryos. Scale bar = 0.1 mm.
Figure 5-3. Zebafish embryotoxicity as described by (a) prevalence and (b) severity of pericardial edema. The data represent the (a) mean percent of embryos displaying edema and (b) mean pericardial area ± SE (N = 1-3 replicates, 19-50 embryos per replicate).
Figure 5-4. Atrium wall thickness, erythrocyte morphology, and girth of retene- and DMSO (0.006%, v/v)-exposed zebrafish embryos at 72 hpf. (a,b) Increased atrial wall thickness and dissimilar erythrocyte morphology in retene-exposed embryos (N = 5). Arrowhead, atrial wall; arrow, erythrocyte. (c,d) Cross-section of zebrafish embryos illustrating the increased girth and brain size in retene-exposed embryos (N = 5). Scale bar = 0.1 mm.
observed is unclear. Furthermore, there was evidence for a disruption in erythropoiesis at 72 hpf. In retene-exposed embryos, only round primitive erythrocytes were apparent, in contrast to the more flattened, ovoid definitive erythrocytes observed in control embryos (Fig 4a,b).

The cross-sections of retene-exposed embryos were noticeably larger in girth than controls (Fig 4c,d). It was not possible to measure the girth of the embryos directly. However, the circumference of the brains, a clearly defined structure, was 1.6-fold larger in retene-exposed embryos than in controls (P = 0.021).

5.3.2 Role of AhR2 and CYP1A

Blockade of AhR2 by ahr2-MO knockdown prevented retene toxicity in zebrafish embryos (Fig. 5h). The prevalence and severity of pericardial edema in AhR2 morphants were significantly lower than those of uninjected embryos exposed to retene (P < 0.001), and were not different from those of control embryos (Fig. 3, 5). Conversely, cyp1a-MO knockdown did not alleviate toxicity in retene-exposed embryos (Fig. 3, 5g), as there was no significant difference in toxicity between CYP1A morphants and uninjected embryos exposed to retene. As expected, the generic standard control morpholinos did not influence toxicity and there was no significant toxicity observed in all DMSO controls (Fig. 3, 5a-d). Thus, retene toxicity is mediated by an AhR2-dependent and CYP1A-independent mechanism.

CYP1A immunofluorescence confirmed the effectiveness of the ahr2- and cyp1a-MOs. There was widespread CYP1A expression in uninjected embryos exposed to retene (Fig. 6a), which was considerably reduced in CYP1A morphants (Fig. 6b). In AhR2 morphants, the ubiquitous expression of CYP1A was reduced; however, CYP1A expression was still observed in the vasculature and eye (Fig. 6c).
Figure 5-5. Effects of retene and DMSO (0.006%, v/v) in uninjected and morpholino-injected zebrafish at 72 hpf. The zebrafish shown are representative of the toxicity observed in each treatment. PE, pericardial edema; JM, jaw malformation. Scale bar = 0.1 mm.
Figure 5-6. CYP1A immunofluorescence in uninjected, *cyp1a*-MO- and *ahr2*-MO-injected zebrafish embryos exposed to retene. Scale bar = 0.1 mm.
5.4 Discussion

5.4.1 Retene embryotoxicity

In agreement with Billiard et al. (1999), retene caused BSD in the embryo-larval stages of zebrafish. The proximate signs of toxicity (pericardial edema, reduced blood flow, and reduced erythrocyte number) were first observed at 36 hpf. Thus, the onset of toxicity occurred sometime between 24-36 hpf, but the primary observable sign remains undetermined.

Two of the proximate signs of toxicity are similar to those observed in TCDD exposures; however, the timing of onset differs. In TCDD-exposed zebrafish embryos, a reduction in blood flow is the first sign of toxicity, but is not observed until ≥ 50 hpf (Dong et al., 2002). TCDD-induced pericardial edema typically does not present until 72 hpf (Belair et al., 2001, Dong et al., 2002; Prasch et al., 2003, Antkiewicz et al., 2005). The first measurable changes after TCDD exposure were a decrease in cardiomyocyte number at 48 hpf (Antkiewicz et al., 2005), which could have lead to a decrease in cardiac output and, subsequently, the observed reduction in blood flow. In the present study, direct cardiac output measurements were not taken; however, some retene-exposed embryos displayed a noticeably smaller ventricular size at 36 hpf {Supplemental video: ventricle_36hpf.mov}. It is uncertain whether the apparent reduction in size is due to an inhibited development or a decreased ventricular filling due to a reduced blood pressure. In addition, a reduction in blood flow could alter the intracardial hemodynamic forces necessary for normal cardiac development (Hove et al., 2003). Specifically, an altered cardiac flow impaired the development of the bulbus arteriosus in zebrafish (Hove et al., 2003), which may, in part, explain the small bulbus arteriosus observed in the retene-exposed embryos at 72 hpf (Fig. 2d) {Supplemental video: heart_72hpf.mov}. 
The proximate signs of toxicity could also result from an effect on the development or function of the peripheral vasculature. Indeed, evidence for a delay in angiogenesis of the tail vasculature was observed in retene-exposed embryos at 52 hpf (Fig. 2b); however, no measurements were taken at earlier times. Altered or inhibited angiogenesis have been previously observed in TCDD toxicity in the embryo-larval stages of fish (Bello et al. 2004; Hornung et al., 1999). In zebrafish, the effects on vasculature development are likely not secondary to altered blood flow or erythropoiesis, as the resulting hemodynamic changes do not substantially affect the formation of the vasculature (Paw and Zon, 2000; Weinstein et al. 1996).

The atrial edema and increase in girth size are notably different signs of end-stage cardiac morphology than those observed in TCDD-exposed zebrafish embryos (Fig. 4). TCDD impairs cardiac looping and regression of the common cardinal vein, which results in a tube-like atrium after 72 hpf (Bello et al. 2004; Antkiewicz et al. 2005). The atrial walls were significantly distended in retene-exposed embryos, which should cause a decreased cardiac output. A decreased output could lead to cardiac edema (i.e., fluid accumulation in the body due to cardiac failure), which may, in part, explain the increased girth of the retene-exposed embryos. The increased girth is presumably a result of excess tissue hydration, as opposed to an increased rate of development. It could result from an increased vascular permeability via a direct insult on the vascular endothelium (e.g., apoptosis), an altered osmoregulation, and/or altered cardiovascular homeostasis (e.g., hypertensive response). Interestingly, changes in vascular permeability have been observed after TCDD exposures. TCDD caused a slight increase in interendothelial spaces in lake trout embryos (Guiney et al., 2000), and apoptosis-induced permeability in medaka embryos (Cantrell et al., 1996).

The evidence for a disruption in erythropoiesis in retene-exposed zebrafish embryos (Fig. 4a,b) is a particularly interesting finding. More direct assessment of erythrocyte morphology is
needed, as the retene-induced erythrocyte shape could also result from cell swelling. However, the general shape of the erythrocytes and reduced erythrocyte number is consistent with that reported in TCDD-induced disruption of definitive stage erythropoiesis (Belair et al., 2001).

Erythropoiesis occurs in two distinct phases in zebrafish, primitive and definitive, which differ both temporally and spatially (see Orkin and Zon, 1997). In addition to the apparent lack of definitive erythrocytes, there was evidence of a decreased number of primitive erythrocytes at 36 hpf in retene-exposed zebrafish embryos {Supplemental video: bloodflow_36hpf.mov}. Whether the lack of erythrocytes is due to a disruption of erythropoiesis or impaired circulation (e.g., pooling of blood cells in the common cardinal vein) is unclear.

Interestingly, the findings of the present study, together with those reported in TCDD exposures, may highlight the need to understand the potential role of erythropoietin (EPO) in toxicity. Erythropoietin is not only the major hematopoietic factor involved in the proliferation and differentiation of erythrocytes, but it plays an integral role in cardiac morphogenesis and angiogenesis (Wu et al., 1999; Kertesz et al., 2004). The phenotypic abnormalities of EPO and EPO receptor (EPOR) knockout mice are strikingly similar to the proximate signs of TCDD and retene toxicity. First, the deletion of Epo and EpoR prevents the initiation of the definitive stage of erythropoiesis, resulting in the absence of definitive erythrocytes (Lee et al., 2001), which has been reported in both TCDD (Belair et al., 2001) and retene exposures (present study; Fig. 4a,b). Second, ventricular hypoplasia is observed in Epo−/− and EpoR−/− mice (Wu et al. 1999), which is the earliest observed effect in TCDD-exposed zebrafish embryos (Antkiewicz et al., 2005). Wu et al. (1999) suggest that EPOR activation may initiate the release of a mitogenic factor involved in cardiomyocyte proliferation during cardiac development. Last, Kertesz et al. (2004) observed an inhibited angiogenesis (not vasculogenesis) in Epo and EpoR null mice during development. Notably, the formation of intersomitic vessels was affected in the knockout mice, which was
observed in the retene-exposed zebrafish embryos in the present study (Fig. 2b). Adding weight to the hypothesis is the finding that a XRE has been found upstream of the Epo gene (Chan et al. 1999); thus, its expression can be AhR-regulated. It is conceivable that: (1) an activation of Epo by AhR during erythropoiesis (i.e., when it is not expressed under normal conditions) could alter hematopoiesis; and/or (2) the activation of AhR could alter Epo expression by interfering with normal Epo-stimulating pathways.

5.4.2 AhR2-dependent and CYP1A-independent toxicity

The mechanism of retene toxicity is AhR2-mediated, but independent of CYP1A activation or activity (Fig. 5). These findings are consistent with those reported in TCDD (Prasch et al., 2003; Teroaka et al., 2003; Carney et al., 2004; Dong et al., 2004; Antkiewicz et al., 2006) and benz[a]anthracene (Incardona et al., 2006) exposures in zebrafish embryos; however, the primary overt signs of toxicity of TCDD (see above) and benz[a]anthracene differ from that of retene. The cardiotoxicity of benz[a]anthracene is similar to that of TCDD (i.e., altered cardiac looping, reduced heart chambers; Incardona et al., 2006). The apparent differences in AhR2-mediated etiologies are difficult to explain. An obvious explanation would be that the differing toxicities may result from different AhR2-mediated effects, and reflect the pleiotropic effects of AhR2 activation. However, it is difficult to understand the toxicities when considering the chemical structure, properties, and pharmacokinetics of the compounds. For example, given that TCDD is more lipophilic and has a higher affinity for AhR than retene, it would be reasonable to assume that TCDD would elicit an AhR2-mediated effect at an earlier time during development than retene. Thus, the findings in the present study suggest that the differences in toxicities cannot be explained by pharmacokinetics alone.
Furthermore, the toxicity of retene differs from that of other tri- and tetracyclic PAHs (Incardona et al., 2004; Incardona et al., 2005). Notably, phenanthrene (unalkylated retene) exposures produce signs characteristic of atrio-ventricular conduction block independent of AhR2 (Incardona et al., 2004), illustrating that the addition of alkyl groups can dramatically alter embryotoxicity.

It is important to note that a CYP1A-independent effect does not rule out the involvement of the byproducts of metabolism (i.e., ROS, reactive metabolites), which have been implicated in the mechanism of retene toxicity (Hawkins et al., 2002; Bauder et al., 2004; Hodson et al. 2007). In addition to CYP1A, the AhR regulates the expression of other CYP enzymes, including CYP1B1, 1C1, and 1C2 (Jönsson et al., 2007), which may contribute to retene toxicity.

5.4.3 Evidence for an additional functional AhR isoform

Karchner et al. (2005) were the first to report a second functional AhR isoform, AhR1B. There were no developmental abnormalities observed in AhR2 morphants (Karchner et al., 2005; present study), suggesting that AhR1B may be the AhR isoform involved in development and controlling the constitutive expression of cyp1a. In retene-exposed zebrafish embryos, CYP1A was detected in the vasculature and eye of AhR2 morphants (Fig. 6c). The finding confirms the existence of a second functional AhR isoform involved in a xenobiotic response, and may be the first in vivo evidence of AhR1B.

In conclusion, the mechanism of retene toxicity is AhR2-mediated and CYP1A-independent, with the cardiovascular system as the primary target tissue. Further, more direct research is needed to investigate: (i) the specific role of AhR2 in retene-induced cardiovascular toxicity; (ii) the nature of the AhR2-mediated pathological differences between retene and TCDD toxicity; and (iii) the role of other functional AhR and CYP isoforms in xenobiotic toxicity.
5.5 Acknowledgements

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Supplemental videos

All supplemental videos are QuickTime movie files (.mov) showing DMSO control embryos in the first half (1-5 sec), and retene-exposed embryos in the second half (5-10 sec) of the videos.

embryos_36hpf.mov. Retene and DMSO-exposed zebrafish embryos at 36 hpf. DMSO control embryos showed no signs of toxicity (1-5 sec). Retene exposures caused pericardial edema and a decrease in blood flow (5-10 sec). Blood flow can be observed in the common cardinal vein which traverses the yolk sac.

bloodflow_36hpf.mov. Blood flood in the tail vasculature of retene and DMSO-exposed zebrafish embryos at 36 hpf. When compared to DMSO control embryos (1-5 sec), blood flow was significantly reduced in embryos exposed to retene (5-10 sec). Vessels shown: dorsal aorta (top) and posterial cardinal vein (bottom).

ventricle_36hpf.mov. Ventricles of retene and DMSO-exposed embryos at 36 hpf. When compared to DMSO control embryos (1-5 sec), the ventricles of retene-exposed embryos appeared reduced in size (5-10 sec).

heart_72hpf.mov. Heart chamber size and function in retene- DMSO-exposed zebrafish embryos at 72 hpf. When compared to DMSO contol embryos (1-5 sec), there was an apparent
reduction in bulbus arteriosus and ventricle size in retene-exposed embryos (5-10 sec). Chambers (right to left): bulbus arteriosus, ventricle, atrium.

5.6 References


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Chapter 6
General Discussion and Summary

6.1 Overview

Alkylphenanthrenes such as retene (7-isopropyl-1-methylphenanthrene) are aquatic contaminants commonly found in anthropogenically-, industrially-, and petroleum-contaminated environments, and have been implicated in crude oil toxicity (Khan, 2008). In the early life stages of fish, exposures to alkylphenanthrenes produce signs of toxicity (i.e., blue sac disease; BSD) typical of those observed in exposures to halogenated aromatic hydrocarbons (HAHs), particularly to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Billiard et al., 1999; Turcotte, 2008). TCDD is the most toxic congener, and as such, serves as the basis of the current mechanism-based risk assessment models of HAH and polycyclic aromatic hydrocarbon (PAH) mixture toxicity (Safe, 1990). The model assumes that congeners that produce TCDD-like toxicity share a common mode of action and act additively. The mechanism of TCDD-like toxicity is assumed to be mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that regulates genes involved in xenobiotic response (e.g. cytochrome P450 1A enzymes; CYP1A) and in normal development (Hahn, 1998; Schmidt and Bradfield, 1996). CYP1A enzymes are not involved in the mechanism of TCDD cardiotoxicity in zebrafish (Carney et al., 2004), likely due its chemical stability and resistance to metabolism.

Alkylphenanthrenes toxic to the early life stages of fish are AhR ligands (Turcotte, 2008) and, in contrast to TCDD, are readily metabolized by CYP1A enzymes (Tabash, 2001). The metabolic products (i.e., reactive metabolites and/or reactive oxygen species; ROS) have been implicated in retene toxicity (Hawkins et al., 2002; Bauder et al., 2005; Hodson et al., 2007). However, the target tissue of retene and the direct roles of AhR and CYP1A in retene toxicity are unknown, but are expected to be similar to those of TCDD. Thus, the following null hypotheses
were tested in this thesis: (1) the proximate signs of retene toxicity are not similar to those of TCDD (i.e., cardiovascular dysfunction, edema); (2) the primary target of retene toxicity is not the cardiovascular system; and (3) the mechanism of retene toxicity is not mediated by AhR2 or (4) CYP1A enzymes.

6.2 Interpretation of findings

The research presented rejected 3 of 4 null hypotheses. The null hypothesis not rejected was: (4) the mechanism of retene toxicity is not mediated by CYP1A enzymes. The interpretation and significance of the findings are discussed in detail herein.

6.2.1 Cardiovascular system is the target of retene toxicity

Collectively, the signs of BSD are a description of end-stage toxicity, which are relatively general toxic responses in the early life stages of fish (see Appendix 1) and could result from a number of mechanisms. Thus, identifying the proximate overt signs of toxicity is the logical place to begin investigating the potential underlying mechanisms of retene toxicity.

Similar to TCDD, the results presented suggest that the primary target of retene is the cardiovascular system. In medaka (*Oryzias latipes*) embryos, the proximate overt sign of retene toxicity was localized pericardial edema, which presented in the late stages of embryonic and cardiovascular development (7 days post-fertilization; dpf) and before a reduction in blood flow. Thus, retene did not affect cardiac development, i.e., chamber formation and/or cardiac looping. Histological analyses of cardiac tissue just prior to the onset of pericardial edema showed no overt differences from controls, which further supports a non-developmental effect and suggests that toxicity may result from a direct insult (e.g., oxidative damage) on cardiac tissue. In
zebrafish (*Danio rerio*) embryos, the proximate overt signs of toxicity were a reduction in blood flow and pericardial edema at 36 hours post-fertilization (hpf).

Although the target of retene appears to be similar to that of TCDD, there were differences in both the time of onset and proximate signs of toxicity. In medaka, TCDD embryotoxicity was associated with an apoptotic-induced cell death in the yolk vasculature prior to 3-5 dpf (stage 26, 2-3 dpf; Cantrell *et al*., 1996), which likely resulted in the overt signs of BSD at 5 dpf (Wisk and Cooper, 1990). In zebrafish embryos, the proximate signs of retene toxicity were similar to those of TCDD, but the time of onset differed. In TCDD-exposed zebrafish embryos, the overt signs of toxicity (i.e., reduction in blood flow, pericardial edema) were first presented between 50-72 hpf (Belair *et al*., 2001; Dong *et al*., 2002; Prasch *et al*., 2003; Antkiewicz *et al*., 2005). Thus, the time of onset of toxicity between retene and TCDD differ between fish species, i.e., the onset of retene toxicity appears prior to TCDD in zebrafish, but after TCDD in medaka.

The expected sequence of the first signs of BSD would be that observed in medaka. TCDD is more lipophilic (i.e., has a greater amount of exposure) and has a higher affinity for AhR than retene (Billiard *et al*., 2002), and as result, should produce its AhR-mediated toxicity (discussed below) earlier. Thus, in zebrafish, the differences in toxicity cannot be explained by exposure and receptor activation alone. This finding may be, in part, explained by ontological or physiological differences between the species. The finding may highlight the inability to extrapolate between fish species in the risk assessment of HAH and/or PAH mixtures (discussed below).

Notably, retene toxicity differed from that of phenanthrene (unalkylated retene), suggesting that alkyl-substitutions can greatly influence the mechanism of toxicity. No arrhythmias were observed in retene-exposed medaka or zebrafish, which is in contrast to the
atrio-ventricular (A-V) conduction block observed in phenanthrene-exposed zebrafish (Incardona et al., 2004).

6.2.2 The mechanism of retene toxicity is AhR-mediated

Antisense morpholino oligonucleotide knockdown of AhR2, the presumed major functional AhR isoform, prevented the signs of BSD in retene-exposed zebrafish embryos, confirming the role of AhR2 in retene toxicity. The finding suggests that the mechanism of retene toxicity is similar to that of TCDD (Prasch et al., 2003; Dong et al., 2004), and that retene fits the major criterion of the current risk assessment model (Safe, 1990; Van der Berg et al., 1998). The differences between retene and TCDD toxicity suggest alternate AhR2-mediated effects.

AhR2-mediated toxicity in the embryonic fish has been observed in exposures to individual (e.g., benz[a]anthracene; Incardona et al., 2006) and mixtures of PAHs (Billiard et al., 2006). However, the A-V conduction block observed in phenanthrene-exposed zebrafish embryos was independent of AhR2, providing further evidence that: (i) alkyl-substitutions can drastically alter toxicity; and (ii) that the differences in toxicity may be directly related to AhR activation. Phenanthrene has no measurable ability to activate AhR (Billiard et al., 2002).

AhR mediated toxicity could result from: (1) a direct tissue insult as a consequence of the xenobiotic response (i.e., oxidative stress via the byproducts of metabolism); and/or (2) a deregulation of normal AhR-regulated physiological processes. There is evidence suggesting the involvement of reactive metabolites and ROS in retene toxicity (Hawkins et al., 2002; Bauder et al., 2005; Hodson et al., 2007); however, the contribution of a disrupted regulation of AhR-responsive genes is unclear. Furthermore, the potential downstream effects of AhR activation on other non-AhR-regulated genes remain uncertain. If the mechanism of retene toxicity is similar
to TCDD, the current mechanistic understanding of TCDD toxicity (Goldstone and Stegeman, 2006; summarized in Fig. 6-1) may provide insights for further directions for investigating the AhR-mediated cardiovascular toxicity of retene.

AhR biology is complex, particularly in fish with the presence of multiple AhR isoforms (Abnet et al., 1999; Incardona et al., 2006), which likely contributes to differences in toxic effects among species for a given compound (discussed below), and between compounds within a species (e.g., retene vs. TCDD; discussed above). The difference could, in theory, result from: (1) differences in structure and ligand affinity between species-specific AhR isoforms (e.g., AhR1s and AhR2 in zebrafish vs. AhR2α and AhRβ in rainbow trout); (2) species-specific AhR expression patterns during development, either in relative timing of AhR inducibility or tissue specific expression of AhR isoforms; and/or (3) different signaling cascades in different species and/or tissues (Kanzawa et al., 2004). Kanzawa et al. (2004) observed the same AhR expression in the liver and heart of TCDD-exposed embryonic chickens, but CYP1A was only induced in liver tissues. Notably, a second functional AhR isoform (AhR1b?; Karchner et al., 2005) was observed in retene-exposed AhR2 morphants (Chapter 5). Thus, to fully understand the mechanism of retene and HAH toxicity, it is imperative to understand the intricacies of the AhR.

Adding to the complexity of understanding the role of AhR in toxicity is the potential for cross-talk with other dimeric partners of the AhR nuclear translocator (Arnt; Chan et al., 1999; Prasch et al., 2004). The AhR belongs to a basic Helix-Loop-Helix PAS family of proteins, which also includes the hypoxia-inducible factor alpha (HIF-α). AhR and HIFα both require dimerization with Arnt (also referred to as HIFβ) to initiate the expression of their responsive genes. It has been suggested that AhR and HIFα can compete for Arnt, resulting in an altered response cascade of one or both transcription factor(s) (Chan et al., 1999). Thus, AhR activation can effectively reduce the hypoxia response, including the expression of prioangiogenic growth
Figure 6-1. Insights from the AhR-mediated cardiovascular toxicity of TCDD in the early life stages of fish (summarized in Goldstone and Stegeman, 2006). HIFα, hypoxia inducible factor alpha; ROS, reactive oxygen species; CYP, cytochrome P450 enzyme; EETs, epoxyeicosatrienoic acids; NADPH, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate.
factors and erythropoietin, mediated by HIFα. However, there is insufficient evidence that the cross-talk between these factors contributes to TCDD toxicity (Prasch et al., 2004; Handley-Goldstone et al., 2005).

6.2.3 The mechanism of retene toxicity is independent of CYP1A enzymes

The involvement of CYP1A has been suggested in PAH exposures (e.g. pyrene; Incardona et al., 2006). CYP1A metabolism can contribute to toxicity via the production of reactive metabolite intermediates and ROS, which can result in adduct formations and oxidative stress, respectively, and an alteration of cellular function (Di Giulio et al., 1995). The early life stages of fish are particularly susceptible to oxidative damage, as they have a limited supply of maternally-derived antioxidants. Indeed, the byproducts of CYP1A metabolism have been implicated in retene toxicity (Hawkins et al., 2002; Bauder et al., 2005; Hodson et al., 2007).

However, a morpholino-induced blockade of CYP1A did not alleviate toxicity in retene-exposed zebrafish embryos, suggesting that the major CYP enzyme induced by AhR activation (Goldstone and Stegeman, 2006; Timme-Laragy et al., 2007) confers protection in retene exposures. Furthermore, larval toxicity increased with a concomitant decrease in ethoxyresorufin-O-deethylase (EROD) activity (in juvenile livers) in rainbow trout (Oncorhynchus mykiss) co-exposed to retene and 2-aminoanthracene (2AA) or α-naphthoflavone (ANF), suggesting that BSD may be produced through a CYP1A-independent mechanism. The parallel concentration-response curves of 2AA and retene + 2AA may infer a common, i.e. CYP1A-independent, mechanism of toxicity for retene in rainbow trout.

It is worth noting that the CYP1A independence observed may not contradict the previously suggested involvement of the byproducts of retene metabolism (Hawkins et al., 2002;
Bauder et al., 2005; Hodson et al., 2007). In addition to CYP1A, AhR activation can lead to exposure-dependent increases in other CYP enzymes, including CYP1B1, 1C1, and 1C2 (Wang et al., 2006; Jönsson et al., 2007; Timme-Laragy et al., 2007), which could contribute to toxicity. Since the mechanism of retene toxicity is AhR2-dependent, further studies are needed to address the potential involvement of these CYP enzymes. Furthermore, other AhR-mediated, CYP1A-independent inputs of ROS have been proposed in TCDD-like toxicity (e.g., mitochondria; Senft et al., 2002; Golstone and Stegeman, 2006).

### 6.2.4 Variably toxicity of mixtures with CYP1A inducers and inhibitors

PAHs are invariably found in complex mixtures, likely containing both inducers and inhibitors of CYP1A enzymes, and/or agonists and antagonists of AhR. The co-exposures of retene with two different CYP1A inhibitors, 2AA and ANF, resulted in drastically different toxicities. Co-treatment of rainbow trout larvae with retene + ANF resulted largely in a synergistic response, while the retene + 2AA co-exposures resulted in exposure-dependent curves characteristic of response addition (i.e., co-exposure curve is parallel but shifted to a lower concentration). Furthermore, there was evidence for multiple mechanisms of toxicity (synergism and response addition) in retene + ANF co-exposures.

The differences in toxicity observed in the co-exposures may be mediated by AhR. There were no measurable changes in CYP1A protein concentrations associated with the concentration-dependent decrease in EROD activity measured in the livers of juvenile trout exposed to retene + ANF, suggesting that ANF was directly inhibiting CYP1A. Conversely, there was a concomitant decrease in CYP1A protein concentrations with the decrease in EROD activity in larvae co-treated with retene and 2AA, suggesting 2AA was antagonizing AhR.
activation. These findings suggest that the synergistic toxicity observed in retene + ANF co-exposures may be AhR-mediated, while the retene + 2AA toxicity may be AhR-independent.

The synergistic response observed in retene + ANF co-exposures was consistent with the mixture toxicity of other CYP1A inducers and inhibitors in Fundulus heteroclitus embryos (Wassenberg and Di Giulio, 2004). In addition, a CYP1A-independent mechanism of mixture toxicity has been observed in other PAH mixtures. In zebrafish embryos, the mixture toxicity of two PAHs, β-naphthoflavone (BNF, CYP1A inducer) and ANF, was reduced after knockdown of AhR2, but was markedly enhanced after a knockdown of CYP1A (Billiard et al., 2006).

6.2.5 Species differences in retene toxicity?

Comparing retene toxicity among three fish species used in this thesis research has raised the concern over whether toxicity data can be extrapolated among fish species. In addition, the aforementioned differences (i.e., opposing sequence of onsets) between retene and TCDD toxicity in zebrafish and medaka embryos provides further evidence of potential problems with cross-species comparisons.

The question of species differences in retene toxicity primarily arose from the observed proximate signs and progression of toxicity, and from ontological and anatomical differences among the species. In retene-exposed zebrafish and medaka embryos, an early sign of BSD was pericardial edema; however, in medaka embryos pericardial edema was not associated with a decrease in blood flow or altered heart morphology. The onset of pericardial edema occurred in the late stages of cardiac development, and a reduction in blood flow resulted from the distension of the pericardium, which pinched the yolk sac vasculature at the sinus venosus in retene-exposed medaka embryos. Furthermore, the tubular heart structure observed in end-stage retene toxicity appears to be due to edema-induced mechanical stretching in medaka embryo (Fig. 2-3 in Chapter 2).
2). The retene exposures in zebrafish presented in this thesis were not carried out long enough to observe the tube heart; however, in TCDD exposures, the tube heart morphology results from an inhibited regression of the common cardinal vein (yolk sac vein; Bello et al., 2004). Thus, the observed differences likely result from the anatomical differences of the embryonic yolk sac vasculature between the species (Fig. 6-2). In zebrafish, there is only one large vessel that traverses the yolk sac (common cardinal vein), which remodels and migrates dorsally post-hatch (72-96 hpf; Isogai et al., 2001). Medaka embryos have three extraembryonic yolk sac vessels (two ducts of Cuvier and a vitello-caudal vein) that enter the embryo at a fixed point of the pericardial sac. This fixed point anchors the heart to the front of the pericardiac sac, making it susceptible to edema-induced cardiac stretching and stenosis in the yolk vasculature.

Retene toxicity in rainbow trout is overtly different than that observed in zebrafish and medaka embryos, which likely results from differing times of exposure and patterns of the yolk sac vasculature (Fig. 6-2). The relatively thick chorion of rainbow trout eggs largely prevents retene exposure until hatch (Brinkworth et al., 2001), when a relatively mature heart and an extensive network of yolk sac vasculature has formed (Isogai and Horiguchi, 1997). While the specific effects of retene on the heart and yolk sac vasculature were not studied, TCDD-exposures in rainbow trout resulted in: (i) a decrease in the complexity (i.e., branching points) of the yolk vasculature attributed to an inhibited angiogenesis and not apoptosis; and (ii) an arrested heart development (Hornung et al., 1999). The proximate and most prominent sign of BSD in retene-exposed trout larvae was yolk sac edema, in contrast to the pericardial edema observed in zebrafish and medaka embryos.

The concentration-dependent increase in 2AA toxicity observed in rainbow trout larvae provides further evidence of species-specific effects. The rationale for using 2AA for CYP1A inhibition to understand retene toxicity was based on the absence of toxicity in 2AA-exposed
Figure 6-2. Differences in the yolk sac vasculature and retene toxicity in the early life stages of 3 fish species: (a,b) zebrafish, (c,d) Japanese medaka, and (e,f) rainbow trout. CCV, common cardinal vein (not defined); CF, craniofacial deformities; H, heart; PE, pericardial edema; VCV, vitello-caudal vein; DC, ducts of Cuvier; YE, yolk sac edema; VV, extensive network of vitelline vasculature. Scale bar = 1.0 mm.
*Fundulus heteroclitus* embryos (Wassenberg and Di Giulio, 2004). Species differences in phenanthrene toxicity between rainbow trout larvae (narcosis; Hawkins *et al*., 2002) and zebrafish embryos (A-V conduction block; Incardona *et al*., 2004) have also been observed.

### 6.3 Significance of findings

Understanding the targets and toxic mechanisms of retene were the primary research objectives of this thesis. However, the results of these studies were intended to provide insight into the appropriateness and/or effectiveness of including alkylphenanthrenes in the current risk assessment model for TCDD-like compounds.

The current mechanism-based model calculates the risk of mixture toxicity of TCDD-like compounds by determining toxic equivalency factors/quantities (TEFs/TEQs) using TCDD as the reference compound (Safe, 1990; Van der Berg *et al*., 1998). The model assumes that TCDD-like compounds share a common mode of action, and their toxicities will be additive in mixtures.

The AhR-mediated, CYP1A-independent mechanism of retene cardiotoxicity in zebrafish is similar to that of TCDD, suggesting that alkylphenanthrenes can be included in the TEF/TEQ risk assessment model. However, the findings presented in this thesis may question the assumptions and effectiveness of this model. The findings suggest: (1) TCDD-like toxicity may result from multiple AhR-mediated effects; (2) extrapolating between or among fish species may be problematic; and (3) mixture toxicity of AhR agonists is not always additive.

In addition, the toxicity of PAHs with no AhR binding affinity can also be influenced by AhR agonists. In rainbow trout larvae, AhR activation by BNF enhanced the toxicity of phenanthrene and changed its mechanism from a narcotic-like effect to a CYP1A-mediated toxicity (Hawkins *et al*., 2002). Taken together, the variable mixture toxicities observed in PAH co-exposures (Chapters 3 and 4; Hawkins *et al*., 2002; Wassenberg and Di Giulio, 2004; Billiard
et al., 2006), suggest that the TEF/TEQ risk assessment model may be drastically oversimplified for PAH mixtures. Furthermore, the potential for mechanistic changes of compounds within a mixture may, in part, explain why exposures to weathered crude oil, which is rich in alkylphenanthrenes, was AhR2-independent in zebrafish embryos (Incardona et al., 2005).

Thus, although alkylphenanthrenes may qualify for TEF/TEQ risk assessment, the inherent problems highlighted with the model require further examination before conclusions can be made on the effectiveness of assessing the early life stage toxicity of other TCDD-like PAHs, and PAH and HAH mixtures.

6.4 Summary of findings

1. A primary target of retene toxicity in the early life stages of fish is the cardiovascular system.
2. Retene toxicity is stage-specific in medaka embryos.
3. The mechanism of retene toxicity is AhR2-mediated and independent of CYP1A enzymes.
4. Multiple EROD (CYP1A)-independent early life stage toxicities can result from exposures to different mixtures of CYP1A inducing (retene) and CYP1A inhibiting (ANF or 2AA) PAHs.
5. Multiple concentration-dependent mechanisms of early life stage toxicity (i.e., synergism and response addition) can occur in co-exposures of a CYP1A inducer (retene) with a range of CYP1A inhibitor (ANF) concentrations.
6. The toxicity of 2AA is species-specific. Although previously considered non-toxic to the early life stages of fish, 2AA exposure causes an exposure-dependent increase in toxicity in rainbow trout larvae.
7. Retene toxicity is mechanistically similar to that of TCDD toxicity. However, depending on the fish species, their toxicity differs in both proximate signs and the onset of BSD, suggesting the existence of multiple AhR-mediated effects.

8. Species differences in the early life stage toxicity of retene exist among medaka, zebrafish, and rainbow trout.

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Appendix A

A guide to scoring the signs of blue sac disease in the embryo-larval stages of Japanese medaka (*Oryzias latipes*)

Introduction

Our focus was to develop a descriptive and quantifiable scoring system for the signs of blue sac disease (BSD) that would allow us to systematically study retene toxicity in Japanese medaka (*Oryzias latipes*), which is presented in Chapter 2. In addition, the secondary objective of developing this scoring system was to: (1) reduce subjectivity and provide consistent and comparable data; (2) provide a functional, user-friendly method of scoring using both a visual and descriptive atlas of signs and severities; and (3) serve as a basis for developing standard scoring systems in other fish species. Furthermore, we wanted to develop a versatile method for both routine testing and mechanistic studies. For that reason, our system focuses on the cardiovascular system, which is believed to be the primary target of toxicity, and it provides criteria for what are likely to be secondary responses.

The hallmark signs of BSD include: pericardial and yolk sac edema, cardiovascular dysfunction (tube-heart formation, hemorrhaging, reduced blood flow), craniofacial and spinal deformities, and fin rot. What characterizes BSD is somewhat ill-defined. The number and appearance of the signs of BSD can vary among species for a given compound, and within species for different compounds. The signs are often collectively referred to as developmental abnormalities. The severities of the signs are typically scored on an arbitrary scale to create indices, making comparative estimates of chemical potencies limited to one observer. Hence, it is critical to have defined criteria to standardize the method of scoring developmental abnormalities.
Blue sac disease has been described in the early life stages of fish for over 130 years, and was aptly named from the buildup of a bluish serous fluid in the yolk sac. Although once thought of as an infectious disease (Atkinson, 1932), BSD has been linked to chemical exposures since the late 1950’s. In an effort to explain developmental effects seen in hatcheries, Wolf (1957) demonstrated that nitrogenous waste products including ammonia could produce BSD. The various signs of BSD have since been described in a variety fish species exposed to an array of environmentally-relevant contaminants including: pesticides (Solomon and Weis, 1979; Hamm and Hinton, 2000; González-Doncel et al., 2004); polychlorinated aromatic hydrocarbons (Wisk and Cooper, 1990; Walker and Peterson, 1991); aliphatic halogenated hydrocarbons (Spencer et al., 2002); polycarbonate plasticizers (Pastva et al., 2001); and polycyclic aromatic hydrocarbons (Billiard et al., 1999; Incardona et al., 2004; Rhodes et al., 2005).

Morphological scoring systems have been developed previously to investigate teratogenic compounds in various fish species, including Japanese medaka. Medaka have been established as a model organism for toxicological and teratological studies due to their clear chorion, well-described ontogeny (Iwamatsu, 2004; González-Doncel et al., 2005), and sensitivity to xenobiotics. Shi and Faustman (1989) described a scoring system in medaka to characterize teratogenesis using methylnitrosourea as a model compound. They systematically scored organogenesis using well-defined developmental parameters that allowed teratogenic effects to be quantified; however, there was no reference to common signs of toxicity including pericardial and yolk sac edemas.

There has also been severity indices proposed to quantify xenobiotic-induced developmental abnormalities. Villalobos et al. (2000) created an effective mathematical formula to calculate a severity index based on an improved scoring system; however, the scoring system was semi-quantitative at most. As with most of the present methods of scoring, the system was
based on ill-defined severities of the actual signs of toxicity. The index of severity of a particular (non-presence-absence) sign was usually based on arbitrary, minimum-maximum, or mild-severe scales. Effects have also been summarized as the prevalence or percentage of embryos displaying particular signs of toxicity (Spencer et al., 2002), which could be very misleading, as most signs have a wide range of phenotypic severities. Quantifying developmental abnormalities and calculating a severity index would be more effective and more comparable among species, test chemicals, and investigators, with more quantifiable measurements of the severity of the signs of toxicity.

**Methods**

Refer to Chapter 2. Tables 1 and 2 are examples of scoring charts and sample calculations of BSD and SI indices for embryos (9 days post-fertilization; dpf) and larvae (12 dpf), respectively.

**Results and Discussion**

The signs of BSD are a seemingly general response to a variety of environmental contaminants, which warrants the need to have a versatile, standardized scoring method that provides consistent data. Current methods are based on arbitrary minimum-maximum scales for scoring the severities of developmental abnormalities. Our method reduces this subjectivity by provide both visual and descriptive atlases of the various signs of toxicity, and also provides an improved analytical technique using a modified severity index. This scoring system has proven to be highly effective and efficient, producing relatively low standard deviation of EC50 values of combined replicates in retene-exposed medaka embryos (see Chapter 2). The time needed for a trained observer to accurately describe and record the developmental stage, stage of heart development, and signs of toxicity averages of 1-1.5 minutes per fish.
1. Scoring the signs of BSD

Pericardial edema (PE)

A build-up of edematous fluid within the pericardial cavity is one of the most prevalent sign of embryotoxicity in medaka and the first observable sign of retene toxicity. In the present model, the severity of PE is scored on a scale of 1 to 5, and should be observed from a lateral view, as other perspectives may be misleading. The first level of severity (PE 1) appears as a bubbling of the pericardial sac with edematous fluid surrounding the heart (Fig. 1a,b). It is important to note that a “bubbling” of the pericardial cavity can present itself in normal embryo; however, the fluid does not completely surround the heart. The key feature of PE 1 is fluid underneath the heart, creating a gap or space between the insertion point of the extraembryonic vessels (ducts of Cuvier, vitellocaudal vein) into the pericardial sac. In other words, the sinus venosus appears to be lifted off the yolk sac or off the bottom of the pericardial sac. As the severity increases, the edematous fluid extends underneath the head region creating a wedge-like pericardial sac from a lateral view. The remaining levels of severity of PE are distinguished by the volume that the pericardial “wedge” occupies relative to the combined volume of the pericardial and yolk sac (pericardial-yolk region). For PE 2, edema extends under the head region and the pericardial sac occupies about 1/4 of the pericardial-yolk region (Fig. 1c). In PE 3, PE 4, and PE 5, the edema-filled pericardial sac occupies 1/3, 1/2, and > 1/2 of the pericardial-yolk region, respectively (Fig. 1d-f). These relative contributions of edema still correspond to the severity of PE in larvae, although the wedge-like appearance changes when the embryo hatches and is not confined within the eggshell.
**Stretched heart (SH)**

Stretched hearts are commonly referred to as a tube, tube-like, or tubular hearts. We have changed the name to discriminate the post-looping cardiac stretching from tube hearts produced as abnormal cardiac development. In the early stages of embryonic development, a tubular heart forms and remains until the formation of the extraembryonic vessels and onset of blood flow (Fig. 2a). The heart then begins to form chambers and loops until the atrium is adjacent to the ventricle (Fig. 2b-e). Teratogens that target cardiac development can halt development during the early stages, preventing cardiac looping. For example, Spencer et al. (2002) reported an improper looping in medaka embryos exposed to 25 mg/L of trichloroethylene.

If cardiac looping occurs (stages 4 and 5; Fig. 2d,e), post-looping cardiac stretching can occur as a result of an edema-induced distension of the pericardial sac. The heart is anchored to the front of the pericardial cavity at the point at which the extraembryonic vessels enter into the sinus venosus; the opposite end is anchored where the outflow tract enters the body of the embryo. Stretching begins in PE 1, and at PE 2, the heart typically has regressed into stage 4 morphology (non-adjacent atrium and ventricle; Fig. 2f). This regression can only be determined if cardiac development is scored and the heart has fully developed. If a stage 5 heart is observed, the regression to stage 4 can be scored as SH 1. As PE increases, the heart is further stretched into what is commonly reported as a tube heart (SH 2 and 3 in the present scoring system; Fig. 2g,h). The name aptly describes the appearance of a tubular atrium, but fails to acknowledge the presence of a ventricle. SH 2 and 3 can be distinguished based on ventricle capacity and contraction, and atrium thickness. In an SH 2 heart, the atrium wall is moderately thick and contracts in a peristaltic motion. The ventricle is reduced in size, presumably due to a decreased lumen size resulting from a decrease in blood flow, and/or cardiac tamponade. An SH 3 atrium appears slightly thinner than SH 2; however, the defining difference is the ventricle, which
appears as a small, dense ball of cardiomyocytes. The ventricle typically appears to be in standstill, likely as a result of cessation of blood flow.

Heart rate

Heart rate measurements are also commonly taken, and alterations in rate have been observed in xenobiotic exposures. González-Doncel et al. (2004) report a 31% increase in mean heart rate in cypermethrin-exposed medaka embryos from 3 to 8 dpf when exposed from fertilization, and a 24% increase when exposed from stage 9 (late morula) of development. Irregular heart beats in medaka have also been observed in carabaryl, parathion, and malathion exposures (Solomon and Weis, 1979). Heart rate can be influenced by a variety of factors, which should be addressed when scoring. It is important to allow the embryos/larvae to acclimate to room temperature and recover from any handling stress. In addition, unanesthetised embryos/larvae should be viewed under the dimmest light setting of the stereomicroscope. Heart rate is typically scored over a 15 to 30 second time frame and reported as beats per minute. Heart rate measurements are effective at not only identifying bradycardia or tachycardia, but also provide an opportunity to detect atrio-ventricular (A-V) conduction block, which has been reported in zebrafish after an exposure to individual PAHs and PAH mixtures (Incardona et al., 2004, 2005). Incardona et al. (2004, 2005) observed both partial (2:1 atrial:ventricular contractions) and complete (ventricular standstill) A-V conduction block in zebrafish exposed to phenanthrene, dibenzothiophene, and weathered crude oil.

Reduced perfusion (RP)

A reduction in blood flow has been commonly observed as the first observable response to dioxin-like compounds in a variety of fish species (Chen and Cooper, 1999; Kim and Cooper,
1999; Hornung et al., 1999; Belair et al., 2001; Dong et al., 2002; Incardona et al., 2004). It remains the most subjective measurement in the present scoring system with no absolute values; the measurement can only be assessed relative to control embryos. Therefore, it is necessary to observe blood flow in water control embryos prior to scoring chemically-exposed embryos. Furthermore, it may not be an appropriate measurement in larvae, as it would require sedation or confinement to a small droplet of water to observe. For a more quantitative approach of measuring blood flow, perfusion rate can be measured using time-lapse digital videography as described by Teraoka et al. (2002).

Scoring reduced perfusion (RP) is a measurement of both flow velocity and red blood cell density. A reduction of blood flow could be a consequence of a reduced heart rate, hemorrhaging, and/or a disruption in erythropoiesis. An RP 1 score is a moderate reduction of blood flow when compared to controls, which is typically a drop in blood velocity, but not blood cell count. In general, RP 2 is associated with a dramatic reduction in both velocity and blood cell count. RP 3 is a complete cessation of blood flow. For the most reliable score, it is best to observe a lateral portion of the duct of Cuvier, which displays continuous blood flow; as opposed to a ventral portion of the vessel where blood flow can be influenced by the normal pulsatile movement of blood through the heart.

In retene-exposed medaka embryos, reduction in blood flow is likely a secondary response to PE. The distended pericardial sac appears to impede blood flow by constricting the opening into the sinus venosus, causing a build up of blood cells at the junction of the extraembryonic vessels. Furthermore, the distension of the pericardial cavity could, in theory, restrict blood flow by compressing the extraembryonic vessels. In affected embryos, RP severities are typically correlated with the respective severities of SH.
Yolk sac edema (YE)

Yolk sac edema is a common sign of toxicity in a variety of fish species. Among species, it can be located in different regions within the yolk sac, presumably based on the extent of yolk sac vasculature. In fish with an extensive network of yolk sac vasculature such as rainbow trout, the edema typically surrounds the yolk in the distal portion of the yolk sac. In medaka, with only 3 meandering yolk sac vessels, the edematous fluid is normally located between the embryo and yolk in the proximal portion of the yolk sac. This anatomical difference among species may give rise to differing mechanisms, etiologies, and BSD scores among species for the same compound.

YE severity in medaka embryos and larvae is scored based on the proportion of edema in the yolk cavity. In an affected fish, there are two observable cavities within the yolk sac region, the pericardial and yolk cavity (Fig. 3b). If the edema occupies up to 1/4 of the yolk cavity, a score of 1 is given (YE 1; Fig. 3c). In YE 2, edematous fluid fills roughly 2/3 to 1/2 of the yolk cavity (Fig. 3d), and in YE 3 it occupies greater than 1/2 of the volume. It is recommended that embryonic YE be scored in a lateral view of the right side of the embryo, where it is not masked by the liver and gall bladder (Fig. 4).

In retene-exposed medaka, YE is more prominent in larvae than embryos. A possible explanation for this is that the embryo is confined within the eggshell, which may provide sufficient pressure to prevent YE or keep it at bay. After hatch, this pressure is relieved and there is less restraint on the progression of YE. This factor could potentially introduce a slight bias if pre-hatch and post-hatch BSD severities are combined to produce a mean SI. In other words, when calculating the mean SI for a treatment, if there are both embryos and larvae within that treatment, combining the scores to produce a mean SI could introduce a bias, even if the scores are limited to only those signs that can be scored in both stages.
**Hemorrhaging**

Hemorrhaging in ocular and peripheral vasculature is a common sign associated with dioxin-like toxicity in various fish species (Cantrell et al., 1996, 1998; Guiney et al., 1997; Billiard et al., 1999; Hornung et al., 1999; Toomey et al., 2001; Brinkworth et al., 2003; Dong et al., 2004; Bauder et al., 2005). Embryonic vasculature in zebrafish and medaka is particularly sensitive to dioxin-like compounds. TCDD has been found to inhibit regression of the common cardinal vein in zebrafish, leading to altered heart morphogenesis (Bello et al., 2004). In addition, a decrease in blood flow and increased vascular permeability has been linked to TCDD-induced apoptosis in brain and yolk sac vasculature in medaka, zebrafish, and mummichogs (Cantrell et al., 1996, 1998; Toomey et al., 2001; Dong et al., 2004).

In medaka, hemorrhaging is often observed around the yolk sac vasculature (Fig. 3d), at the heart, and at the base of the tail in larvae. The site of a hemorrhage appears reddish in color, as blood pools in the interstitial tissues. In addition, the presence of blood cells is often observed.

**Spinal deformities (SD)**

A spinal deformity can be observed as a dorsal, ventral, or scoliotic curvature of the spine. The specific nature of the curvature should be recorded, as it could be related to different mechanisms. Incardona et al. (2004) associated dorsal curvature with an edema-induced distention of the neural tube in zebrafish after PAH exposure, which was not consistent with a structural or developmental abnormality of the spine. Dorsal curvatures have also been seen in PAH-exposed rainbow trout, which only presents itself in affected larvae with severe pericardial and yolk sac edema and a reduction in blood flow. Ventral curvatures have also been observed in both control and exposed trout, typically in larvae showing no other signs of toxicity, suggesting it is likely a random developmental/genetic abnormality. Spinal deformities are commonly observed post-
hatch; however, it can manifest itself in ovo in response to exposure to severely dysmorphogenic teratogens (e.g. α-naphthoflavone; Scott unpub.).

**Craniofacial deformities (CF)**

Craniofacial deformities is a collective term that includes: a blunt nose, jaw deformities and often the appearance of a protuberance on the top of the head (also referred to as a crowned skull; Fig. 3e). These malformations may be secondary to cardiovascular dysfunction and are affected in the latter stages of skeletal development of the head and jaw (Hornung et al., 1999; Incardona et al., 2004). In TCDD-exposed trout, the major structures contributing to the affected phenotypes were the skeletal structures that develop post-hatch, and the deformities paralleled a reduction in blood flow (Hornung et al., 1999).

**Fin rot (FR)**

Fin rot appears to have different manifestations in different fish species. In rainbow trout, fin rot is characterized by the absence or seemingly degraded, rayless caudal fin. In medaka, fin rot is typically defined by the appearance of uncharacteristic circles in the membranous portion of the tail and caudal fin, and absence of fin rays (Fig. 3f).

**2. Scoring development**

The stages of embryonic development in medaka have been well-described and illustrated (Iwamatsu, 2004). The early atlases provide a detailed description of medaka ontogeny; however, the 2-dimensional line drawings are limited in their use for an untrained observer. González-Doncel et al. (2005) created a practical tool for stage identification using a photographic atlas, which was used in the present study. The atlas is a user-friendly guide that
provides both pictures and descriptions of key features identifying various stages of medaka development. Using the developmental guide of González-Doncel et al. (2005) in conjunction with the present guide can provide valuable additional information for mechanistic studies.

3. Mechanistic vs. endpoint analysis

For preliminary mechanistic studies, it is recommended to score developmental stage using González-Doncel et al. (2005) to identify the primary sign(s)/proximate cause(s) of toxicity and their time of onset, which may identify target tissues. The timing of the onset of BSD can also provide valuable information to assess the potential involvement, mechanistically, of various processes (e.g. metabolism). For example, metabolic activation of various xenobiotics by cytochrome P450 1A enzymes (CYP1A) have been implicated in toxicity of a variety of compounds (Hamm and Hinton, 2002; Brinkworth et al., 2003; Rifkind, 2006). CYP1A becomes inducible and functional in medaka at 5 dpf (Wisk and Cooper, 1992), suggesting its involvement in chemically-induced signs of toxicity presenting themselves at or beyond 5 dpf. Identifying the nature of the onset and progression of the signs of BSD using this guide could provide valuable information for mechanistic comparisons among teratogens. In addition, knowing the onset can provide the basis for examining structural or ultrastructural changes prior to the observable signs of toxicity (e.g. edema).

This guide provides a fast, reliable, and efficient method of scoring various endpoints and at various times during development. For endpoint analyses, it is recommended that medaka larvae be scored at swim-up (12-14 dpf at 25°C) if interested strictly in the overall toxicity, as described by the viability of the fish. However, if toxicity is to be attributed to BSD (or other diseases or syndromes), it is recommended that embryos be scored at an embryonic stage that is displaying signs of BSD with little to no mortality. It is difficult to link mortality with BSD when mortality
confounds the identification of fish with pathology. Assessing BSD at the embryonic stage will add weight to the conclusion.

Our scoring system reduces subjectivity by providing defined criteria in place of arbitrary scales of severity when scoring signs of toxicity in medaka. With the scoring system and severity index, it is now possible to compare embryo-larval toxicities among compounds and observers. This will provide valuable information for understanding mechanisms and developing quantitative structure-activity relationships. The shortfalls of the method arise from the inability to score the same number of signs of toxicity in ovo, as compared to the larval stages. During some days of development, there are both embryos and larvae in a particular treatment, which are scored based on a different number of signs. In ovo, craniofacial and spinal deformities, eye edema, fin rot, hemorrhaging on the body, failure of swim bladder formation, and no movement cannot be scored accurately. Therefore, the maximum BSD score differs between embryos and larvae, which can make statistical comparisons among treatments difficult at both stages of development. That aside, our scoring system effectively provides consistent data, and a basis for developing standard scoring methods in other fish species.

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Table 1. Sample scoring chart for embryonic BSD. The embryo stages were scored daily for signs of BSD in each treatment. At 9 dpf, embryos exposed to 560 µg/L retene showed signs of reduced perfusion (RP), a stretched heart (SH), pericardial edema (PE), and yolk sac edema (YE). No arrhythmias (AR) or ventricular standstill were observed. RP, SH, YE are scored on severity scales from 1 to 3, and PE severity is scored on a 1 to 5 scale. If present, VS and AR are scored as presence-absence (0-1). The BSD index of 0.12 was calculated by dividing the BSD score (the sum of the severity scores for an individual) by the maximum BSD score possible (16). The severity index (0.08) is the sum of the number of fish displaying a severity of each sign divided by the maximum SI score possible (352.5). The stage of development was determined according to González-Doncel et al., (2005).

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<th>SH (0-3)</th>
<th>VS (0-1)</th>
<th>AR (0-1)</th>
<th>PE (0-5)</th>
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|                | 1.87 | 0.12 | 0.079 |
|                | Average | Total |       |
Table 2. Sample scoring chart for larval BSD at 12 dpf. In the larval stages, craniofacial deformities (CF), eye edema (EE), spinal deformities (SD), hemorrhaging (HE), fin rot (FR), failure of swim bladder inflation (NSB), and no movement (NMOV) can be scored accurately in addition to the signs identifiable in the embryonic stages. A value of 23.5 was assigned to those larvae that died with signs of BSD.

| Treatment | Stage (0-38) | RP (0-3) | SH (0-3) | VS (0-1) | AR (0-1) | PE (0-5) | YE (0-3) | CF (0-1) | EE (0-1) | SD (0-1) | HE (0-1) | FR (0-1) | NSB (0-1) | NMOV (0-1) | Dead (23.5) | BSD Score | BSD Index | SI |
|-----------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|------------|------------|----------|----------|-----|
| 560 µg/L  | 38           | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0          | 0          | 0.00      | 0.000     |     |
| retene    | 38           | 3        | 3        | 0        | 0        | 5        | 1        | 1        | 1        | 1        | 1        | 1        | 1        | 1        | -          | 18         | 0.78      | 0.051     |     |
|           | 38           | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 1        | 0        | 0        | -          | 1          | 0.04      | 0.003     |     |
|           | 38           | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 1        | 0        | 0        | -          | 1          | 0.04      | 0.003     |     |
|           | 38           | 2        | 0        | 0        | 0        | 1        | 2        | 1        | 1        | 0        | 1        | 1        | 1        | 0        | -          | 10         | 0.43      | 0.028     |     |
|           | 38           | 3        | 3        | 0        | 0        | 5        | 2        | 1        | 1        | 1        | 1        | 1        | 1        | 1        | -          | 20         | 0.87      | 0.057     |     |
|           |              | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -          | -          | -        | -        | 23.5 |
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|           |              | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -          | -          | -        | -        | 23.5 |
|           |              | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -          | -          | -        | -        | 23.5 |
|           |              | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -          | -          | -        | -        | 23.5 |
|           |              | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -          | -          | -        | -        | 23.5 |
|           |              | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -          | -          | -        | -        | 23.5 |
|           |              | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -        | -          | -          | -        | -        | 23.5 |

Average: 7.29 Total: 0.32 SI: 0.611
Fig. 1. Scoring pericardial edema (PE). (a) and (b) PE 1: a bubbling of the pericardial sac underneath the jaw (arrow); edematous fluid surround the heart (asterisks). (c) PE 2: edematous fluid extending under the head region (arrow), occupying approximately 1/4 of the yolk sac volume. (d) PE 3: the edema fills approximately 2/3 of the yolk sac volume; as PE increases, the anchored heart stretches the atrium (A) into a tube-like structure; there is also the presence of a shrunken ventricle (V); (e) PE 4 and (f) PE 5: edematous fluid occupies 1/2 and > 1/2 of the yolk sac volume, respectively. In all frames: arrowhead, point where extraembryonic vessels enter the pericardial cavity (where heart is anchored); dotted line, bottom of pericardial cavity; V, ventricle; A, atrium; E, edema. Scale bar = 0.1 mm. Note: this figure is a modified version of Fig. 3 in Chapter 2.
Fig. 2. Stages of normal heart development and stretched heart morphology. (a) Stage 1 of heart development (H1): tube heart (TH) extending from the head region; DC, duct of Cuvier. (b) Chamber formation begins in Stage 2 (H2): the linear atrium (A) extends under the head; the atrio-ventricular junction appears as a kink in the tube, and an apex is apparent in the ventricle (V); the bulbus arteriosis (BA) begins to take shape. (c) Stage 3 (H3): the chambers begin to develop in size; the ventricle begins to extend to a position closer to the underside of the jaw; the atrium is still extended and has thin walls. (d) Stage 4 (H4): the heart continues looping, drawing the ventricle forward under the jaw; the atrium is more compact than Stage 3 due to a thicker atrial wall, although it still appears slightly extended. (e) Stage 5 (H5): the completion of cardiac looping; the atrium is adjacent to the ventricle and resembles its shape. (f) SH1: slight pulling of the heart out of a position of adjacent chambers; typically resembles a stage 4 heart; stretch only evident in the atrium; no visible reduction in chamber size; typically associated with PE1 and PE2. (g) SH2: the heart is stretched into a position similar to a stage 3 heart; both chambers have appeared to lose mass; the ventricle still appears to have a lumen; typically associated with PE 3 and a decreased or ceasing of blood flow. (h) SH3: The heart is stretched into a position where the atrium appears like a string-like structure; ventricle appears to be a small mass of cells; atrium will typically keep beating, but ventricular standstill is often observed; typically associated with PE 4-5; blow flood has typically ceased.
Fig. 3. Scoring larvae. (a) Normal larvae, swim bladder has not fully inflated. (b) In an affected fish there are two observable cavities within the yolk sac: the pericardial cavity (PC) and yolk cavity (YC). Scoring of pericardial edema is done as previously described; yolk sac edema (YE) is scored based on the proportion of edema in the yolk cavity. (c) YE 1: edema occupies approximately 1/4 of the yolk cavity. (d) YE 2: approximately 1/3 to 1/2 of the yolk cavity is filled with edematous fluid; hemorrhages (HE) and a stretched heart (SH) are present. A YE 3 severity would be scored if the edema occupies > 1/2. (e) Craniofacial deformities often include: a cranial protuberance (PR); a blunt nose (BN); and jaw deformities (JD); eye edema (EE) is often observed in conjunction with craniofacial deformities. (f) Fin rot (FR): the appearance of uncharacteristic circles on the membranous portion of the tail and caudal fin in medaka. Scale bar = 0.1 mm.
Fig. 4. Scoring embryonic yolk sac edema (YE). Similar to larval YE, embryonic YE is scored based on the proportion of edema relative to the yolk sac area. (a) YE 1: edematous fluid occupies approximately 1/4 of the yolk cavity, and in (b) YE 2 the edema occupies 1/3 to 1/2 of yolk cavity. YE 3 is typically not seen in embryos, but would be the presence of edema in > 1/2 of the cavity. The dotted lines outline the edematous area. Scale bar = 0.1 mm.