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Energetic costs of toxicant metabolism in rainbow trout

(*Oncorhynchus mykiss*)

Samar Al-Hameedi

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ABSTRACT

Aquatic ecosystems are continuously exposed to a variety of environmental toxicants including polychlorinated biphenyls (PCBs). A portion of the energy assimilated by the fish is dedicated to detoxification. This study investigates the changes in energetic demands associated with increased detoxification.

Two CYP1A inducers were employed, PCB-126 and BNF. Resveratrol, a documented inhibitor of CYP1A activity and gene expression, was used to correlate metabolic changes in CYP1A induction with any metabolic changes. PCB-126 and BNF effectively induced CYP1A activity *in vitro* and *in vivo*. Energy metabolism was measured as changes in oxygen consumption rates and changes in plasma glucose and triglyceride concentrations as well as liver glycogen. PCB-126 and BNF treatments resulted in significant changes in metabolic parameters. Treatments with RVT were inconclusive. Although results from this study imply possible energetic changes associated with exposure, further investigation is required to obtain more conclusive results on energetic costs of exposure and detoxification.

RÉSUMÉ

Les écosystèmes aquatiques sont continuellement exposés à une variété de contaminants environnementaux incluant les biphényles polychlorés (BPCs). Une portion de l'énergie assimilée par le poisson est consacrée à la détoxification et cette étude enquête sur les changements dans les demandes énergiques associées à l'augmentation de la détoxification.

PCB-126 et BNF , deux inducteurs de CYP1A ont été utilisés. Resveratrol, un inhibiteur connu de l'activité et de l'expression génique de CYP1A ont été utilisés pour corréler l'induction de CYP1A avec les changements métaboliques. PCB-126 et BNF ont augmentés l'activité EROD *in-vitro* et *in-vivo.* Les variations des taux de consommation d'oxygène et des niveaux de glucose plasmique, les concentrations plasmique de triglycérides ainsi que le niveau de glycogène dans le foie ont été utilisés comme indicateurs de l'énergie métabolique. L'exposition à PCB-126 et BNF a significativement modifié les paramètres métaboliques contrairement à RVT qui n'était pas concluant. Les résultats de cette étude suggèrent un prix énergique associé à l'exposition mais des études plus poussées seront nécessaires pour connaître les coûts associés à l'exposition et la détoxification.

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LIST OF ABBREVIATIONS

Abbreviation	Description
7-ER	7-Ethoxyresorfin
AHR	Aryl hydrocarbon receptor
ANF	α - naphthoflavone
ARNT	Aryl hydrocarbon nuclear translocator
BNF	β - naphthoflavone
CEPA	Canadian Environmental Protection Act
CYP	Cytochrome P450
EROD	Ethoxyresorufin-*O*-ethylase
GAS	General Adaptation Syndrome
PAHs	Poly Aromatic Hydrocarbons
PCBs	Polychlorinated biphenyls
POPs	Persistent Organic Pollutants
RVT	Resveratrol
TEF	Toxic Equivalency Factor

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CHAPTER 1 - General Introduction

1. Rationale

The environment is continuously receiving chemicals released by human activities and the ultimate sink for many of these chemicals is the aquatic environment (van der Oost *et al.*, 2003). These include pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins (PCDDs). Many environmental contaminants induce cellular oxidative damage that may lead to lipid peroxidation, DNA and protein damage and eventually cell death (Otto *et al.*, 1999; Katynski *et al.*, 2004). Ultimately such damage may disrupt the development, reproduction and survival of wildlife including fish, birds and mammals. In addition, the highly lipophilic nature of these toxicants can alter membrane characteristics such as membrane fluidity which eventually compromises membrane integrity (Katynski *et al.*, 2004).

The aquatic ecosystem is contaminated with a wide variety of toxicants that may bioaccumulate at high levels in fish tissues (Wirgin and Waldman, 2004); hence, fish serve as representative models for determining the toxic effects and mechanisms of toxicity of aquatic contaminants. Various biomarkers are recognized to assess exposure and toxicological impacts of environmental pollutants (Behnisch *et al.*, 2001; van der Oost *et al.*, 2003). The most extensively used biomarker is the induction of cytochrome P450s that metabolize or attempt to metabolize these foreign chemicals in living tissues (Kennedy *et al.*, 1996; Lorenzen *et al.*, 1997; Otto *et al.*, 1999; Miller *et al.*, 2003).

Other potential biomarkers to consider include energy reserves and metabolic enzymes. As a result of toxicant-induced stress, organisms can mount a number of specific

responses. Many of these responses may have energetic consequences. These consequences may be a direct result of toxicants interfering with metabolic pathways or eliciting changes in energy-related substrates in the plasma (Rissanen *et al.*, 2003). When the energy demands of the organism increases, for instance during stress, energy stores are mobilized (Wendelaar Bonga, 1997).

Detoxification is an essential cellular process to ensure the survival of an organism. The question addressed in this thesis is what costs are associated with detoxification? Organisms have a fixed energy budget that they obtain through ingested food that is then allocated to maintenance, growth and reproduction (Brett and Groves, 1979). Detoxification requires energy and the question becomes if there is a fixed energy budget, is there energy reallocation from other processes to compensate for the energy demands of detoxification? If it is an issue of survival, intuitively survival would rank higher than other factors such as growth and reproduction and hence energy would be directed towards survival.

Some studies have looked at detoxification costs on growth (Cresswell *et al.*, 1992), feeding (Mathers *et al.*, 1985; Palanivelu *et al.*, 2005) and cellular metabolism (Rissanen *et al.*, 2003). However, none of these studies provided unequivocal results on differentiating between the costs detoxification might have on overall cellular energetics and the impact resulting from toxic injury and its associated costs including repair of damaged systems (Beyers *et al.*, 1999a; Bains and Kennedy, 2004). It is perhaps not possible to completely ascertain if changes in cellular processes are a result of an additional cost of metabolizing these chemicals and not a result of the potential toxic effects of the chemical on these processes. For instance, some studies were unable to determine if the energetic costs of detoxification were a result of reallocation of energy from growth, or that the reduction in

growth was a result of suppression in appetite, an endpoint of chemical toxicity (Cresswell *et al.*, 1992).

It has been proposed, as mentioned above, that detoxifying chemicals are associated with energetic and metabolic costs (Handy *et al.*, 1999; Bains and Kennedy, 2004). This leads to two interesting questions: *i*) what is the amount of energy spent on detoxification and the source of this energy and, *ii*) whether there are changes in the metabolism of energy reserves (lipid, glycogen and protein) to compensate for the energy demand to drive detoxification.

The following sections briefly describe the mechanisms of xenobiotic metabolism and the energetics concept, and an overview of the studies that have attempted to determine costs of toxicant exposure and detoxification.

2. Pollutants in the environment

The majority of chemical substances that persist in the environment belong to a class of pollutants called persistent organic pollutants or POPs. These are a carbon-based set of chemicals with a range of by-products derived from industrial processes or combustion. These include polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and pesticides such as dichlorodiphenyltrichloroethane (DDT). There is concern over the presence of these chemicals, their distribution and fate in the aquatic environment and their effects on species within aquatic ecosystems. The toxic effects of this ubiquitous group of environmental chemicals on metabolism, growth, development and reproduction are well documented (Corsolini *et al.*, 2005; Vaccaro *et al.*, 2005).

Most of these chemicals are highly persistent within the environment because of their high resistance to degradation or biotransformation regardless of the environmental compartment they are in, i.e. air, water, or sediment (Eljarrat and Barceló, 2003). In addition, these compounds have a tendency to bioaccumulate in living tissue as a consequence of their hydrophobic nature and their high affinity for fat (Safe *et* al., 1995; Corsolini *et al.*, 2005; Blais *et al.*, 2006). Evans *et al.* (2005) showed that organochlorine concentrations in five fish species from Canadian lakes were higher in the liver than the muscle which was related to differences in lipid content between the two tissues. For example, burbot muscle with ~1% lipid had a PCB concentration of approximately 5 ng/g (wet wt) compared with the burbot liver with ~40% lipid and 100 ng PCB per g (wet wt). These pollutants can biomagnify across the food chain (Safe *et* al., 1995; Fisk *et al.*, 2005; Blais *et al.,* 2006) and pose a considerable risk of causing adverse effects to both environmental and human health. Another source of concern with these pollutants is their high potential of long range transport from the source of release to regions where they are not in use or produced (Breivik *et* al., 2004; Corsolini *et al.*, 2005; Evans *et al.*, 2005; Fisk *et al.*, 2005).

Effects on fish species are extensively studied as these organisms are widely distributed in the aquatic environment and hence represent an ideal model to study the impact of environmental contaminants on aquatic wildlife. In addition, the study of fish responses to a chemically challenged environment is of key ecological importance given their function as key components of aquatic food webs and a major component of the human diet (Jobling, 1994; van der Oost *et al.*, 2003; Evans *et al.*, 2005).

Polychlorinated biphenyls (PCBs) represent a common example of a ubiquitous environmental pollutant. This class of compounds comprises 209 structurally similar

congeners that vary in the number of chlorine substitutions on the biphenyl structure (Figure 1.1). The number and location of chlorine atoms determine the physical and chemical properties of the PCB molecule as well as their environmental toxicity (Gdaniec-Pietryka *et al.*, 2007).

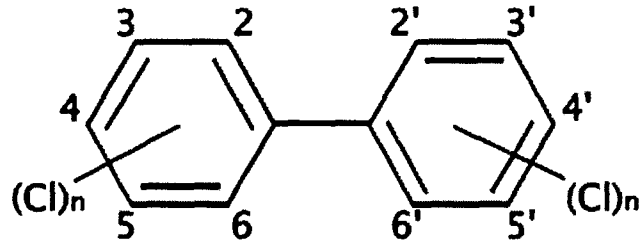


Figure 1.1: Chemical structure of polychlorinated biphenyls (PCBs) comprises two benzene rings able to rotate around a carbon-carbon bond.

The physical and chemical characteristics of PCBs such as low water solubility, high thermal and chemical resistance have deemed them suitable in a broad range of industrial applications. However, these characteristics are what make PCBs persistent in marine ecosystems, resistant to degradation and therefore have relatively long half-lives (Stapleton *et al.*, 2001). Different isomers have different effects as a result of their degree of chlorination. The higher the chlorine content, the lower their water solubility and the higher their affinity for fatty tissues (Borja *et al.*, 2005). The toxicity of PCBs varies amongst the different congeners; the most toxic are those with non-*ortho* chlorine substitutions (i.e. *meta* and *para*

positions) such as 3,3',4,4' – tetrachlorobiphenyl, (PCB-77), 3,3',4,4',5 - pentachlorobiphenyl (PCB-126) and 3,3',4,4',5,5' – hexachlorobiphenyl (PCB-169) (Bastien *et al.*, 1997; Borja *et al.*, 2005; White *et al.*, 2000). These tend to have dioxin-like properties and this is further supported by their assigned TEF (toxic equivalency factor) values as explained in the following section.

The PCB congener PCB-126 has been selected as the model PCB congener in this study for its ability to effectively induce the hepatic detoxification system in rainbow trout (Newsted *et al.*, 1995; Hahn *et al.*, 1996).

2.1 Consequences of pollutant exposure

External stimuli such as the presence of contaminants trigger specific physiological processes that influence the normal behavior of individuals in an ecosystem and any alteration in these responses as a result of toxicity could have potentially detrimental impacts on behavior and ultimately survival. Even if the exposures are non-lethal as determined for various chemicals by their low LC_{50} value (lethal concentration causing 50% death of the individuals when exposed), lower concentrations of contaminants may elicit toxic effects in organisms including interference with endocrine systems leading to irreversible biochemical and physiological changes (van der Oost *et al.*, 2003; Katynski *et al.*, 2004; Van den Berg *et al.*, 2006; Medina *et al.*, 2007).

The adverse impacts of POPs including PCBs on marine animals have been studied and documented (Gooch *et al.*, 1989; White *et al.*, 2000; Stapleton *et al.*, 2001; Montie *et al.*, 2008). This poses a major health concern for human populations and in particular those with diets dependent on fish stocks (Aoki, 2001; Karl *et al.*, 2003; Ross, 2004; Domingo and Bocio, 2007). Various classes of toxicants, including PCBs and metals such as mercury are

implicated in cases of severe decline in human health and poisonings (Safe *et* al., 1995; Aoki, 2001).

2.2 Screening and pollution monitoring

A number of regulatory guidelines are in place to monitor the release and impact of these chemicals into the environment. The Stockholm Convention on Persistent Organic Pollutants is an example of an international effort that has taken action on 12 pollutants (Table 1). The objective of such international agreements is to regulate the release of these pollutants into the environment and ultimately eliminating or reducing the toxic environmental burden in a cost-effective manner (Eljarrat and Barceló, 2003; Breivik *et al.*, 2004).

Canada was the first country to both sign and to ratify the Stockholm Convention to eliminate the 'dirty dozen'. Beginning with PCBs in the late 1970s, use of POPs has been restricted. The Canadian government oversees the 'Federal Chlorobiphenyls Regulations' under the Canadian Environmental Protection Act (CEPA, 1999), that addresses handling and disposal of PCBs in Canada as part of the international agreement to phase out and destroy PCBs in service or in storage. Concerns over the effects on ecosystems first surfaced in 1966 when PCBs were discovered in bird populations in Switzerland. The concern was their persistence in the environment and specifically in fatty tissue of animals (Ross, 2004; Borja *et al.*, 2005; Evans *et al.*, 2005). In the mid-1970s, Canada and the US voluntarily made the production, import and distribution of PCBs illegal after a growing body of evidence found that these compounds had an impact on the environment and human health (Ross, 2004; Montie *et al.*, 2008).

Toxicological impacts on development, growth and reproduction are used as indicators of toxicity of these chemicals. A number of bioanalytical tools were developed for the screening of dioxins and dioxin-like compounds including PCBs in order to close the gap between environmental pollution and its impact on wildlife; such indicators include induction of specific enzymes as a biological response, bioassays, DNA binding assays, immunoassays, monitoring of physiological responses, etc (Behnisch *et al.*, 2001; van der Oost *et al.*, 2003). Assessment of the toxic potential of these chemicals is complicated by several factors. Different chemicals differ in their relative concentrations across environmental compartments as a result of their different solubilities and rates of degradation which influence their fates in the environment (Gdaniec-Pietryka *et al.*, 2007). Risk assessment of these contaminants is further complicated by the fact that individual effects of congeners are masked by the synergistic effects from combined chemicals (Van den Berg *et al.*, 2006). In addition, difference in species, gender and developmental stage responsiveness and sensitivities that range over orders of magnitude, further complicate the quantification of hazardous effects of POPs. Kennedy *et al.* (1996) demonstrated species-specific differences in the sensitivity of avian embryos to PCBs. In another study, after a dietary exposure for a 2 year period of female and male rats to low doses of 2, 3, 7, 8-tetrachloro-dibenzo-*p*-dioxin (TCDD), females developed hepatocellular carcinomas whereas males did not (Safe *et al.*, 1995). A study by Bosveld *et al.* (1997) reported decreasing sensitivities to aromatic hydrocarbons (dioxins and PCBs) at progressive developmental stages of the chicken.

In order to facilitate risk evaluation of these compounds and estimate toxic potential of environmental mixtures, the toxic equivalency factor (TEF) was developed (Van den Berg *et al.*, 2006). This concept is based on an understanding of the common mechanism of action

of these dioxin-like compounds which is mediated through their binding to the cytosolic aryl hydrocarbon receptor (AhR) inducing the transcription of a group of genes including the oxidizing enzyme cytochrome P4501A1.

Table 1.1: Lists of POPs selected for environmental and toxicological studies (Eljarrat and Barceló, 2003). TEF: Toxic Equivalency Factor; REP: Relative Potency.

POPs selected at the Stockholm Convention (2001)	**POPs with an assigned TEF or REP**	**Emerging POPs**
Aldrin		
Chlordane		
DDT		
Dieldrin		
Endrin		
Heptachlor		
Hexachlorobenzene		
Mirex		
Toxaphene		
PCBs	PCBs	
PCDDs/PCDFs	PCDDs/PCDFs	
	PCNs	
	PBDEs	PBDEs
	PBDDs/PBDFs	PBDDs/PBDFs
	PBBs	PBBs
	PAHs	

The toxic potency of these chemicals is derived and ranked from comparison of dose-response curves for toxic endpoints of individual congeners (usually, CYP1A induction) and relative to the most potent inducer of the AhR signal transduction pathway, TCDD. The TEF of TCDD is set to 1.0 and each compound is attributed a numerical value that reflects their toxicity relative to TCDD. For instance, Newsted *et al.* (1995) dervided TEF values for PCB congeners based on their ability to induce CYP1A activity in rainbow trout and were 0.0006, 0.00035 and 0.0014 for PCB congeners 77, 169 and 126, respectively. TEFs are species specific and this approach is used to characterize toxicity of aromatic hydrocarbons for CYP1A induction in both mammalian and teleost cell systems (Clemons *et al.,* 1994; Behnisch *et al.,* 2001; Billiard *et al.*, 2004). TEFs for fish were derived using endpoints mediated by the AhR (Newsted *et al.*, 1995).

3. Xenobiotic metabolism

The impact of environmental chemicals on chemical-biological reactions requires an understanding of their biotransformation within a biological system since these processes determine the biological activity of these compounds. The liver of vertebrates including fish is the organ most involved in the detoxification of foreign compounds (Jobling, 1994). Xenobiotic metabolism or biotransformation is a process catalyzed by enzymes that convert xenobiotic or foreign compounds which are normally hydrophobic, to more water-soluble compounds allowing their excretion from the organism. This process is generally divided into two phases. In phase I, the xenobiotic undergoes hydrolysis, oxidation or reduction reactions, which is then conjugated by phase II enzymes (glucuronosyltransferase,

sulphotransferase) by the addition of polar groups such as glucuronic acid to render it more hydrophilic facilitating its elimination (Buhler and Wang-Buhler, 1998; van der Oost *et al.*, 2003; Široká and Drastichová, 2004; Sagredo *et al.*, 2006). Metabolism of AhR-ligands may result in the formation of carcinogenic metabolites (e.g. epoxides) that may form DNA and protein adducts, the first step in initiating chemical-induced carcinogenesis (Janošek *et al.*, 2006).

3.1 Cytochrome P-450s

Cytochrome P450 (CYP) enzymes are a large family of heme-proteins that are found in microorganisms, plants, invertebrates and vertebrates. Cytochrome P450s catalyze a diverse range of oxidation reactions including hydroxylation, dealkylation and dehalogenation. These enzymes play two main roles in living organisms. One class catalyzes oxidation reactions involved in the biosynthesis or biodegradation of endogenous compounds like steroids or fatty acids. Another class of CYPs has a key function in the oxidative biotransformation of xenobiotics from the environment eliminating them from living tissue (Buhler and Wang-Buhler, 1998; Mansuy, 1998; Miller *et al.*, 2003; van der Oost *et al.*, 2003; Široká and Drastichová, 2004). These enzymes catalyze phase-I biotransformations of lipophilic compounds into more soluble products, according to the following reaction:

$$RH + O_2 + 2e^- + 2H^+ \xrightarrow[\text{CYP1A1}]{\text{NADPH} \quad \text{NADP}^+} ROH + H_2O$$

More than 500 different cytochrome P450 genes are cloned and sequenced. These are classified into families and subfamilies based on specific functions or their presence in

certain species. Isoforms whose primary function is xenobiotic metabolism belong to the CYP1, CYP2 and CYP3 families (Široká and Drastichová, 2004).

Cytochrome P450s are also detected and characterized in fish species; these are found predominately in the liver but in lower concentrations in other tissues (intestine, kidney and brain) (Jobling, 1994). Induction of cytochrome P450s is extensively studied in fish as a biomarker indicating pollution of the aquatic environment. In fish it is mainly the CYP1A family comprising two genes CYP1A1 and CYP1A2 that are responsible for phase-I reactions (Buhler and Wang-Buhler, 1998; Stegeman *et al.*, 2001; Miller *et al.*, 2003). Responses to foreign chemicals in fish may differ from those in other species. Results of metabolic studies using a specific toxicant in lab animals are not always applicable to fish. For instance, CYP 2B fails to be induced in fish (Široká and Drastichová, 2004).

Cytochrome P450 induction can serve as an indicator of the extent of toxic burden. An interesting aspect of these enzymes that has strong toxicological relevance is that several substrates including PCBs, PCDDs and PAHs increase their mRNA expression, a response mediated by the AhR (Stegeman and Lech, 1991; Celander and Forlin, 1995; Otto *et al.*, 1999; Miller *et al.*, 2003). Therefore estimating the catalytic activity of the CYP1A enzymes (ethoxyresorufin-*O*-deethylase, EROD) is considered a useful biomarker of exposure to common organic pollutants (Stegeman and Lech, 1991; Hahn *et al.*, 1996; Kennedy *et al.*, 1996; Lorenzen et al., 1997). However, Kennedy *et al.* (2003) reported a negative correlation between EROD activity and total PCB concentrations in adult herring gulls, suggesting that hepatic EROD activity may not be a useful biomarker of exposure to toxicants in all situations or in all species.

The toxic potential of many AhR-ligands is evaluated as their induction of CYP1A enzyme activity. A greater induction of these enzymes could potentially lead to a higher level of carcinogenesis and consequently to a higher degree of oxidative DNA damage (Stegeman and Kloepper-Sams, 1987; Stegeman and Lech, 1991; van der Oost *et al.*, 2003). The metabolic function of CYP1A is implicated in transforming some of the chemicals into carcinogens in fish, an activity that is mediated by the AhR (Stegeman and Lech, 1991; Janošek *et al.*, 2006). Another indicative endpoint of toxicant exposure is the measurement of the deregulation of the heme biosynthetic pathway associated with exposure. There is evidence that CYP1A enzymes are involved in porphyrin accumulation mediated by CYP1A-inducers such as dioxins and PCBs (Kennedy *et al.*, 1993; Lorenzen *et al.*, 1997).

3.2 AhR signaling pathway

Animals are able to mount a number of adaptive responses when chemically challenged. These responses include the activation of enzyme systems that target these chemicals. However, as mentioned above, these same systems mediate reactions that lead to toxic effects hence displaying a duality in their mode of actions. One of the adaptive responses of organisms to chemical challenge is the induction of genes for xenobiotic metabolizing enzymes by the AhR. The AhR is a ligand-dependent intracellular receptor that stimulates gene transcription in the presence of many xenobiotics (Barron *et al.*, 2004). In its non-liganded state, the cytosolic AhR exists as a multi-protein complex with two molecules of the chaperone heat shock protein (Hsp90), the X-associated protein 2 (XAP2), as well as a 23 kDa co-chaperone protein referred to as *p*23 (Figure 1.2).

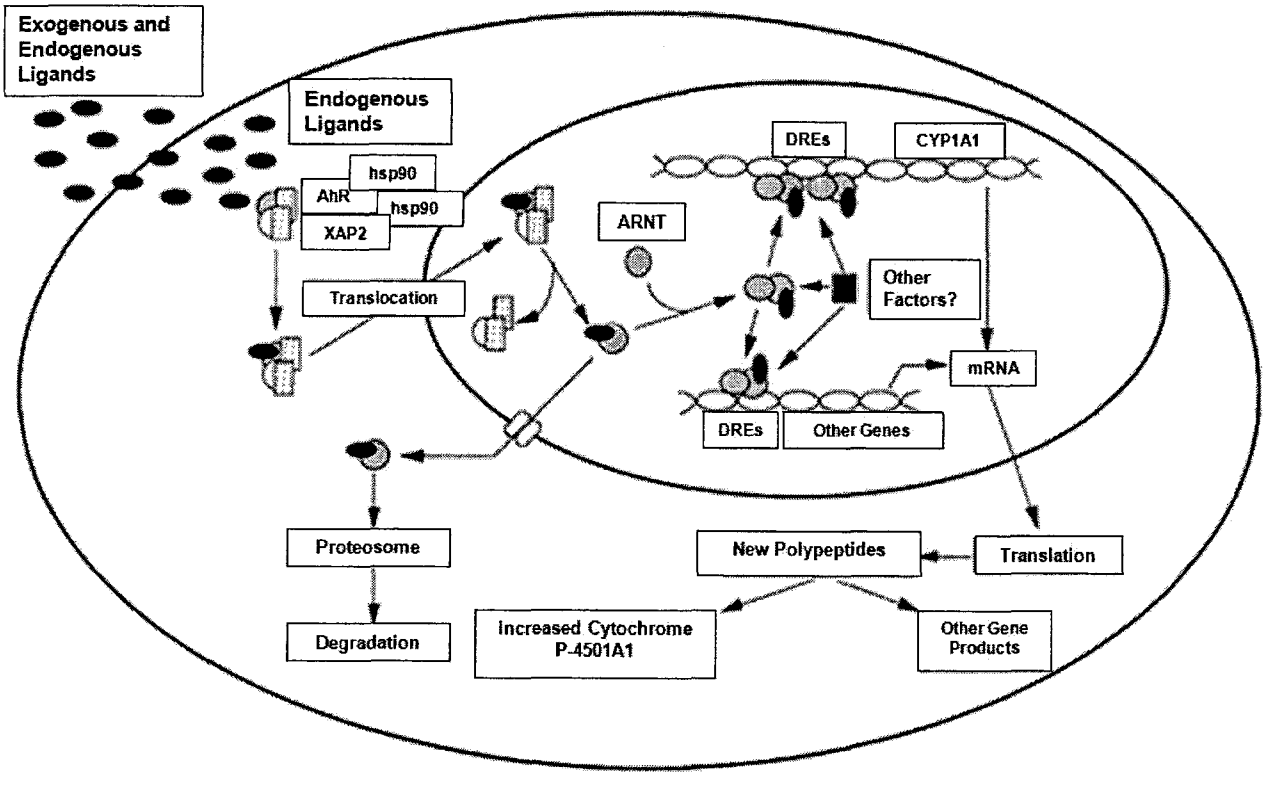


Figure 1.2: Current model for the mechanism of gene activation by the aryl hydrocarbon receptor, AhR (from Denison and Nagy, 2003).

Following ligand binding, the protein complex undergoes a conformational change that results in the release of the Hsp90 dimer and associated proteins followed by the translocation of AhR-ligand complex into the nucleus. The AhR-ligand complex then dimerizes with a nuclear protein, ARNT. The heteromeric AhR-ligand-ARNT complex then binds to a DNA recognition sequence known as the dioxin response element upstream of the CYP1A1 gene, conjugation (Phase II) enzymes and other AhR-responsive genes hence

stimulating their transcription as a group (Schmidt and Bradfield, 1996; Besselink *et al.*, 1998; Denison and Nagy, 2003).

Since AhR ligands are typically good substrates for and are readily metabolized by CYP1A, the induction of the CYP1A enzyme system through AhR activation is an efficient mechanism where fish can counter the bioaccumulation tendencies of toxicants. This very system also contributes to the toxic and mutagenic potency of xenobiotics (Safe *et al.,* 1995; Schmidt and Bradfield, 1996; Billiard *et al.*, 2002). Hahn (1998) reviewed the available information on the toxic effects of AhR agonists in fish; however few studies address the mechanism through which AhR induces these toxicities (Billiard *et al.*, 2002).

Toxicological analyses and bioanalytical tools focus on direct indicators of toxic exposure. Scientific research also focuses on the effects of these contaminants on the physiology and survival of exposed animals. Additional and more complex indicators need to be assessed which may describe disruptions of fish behavior that result from minimal exposures or disruptions in metabolism. Although, some studies do shed some light on these issues, there is a considerable lack of data on the physiological mechanisms that govern metabolic changes associated with exposure.

4. Energetic costs of detoxification

This section aims to provide an overview of the possible implications of environmental pollution on the overall energetics of an animal. An understanding of the concept of bioenergetics is necessary to provide an insight into the impact of added costs to organisms under chemical challenge. To this end, the basics of energy metabolism and

animal energetics are presented followed by a review of scientific evidence on costs of toxicant exposure.

4.1 Toxicant Stress and Bioenergetics

Stress can be simply defined as a stimulus that leads to a disturbance or loss of homeostasis. A stressor, be it natural (seasonal/temperature changes) or anthropogenic (e.g. environmental pollutants), can act in two ways: (1) elicit effects that threaten homeostasis of the organism and/or (2) stimulate compensatory/adaptive responses that could be behavioral or physiological in nature (Odum, 1985; Calow, 1991; Jobling, 1994; Wendelaar Bonga, 1997; Knops *et al.*, 2001; Beyers and Rice, 2002; Francis-Floyd, 2002). These responses are generally energy-demanding and are emphasized by a pattern referred to by Selye (1976) as the general adaptation syndrome (GAS) (Jobling, 1994; Beyers *et al.* 1999a). GAS provides a description of the short- and long-term physiological changes that occur following a prolonged exposure to a non-specific stressor, and these are characterized by three phases: alarm, resistance and exhaustion (Figure 1.4). The concept was developed after monitoring animal responses to a number of external influences and the coupled biochemical and physiological changes in the individuals. Stress resulting from exposure to contaminants may result in a loss of appetite and behavioral changes may occur (Handy *et al.*, 1999; Palanivelu *et al.*, 2005). The second phase is associated with an increased metabolic rate as the animal compensates for the effects of the stressor and achieves adaptation (Calow, 1991; Knops *et al.*, 2001). However, prolonged exposure or exposure to a second stressor could result in exhaustion and depletion of resources (phase 3) (Calow, 1991; Beyers *et al.*, 1999a). At this point the animal is no longer able to maintain homeostasis, compromising the immune system and making it susceptible to disease and the risk of pathogenic infections and

eventually leading to mortality (Jobling, 1994; Lochmiller and Deerenberg, 2000; Francis-Floyd, 2002).

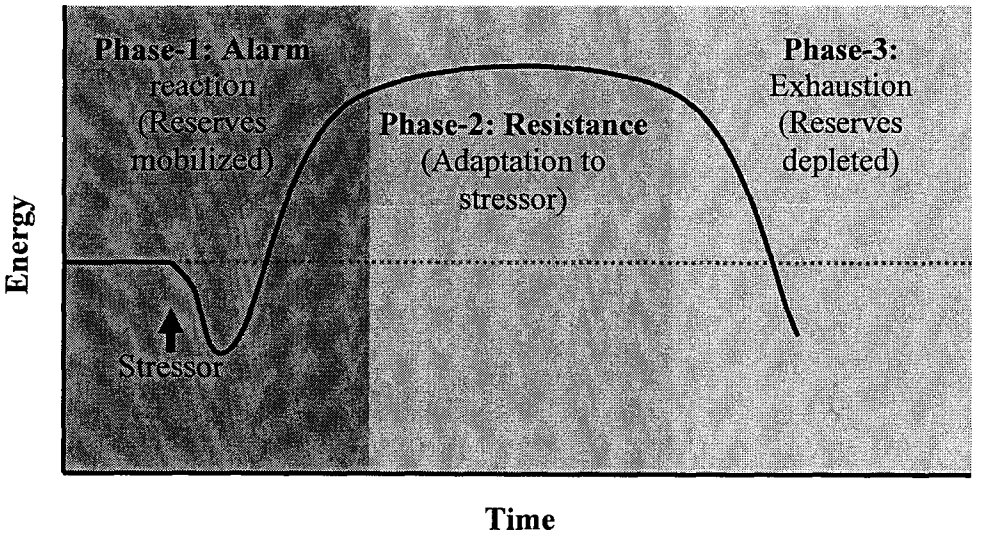


Figure 1.3: Cartoon illustrating the three phases of the general adaptation syndrome (Selye, 1976) as a function of time.

In addition to the usual biomarkers to assess stress, such as changes in cortisol and catecholamine levels and the subsequent metabolic/physiological changes (Jobling, 1994; Wendelaar Bonga, 1997), stress can be quantified by measuring energy intake versus expenditure and any changes in animal bioenergetics (Beyers *et al.*, 1999a, b; Knops *et al.*, 2001; Beyers and Rice, 2002).

The basic premise of animal bioenergetics is that of energy balance. An animal such as a fish gains energy in the form of assimilated food which is then allocated to catabolic and

anabolic processes, providing energy for maintenance and activities including growth, reproduction, locomotion, waste excretion, etc. (Beamish *et al.*, 1975; Brett and Groves, 1979; Jobling, 1994; Beyers *et al.*, 1999a; Knops *et al.*, 2001). Intake from food consumption against the costs of maintenance and growth is balanced in the following energy budget equation:

$$R = F + U + M + P$$

When the intake of energy, R, exceeds maintenance and activities (metabolism, M; waste excretion, F; products of metabolism, U), energy is stored in the form of reserves that include glycogen and triacylglycerides, P (Jobling, 1994; Beyers *et al.*, 1999a). Vital bodily functions such as growth, reproduction, and maintenance (including locomotion) constitute the majority of energy expenditure. Activities such as growth may be affected by a number of variables including: size, gender, age and environmental conditions (temperature, toxicant exposure, etc.) (Brett and Groves, 1979; Jobling, 1994). In addition, due to the limited availability of energy resources, any additional metabolic costs to compensate/adapt to toxic stress could result in energy reallocation/trade-offs at the expense of other processes essential for the survival of an organism. Any of these variables may affect the partitioning of energy between the components of the energy budget equation (Calow, 1991; Jobling, 1994; Beyers *et al.*, 1999a, b; Lochmiller and Deerenberg, 2000). Therefore, in order to gain a better understanding of the energy allocation between the various components, there exists an extensive amount of literature on energetic costs of these components in association with immune responses, swimming and growth. However, there is insufficient literature on the energetic costs of defense mechanisms in fish.

4.2 Literature Review

Lochmiller and Deerenberg (2000) reviewed the existing evidence on the costs of maintaining a functional immune system, and concluded that there is sufficient literature to support that an up-regulation of the immune system is associated with metabolic costs and energy reallocation (e.g. growth) to adjust for the increase in energy demand.

Costs associated with chemical exposure were examined in a number of studies. Bains and Kennedy (2004) studied the costs of pyrene exposure and biotransformation in rainbow trout (*Oncorhynchus mykiss*). The authors showed a concentration-dependent increase in oxygen consumption of hepatocytes treated with pyrene *in vitro*. They also showed an increase in respiration rates with the amount of the main metabolite of pyrene detoxification/metabolism, hydroxypyrene. Rissanen *et al.* (2003) demonstrated that rainbow trout hepatocytes experienced changes in cellular energetics and increased respiration in the presence of dehydroabietic acid (DHAA), a resin acid found in pulp mill effluents. Metabolic disruption caused by toxicant/pollutant stress is also demonstrated by alterations in resting metabolic states, measured as oxygen consumption rates (Holmberg *et al.*, 1972; Grobler *et al.*, 1989; Wendelaar Bonga, 1997; Palanivelu *et al.*, 2005).

In addition to metabolic disruption caused by exposure, energetic trade-offs between detoxification processes and essential bodily functions are likely to occur (Handy *et al.*, 1999; Marchand *et al.*, 2004). Handy *et al.* (1999) studied a number of biochemical, morphological and physiological parameters and integrated the observed data to establish an understanding of fish responses and compensations to sub-lethal oral exposure of copper. The exposures employed in their study were such that they did not exact any toxic effects on the fish. They reported possible trade-offs between the metabolic cost of detoxification and

physiological processes essential for the survival of the fish. Exposed rainbow trout demonstrated a reduction in swimming rate compared to control fish in response to an increased metabolic rate associated with the detoxification of copper.

Sherwood *et al.* (2000) and Levesque *et al.* (2002) demonstrated that yellow perch (*Perca flavescens*) from Quebec lakes with varying degrees of heavy-metal pollution had significantly reduced growth rates in comparison with reference lakes with no metal pollution. In addition, this growth reduction was not associated with decreased food intake. Cresswell *et al.* (1992) demonstrated that the presence of a dietary toxicant, nicotine, had a negative effect on the growth rate of the southern armyworm (*Spodoptera eridania*). These studies concluded that the net reduction in growth rate resulted from a reduced efficiency of energy metabolism and was not due to a decrease in the amount of food assimilated. It is often difficult to determine if growth reduction is a result of reduced/reallocated energy supply or a result of physiological stress or perhaps a combination of the two (Beyers *et al.*, 1999a, b).

Some experiments demonstrate that with continued exposure to toxicants, fish eat less (Mathers *et al.*, 1985; Bains and Kennedy, 2004; Palanivelu *et al.*, 2005). When energy obtained from the diet is insufficient to meet maintenance requirements and under increased energy demand resulting from acute stress, endogenous sources (energy reserves) are mobilized (Brett and Groves, 1979; Mayer *et al.*, 1992; Vijayan and Moon, 1992; Wendelaar Bonga, 1997; Levesque *et al.*, 2002). Glycogen and lipid contents in freshwater and marine invertebrates are reported to decrease with exposure to toxicants (Rao and Rao, 1984; Sancho *et al.*, 1998; Handy *et al.*, 1999).

These studies suggest that a number of adaptive responses occur when organisms are exposed to contaminants including increases in detoxifying enzymes and increases in metabolic rates. In addition, a decrease in some aspects of fish physiology such as locomotor activities may occur (Handy *et al.*, 1999). This supports the primary assumption that the costs of detoxification processes may be associated with increases in energy required to drive metabolic processes. It also suggests that energetic costs of maintaining increasing detoxification reactions could constitute a significant component of an organism's energy budget.

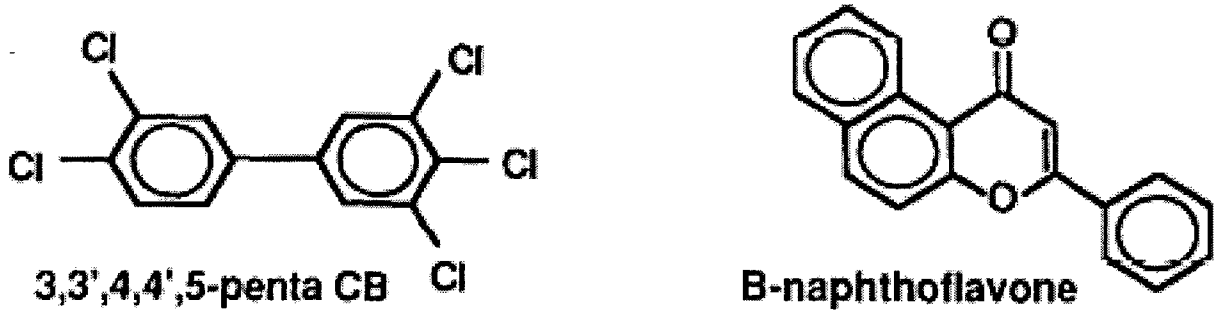
5. Hypothesis

The question addressed by my work is whether the processes required to detoxify xenobiotics by an exposed organism would result in an increase in energy demand which would result in a repartitioning of energy within that organism. It is proposed that the induction of xenobiotic metabolism in toxicant-stressed tissues could result in the mobilization of energy reserves. Fish were used in these studies as they are widely distributed in the aquatic environment and hence represent an ideal model to study the impact of environmental contaminants. In addition, fish are of key ecological importance given their function within aquatic food webs (van der Oost *et al.*, 2003). To this end, I focused on the rainbow trout, *Oncorhynchus mykiss*.

Therefore the hypothesis for my work is that the detoxification of xenobiotics in animal tissue has energetic costs resulting in higher metabolic demands. In addition, maintaining induction of the detoxifying enzymes in the presence of xenobiotics will result in an increased mobilization of energy reserves in the trout.

6. Objectives

The objectives of this study were investigate the costs of detoxification by quantitatively determining if the energy of detoxification is significant enough to affect the overall energy budget of the fish. Rainbow trout were used as the experimental animal in this study as this species is frequently used as a model for toxicological research and investigations of physiological processes or is toxicities involving P450 metabolism. The effects of two AhR agonists were investigated in this study: the PCB 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB-126) and the PAH β-naphthoflavone (BNF). The structures of these two compounds are shown below.



(a) (b)

Figure 1.4: Chemical structures of (a) 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB-126) and (b) β-naphthoflavone (BNF).

Both chemicals induce CYP1A activities but PCB-126 is a stronger inducer than BNF (Zhang *et al.,* 1990; Newsted *et al.*, 1995; Hahn *et al.,* 1996; Navas and Segner, 2000; Gravato and Santos, 2002). The two chemicals were employed to delineate any relationship between the potency of the agonist and the observed parameters studied.

The specific objectives of my work were:

1. To measure CYP1A activity as EROD activity following exposure to the two agonists;
2. To determine metabolic changes at the whole fish level by measuring oxygen consumption rates of exposed animals;
3. To determine changes in metabolic endpoints including plasma glucose and tissue glycogen contents; and,
4. To correlate changes in CYP1A activities with the metabolic changes. To this end, resveratrol, an AhR antagonist was employed. Resveratrol is a phytoalexin compound found in grape juice and wine that is an AhR antagonist in mammals. Aluru and Vijayan (2004) reported that resveratrol was also an antagonist in rainbow trout.

CHAPTER 2 - Resveratrol Inhibits CYP1A1 Activity in Liver Microsomes of Rainbow Trout (*Oncorhynchus mykiss)*

1. Introduction

The aquatic environment is a continuous receiver of environmental chemicals that have a wide range of reported responses (behavioral, endocrine, reproductive, etc.) in a number of aquatic species (Scott and Sloman, 2004). Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) are known to be responsible for a diverse range of physiological, biochemical and toxic effects within aquatic species. The aryl hydrocarbon receptor (AhR), a cytosolic ligand-activated transcription factor, is shown to mediate the toxic effects of some PCBs(i.e., the co-planar PCBs; Nebert *et al.*, 1993; Billiard *et al.*, 2002). The AhR protein undergoes a conformational change following the binding of a ligand and translocates to the nucleus forming a heterodimer complex with the aryl hydrocarbon nuclear translocator (ARNT). This complex in turn interacts with a specific DNA recognition sequence regulating the transcription of a number of genes in the AhR gene battery including cytochrome P450 1A1 (CYP1A1) (Denison and Nagy, 2003). The induction of CYP1A1 gene and protein expression is an AhR-mediated cellular response that is extensively used as a toxicological indicator of exposure to chemicals activating AhR (Buhler and Wang-Buhler, 1998; Stegeman *et al.*, 2001). The induction of the CYP1A1 system also serves as a detoxification mechanism since many of these AhR ligands are also CYP1A1 substrates (van der Oost *et al.*, 2003; Sagredo *et al.*, 2006). A considerable amount of research has gone into elucidating the various toxic effects of AhR ligands on biochemical

and physiological processes of aquatic species, however, the specific mechanisms of these toxic actions are yet to be completely elucidated.

The premise of this thesis focuses on establishing a cause-effect relationship between CYP1A1 activation and metabolic changes in a piscine model. Therefore, a reliable tool is needed to separate metabolic changes resulting from the activation of AhR-mediated signaling and CYP1A1 induction.

Several chemicals including phytochemicals such as flavonoids, curcumin and natural plant extracts act as AhR antagonists and are able to suppress events downstream of AhR signaling. Although the exact mechanism of suppression is not fully understood, there is evidence to suggest that some of these chemicals inhibit the DNA binding of the ligand-activated AhR, whereas others such as the flavanoid, quercetin, compete with the agonist for binding to the AhR (Miranda *et al.*, 1998; Ciolino and Yeh, 1999; Zhang *et al.*, 2003; Fukuda *et al.*, 2007). Another commonly used AhR antagonist used in fish models, α-naphthoflavone (ANF) is an isomer of β-naphthoflavone (BNF) which is a prototype agonist of the AhR (Zhang *et al.*,1990; Gasiewicz and Rucci, 1991; Miranda *et al.*, 1998; Wilson *et al.*, 1998). ANF is shown to inhibit BNF-induced CYP1A1 activity in fish (Navas and Segner, 2000) and TCDD–induced CYP1A1 induction in mouse hepatoma cells (Gasiewicz and Rucci, 1991). This involves ANF binding to the AhR resulting in a ligand-receptor complex with a lower affinity than typical stronger agonists (Gasiewicz and Rucci, 1991).

In recent years, resveratrol (3, 5, 4'-trihydroxystilbene) has received attention for its use as an AhR antagonist in mammalian models including cell lines. Resveratrol is a natural polyphenolic phytoalexin that is synthesized in response to fungal attack or microbial infection and found in dietary sources such as grapes and peanuts (Casper *et al.*, 1999; Chun

et al., 1999; Ciolino and Yeh, 1999; Singh *et al.*, 2000). In addition, it is a component of red wine which triggered initial interests in its potential role in the low incidence of cardiovascular diseases in countries with high wine consumption. A number of studies report RVT to have anti-oxidant and anti-inflammatory effects and to be a chemopreventative agent inhibiting cellular events associated with tumor initiation, promotion and progression (Ciolino *et al.*, 1998; Chang *et al.*, 2001; Lee and Safe, 2001). RVT, by inhibiting the AhR signal transduction pathway and CYP activity, has successfully reversed toxic effects of AhR ligands that lead to carcinogenesis, inhibition of bone formation, apoptosis and reproductive abnormalities, by inhibiting the AhR signal transduction pathway and CYP activity (Ciolino and Yeh, 1999; Singh *et al.*, 2000; Revel *et al.*, 2001; Chen *et al.*, 2004; Wang *et al.*, 2006). Interestingly, RVT is also shown to have neuroprotective effects (Dasgupta and Milbrandt, 2007), extension of life span in diverse species including mice (Baur *et al.*, 2006), improvement of mitochondrial function and protection against metabolic disease (Lagouge *et al.*, 2006).

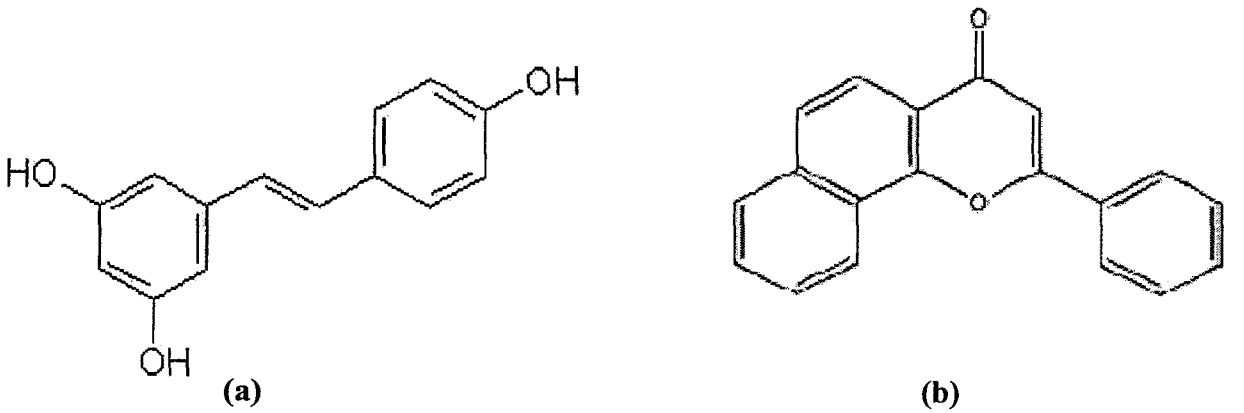


Figure 2.1: Chemical structures of (a) 3, 5, 4' – trihydroxystilbene or resveratrol, and (b) α-naphthoflavone.

The objective of this study was to examine the effect of RVT on CYP1A1 activity, measured as EROD, in rainbow trout microsomes and to determine the capacity of RVT to inhibit CYP1A1 activity of rainbow trout microsomes *in vitro*. The inhibitory effects of resveratrol on AhR function and CYP activity are well studied in mammalian systems but not in piscine models (Ciolino and Yeh, 1999; Lee and Safe, 2001; Wang *et al.,* 2006). Aluru and Vijayan (2006) did demonstrate that RVT was a potent AhR antagonist in rainbow trout hepatocytes. They showed that RVT significantly inhibited BNF-induced CYP1A protein expression. In this study, inhibitory effects of varying concentrations of RVT on EROD activity were compared to that of ANF in the presence or absence of two AhR ligands, 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB-126) and BNF. It is expected that RVT will effectively inhibit EROD in the presence of BNF and PCB-126.

2. Materials and Methods

2.1. Chemicals

3, 3', 4, 4', 5-Pentachlorobiphenyl (PCB-126) was provided by Dr. Sean Kennedy at the National Wildlife Research Centre (NWRC, ON, Canada). β-Naphthaflavone (BNF; N3633), α-naphthaflavone (ANF; N5757), resveratrol (RVT; R5010), resorufin (R3257), ethoxyresorufin (E3763), NADPH (N6505), bicinchoninic acid (BCA) were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest purity and provided by local suppliers.

2.2. Fish

Sexually immature female rainbow trout (*Oncorhynchus mykiss*), weighing approximately 75 g were purchased from Linwood Acres Trout Farm (Campbellcroft, ON,

Canada). Fish were maintained for at least 2 weeks in 1185 L tanks at the University of Ottawa Aquatic Care Facility supplied with flowing and dechloraminated City of Ottawa water. Water was oxygen saturated and maintained at a temperature of 13°C. Fish were maintained under a constant 12:12 light-dark photoperiod and fed a commercial diet at 1% body weight daily. Fish feeding, handling and experiments were conducted under a protocol approved by the University of Ottawa Animal Care Committee (protocol number BL-190) under guidelines for the use of animals in research established by the Canadian Council on Animal Care.

2.3. Preparation of microsomal fractions

Livers were excised and homogenized in cold 50 mM Tris-HCl buffer containing 0.25 M sucrose and 1 mM EDTA (pH 7.5) using a straight Potter-Elvehjem-type glass homogenizer. Homogenates were centrifuged at 10,000 x g at 4°C for 15 min using a Beckman-Coulter microfuge R centrifuge. The resultant supernatants were centrifuged at 105,000 x g at 4°C for 60 min using a Beckman-Coulter Optima TL Ultracentrifuge. Resulting microsomal pellets were suspended by homogenization in a resuspension buffer comprising 50 mM Tris-HCl with 0.25 M sucrose, 5 M EDTA and 20% glycerol (pH 7.5). Microsomal protein concentrations were determined by the Bicinchoninic acid (BCA) assay (Sigma) with BSA as a protein standard. Microsomes were stored at -80°C until analyzed for CYP1A1 activity.

2.4. Experimental treatments

Toxicant exposures were carried out in 48-well plates and each reaction well contained 50 µg microsomal protein and 135 µl 50 mM sodium phosphate buffer (pH 8.0). Dose response curves for each of PCB-126 (10^{-3} to 10^{3} nM), BNF (10^{-4} to 10^{2} µM), RVT (10^{-}

^{1} to 10^{2} μM) and ANF (10^{-4} to 10^{2} μM) were established. Microsomes were incubated for 10 min with 5 μl CYP1A1 inducers PCB-126 and BNF, or the antagonists RVT and ANF prior to the EROD assay. To elucidate the effect of RVT exposure on CYP1A1 catalytic activity, microsomes were exposed to 2.5 μl PCB-126 (1 nM) or BNF (1 μM) for 10 min and incubated a further 10 min with 2.5 μl of varying concentrations of RVT (10^{-1} to 10^{2} μM). This was repeated with ANF in order to compare the mode of action of RVT with that of ANF.

2.5. EROD assay

Determination of EROD activity was adapted from the method established by Kennedy *et al.* (1993). EROD activity was estimated as the rate of production of the fluourescent product resorufin using a SPECTRAmax GEMINI XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) against a resorufin standard curve. To each reaction well, 10 μl 7-ethoxyresorufin (final concentration, 2 μM) was added and plates were incubated for 5 min at room temperature. Reactions were initiated by adding 40 μl NADPH (final concentration, 0.5 mM) and left to stand for 15 min (preliminary experiments showed linear reactions for at least 25 min). Reactions were terminated by addition of 100 μl cold acetonitrile. Resorufin concentrations were determined fluorometrically at 530 nm excitation and 590 nm emission wavelengths.

2.6. Statistical Analysis

All results are presented as means ± standard error of the mean (SEM). Results are graphed using SigmaPlot ^{TM} 10 (Systat Software, Inc.). All statistical analyses were done using SigmaStat^{TM} 3.1 (SPSS Corp., Chicago, IL, USA). Statistical significance was tested

using a One-way ANOVA. An appropriate post-hoc test was used where statistical differences were detected. A value of $p < 0.05$ was accepted as significant.

IC_{50} values (concentration resulting in 50% inhibition of EROD activity) were calculated for each separate experiment by linear regression curve fitting and determining the RVT concentration corresponding to 50% activity; a mean ± SEM was determined.

3. Results

3.1. Effect of Resveratrol on CYP1A1 activity

The effect of RVT on CYP1A1 activity was determined in rainbow trout microsomal preparations. To investigate whether RVT is a strong inhibitor of EROD activity, it was compared to the known CYP1A1 inhibitor ANF under the same experimental conditions. Comparisons of EROD activities are also examined in the presence of two CYP1A1 substrates BNF and PCB-126. All activities are represented as 100% of DMSO control. Average activity of the control values in all experimental groups was 3.7 ± 0.25 pmol/min/mg protein.

3.1.1. Effect of inhibitors in absence of AhR agonists/CYP1A substrates

RVT inhibited microsomal EROD in a dose-dependent fashion with an IC_{50} value of 43.9 ± 4.7 μM (Figure 2.2B). In comparison, ANF had an IC_{50} value of 51.6 ± 17.7 μM. Statistical analyses showed that approximately 100 μM ANF significantly inhibited EROD activity by 50% (Figure 2.2A).

3.1.2. Effect of PCB-126/BNF on microsomal CYP1A activity

Microsomal EROD activities were determined in the presence of PCB-126 and BNF (Figure 2.3A, B). BNF decreased EROD activity in a dose-dependent manner with 100 μM inhibiting more than 50%. No significant changes in EROD activity were observed at PCB-126 concentrations ≤ 1 nM. At both 10 and 1000 nM but not 100 nM PCB-126, there was a significant reduction in EROD activities compared to the DMSO control.

3.1.3. Effect of inhibitors in presence of AhR agonists/CYP1A substrates

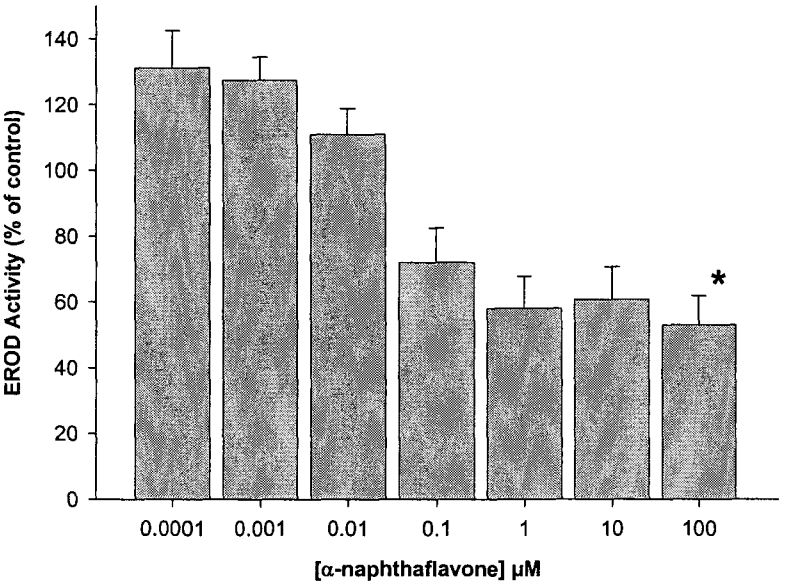
3.1.3.a. BNF

A concentration of 1 μM BNF was used for microsomal preparations. Two sets of experiments were conducted to compare inhibitory effects of ANF and RVT on BNF-treated microsomes (Figure 2.4A, B). ANF produced no significant inhibition or reduction in EROD activity compared with the DMSO control (Figure 2.4A). Concentrations of 50 and 100 μM RVT significantly inhibited EROD activity ($p<0.05$) (Figure 2.4B) with an IC_{50} value of 65.4 ± 1.8 μM.

3.1.3.b. PCB-126

A concentration of 1 nM PCB-126 was used for inhibition studies. As above, the inhibitory effects of ANF and RVT on PCB-126-treated microsomes were compared. Statistically significant ANF-inhibition of EROD activity is demonstrated in the presence of PCB-126 (Figure 2.5A). As shown in Figure 2.5A, approximately 50% inhibition was observed for ANF at 0.1 μM; no further decrease in activity occurred at higher ANF concentrations. In comparison and as observed from Figure 2.5B, RVT demonstrated more than 50% inhibition of EROD activity at 25 μM and activity continued to decrease at 100 μM (Figure 2.5B). IC_{50} values as determined from the graphs were 55.1 ± 6.8 μM and 49.3 ± 1.1 μM for ANF and RVT, respectively.

(A)



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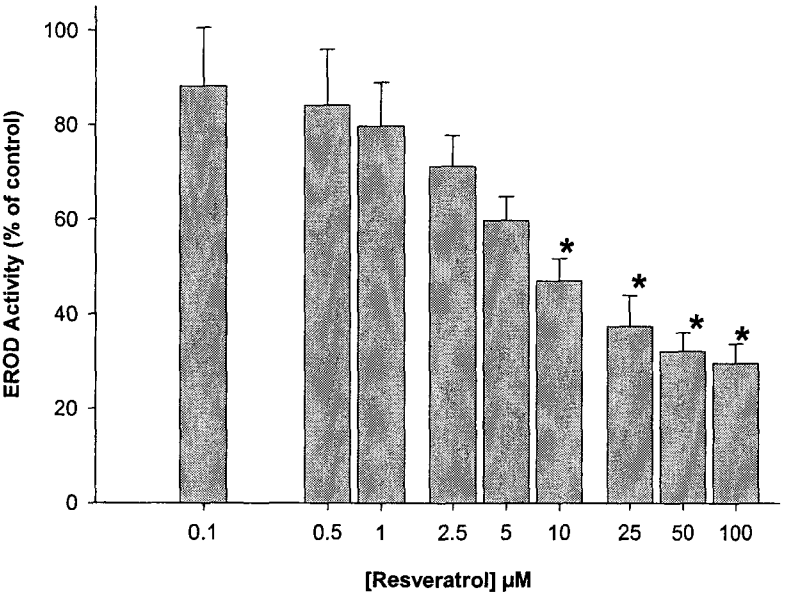
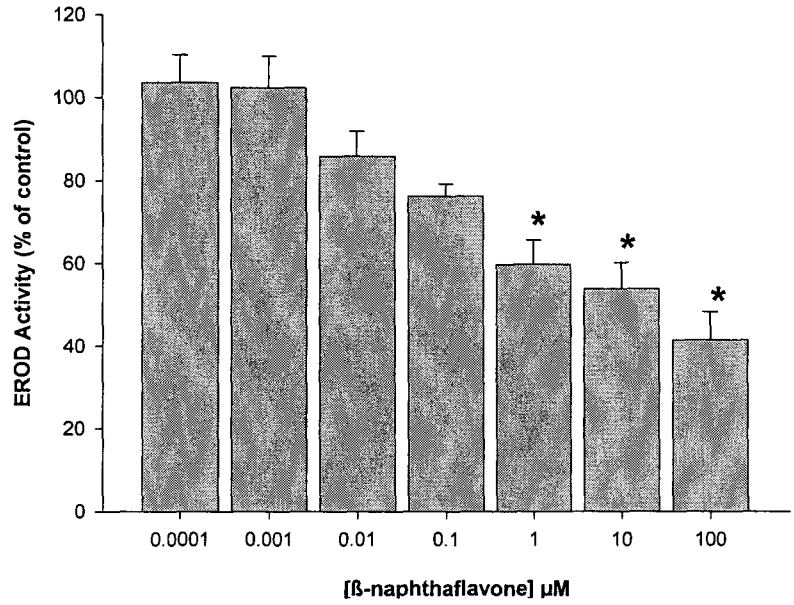


Figure 2.2: Inhibition of microsomal EROD activities in the presence of increasing concentrations of ANF (A) and RVT (B) relative to the DMSO control. Incubation and experimental conditions are as indicated in Materials and Methods. Data represent means + SEM (n = 6). One-way ANOVA on ranks followed by Dunn's test show a significant effect ($p < 0.001$) of treatments; stars indicate a significant effect compared to the DMSO control. IC_{50} values for ANF and RVT were calculated from linear regression curve fitting to be 51.6 ± 17.7 μM and 43.9 ± 4.7 μM, respectively.

(A)



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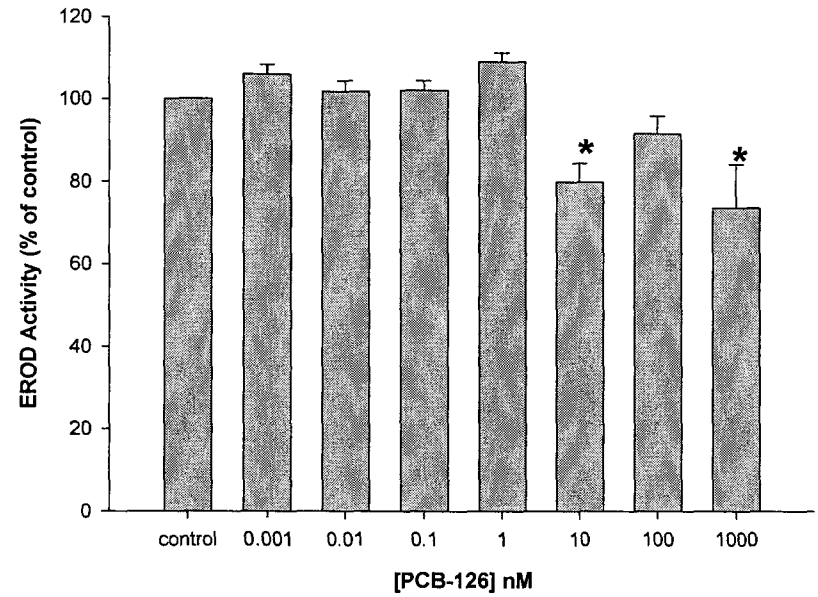
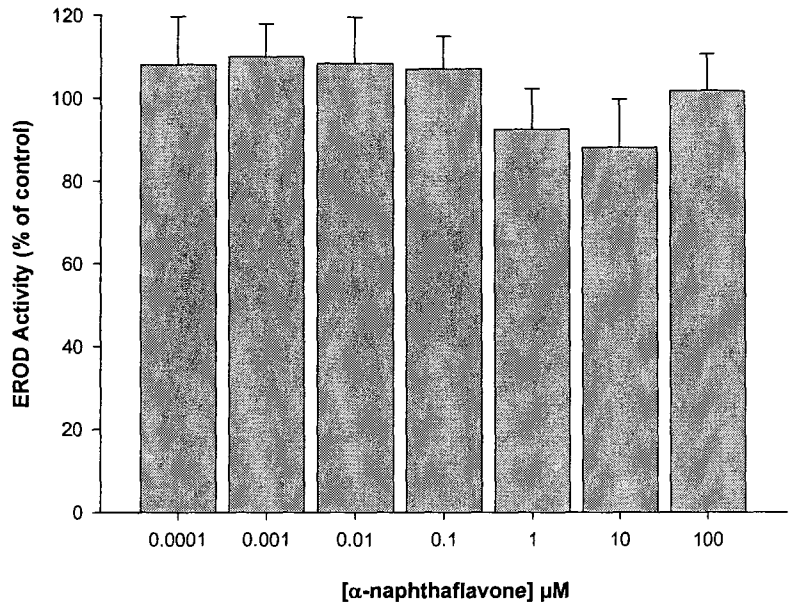


Figure 2.3: EROD activities of microsomes incubated with increasing concentrations of (A) BNF and (B) PCB-126. See Figure 2.2 legend for details; n = 5-6, stars indicate a significant effect ($p \leq 0.001$) compared to the DMSO control.

(A)



(B)

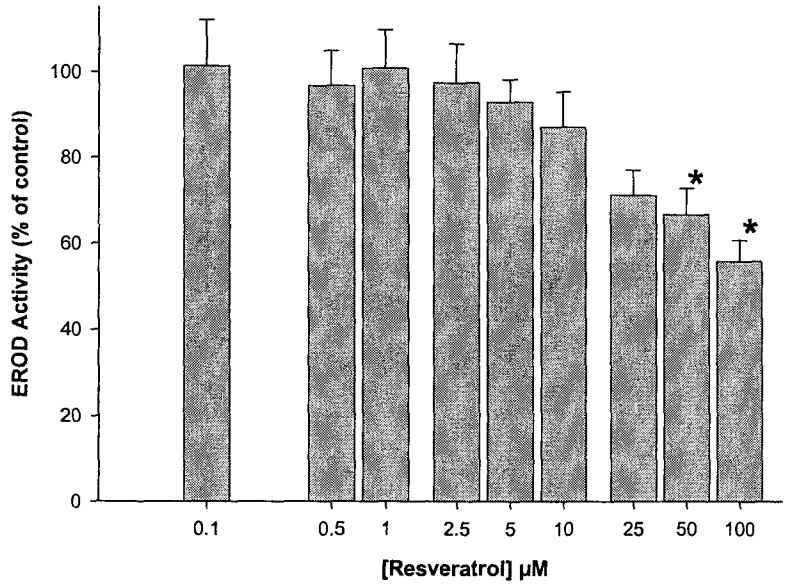
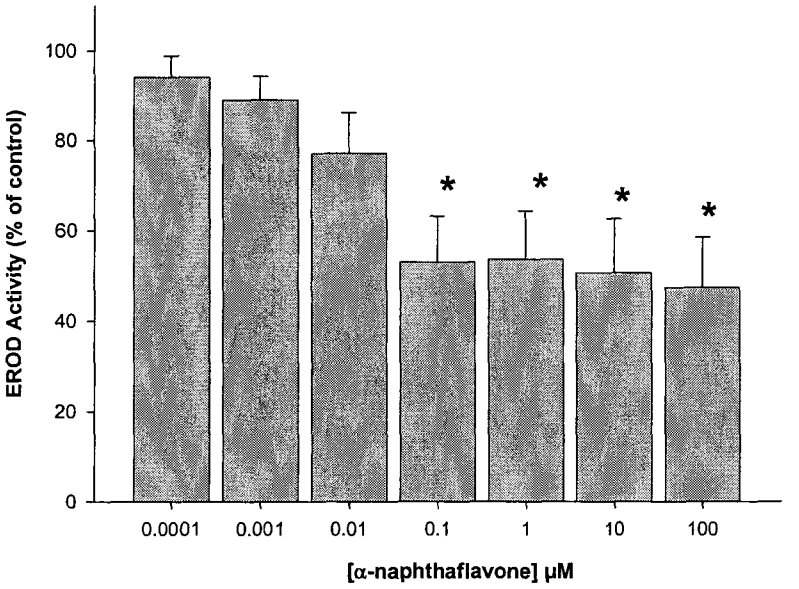


Figure 2.4: Inhibition of CYP1A activity in microsomal preparations treated with 1 μM BNF and increasing concentrations of (A) ANF and (B) RVT. See Figure 2.2 legend for details; n = 5-8). (A) No statistical difference was found with BNF+ANF co-exposure. (B) A significant effect compared to DMSO control ($p < 0.001$) was found for BNF+RVT.

(A)



(B)

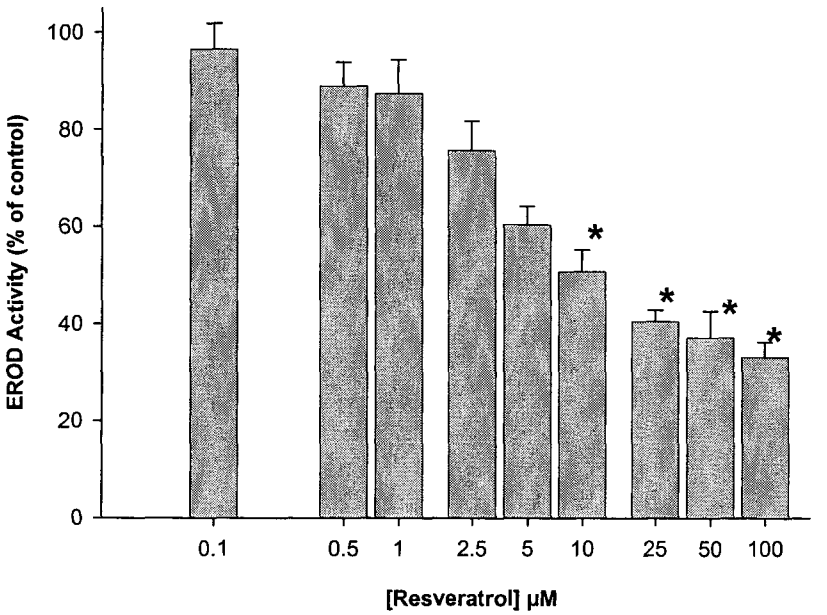


Figure 2.5: Inhibition of CYP1A activity in microsomal preparations treated with 1 nM PCB-126 and increasing concentrations of ANF (A) and RVT (B). See Figure 2.2 legend for details; n = 6, stars indicate a significant effect compared to the DMSO control.

3.2. Enzyme kinetic assay

Enzyme kinetic analysis was undertaken to investigate the responses of EROD activity to RVT inhibition and to determine the mode of RVT inhibition. Three concentrations (0, 25 and 100 μM) of RVT and 8 concentrations (0, 0.5, 1, 2, 2.5, 3, 4 and 5 μM) of the substrate 7-ethoxyresorufin (7-ER) were used. Analysis of inhibition kinetics by Lineweaver-Burk plots demonstrated that in the presence of RVT, the K_m values for 7-ER increased (Figure 2.6). Slope values also increased with a simultaneous reduction in V_{max}. A subplot of reaction rate versus substrate concentration shows increasing V_{max} values with increasing 7-ER concentration (Figure 2.6, subplot-b) supporting a mixed-inhibition kinetics model.

The plot (Figure 2.6) indicates that RVT inhibited trout microsomal EROD activities in a mixed-type inhibition (Chang *et al.,* 2001; Wang *et al.,* 2006). However, statistical analysis of individual K_m and V_{max} values (Figure 2.6) did not reveal a significant difference, therefore implying a competitive inhibition. This could be a result of the small sample size (n = 3). A mixed-type inhibition is a more acceptable scenario as determined from the graphical analysis using the Enzyme Kinetics Software in SigmaPlot. To calculate the apparent K_i value, a subplot of slope values (ratio of apparent K_m / V_{max}) against RVT concentrations was plotted and a K_i value of 46.7 μM was determined from the x-intercept (Figure 2.6, subplot-a).

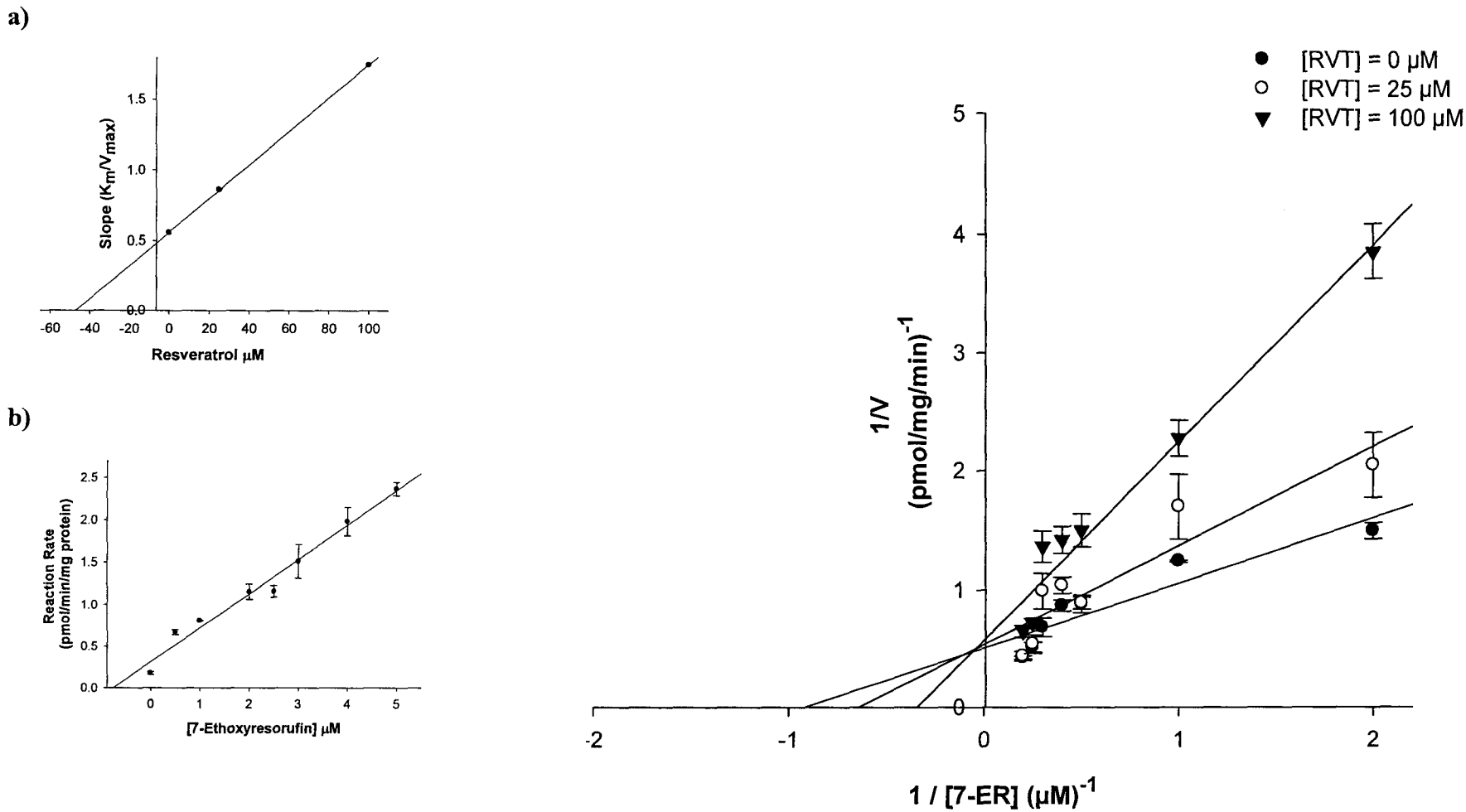


Figure 2.6: Inhibition kinetics analysis of RVT on EROD activities using a Lineweaver-Burk plot. EROD activity using 50 µg of rainbow trout microsomes was determined in the presence of no inhibitor (●), 25 µM (○) or 100 µM (▼) RVT. Values are means ± SEM (n = 3). Each data point represents the mean of three different microsome preparations, with each preparation done in triplicate. The insert (a) presents the graphical determination of $K_{i(RVT)}$. Saturation curve showing increasing V_{max} is shown in insert (b).

4. Discussion

Selective chemical inhibitors are important diagnostic tools for evaluating the contribution of particular cytochrome P450s in the metabolism of xenobiotics. In addition, specific receptor antagonists/CYP inhibitors play a key role in elucidating the consequential effects of receptor activation and their role in toxicity. Although a number of AhR antagonists have been identified, these also exhibit AhR-agonistic activities (Casper *et al.,* 1999; Zhang *et al.,* 2003). ANF is commonly used in fish models as an antagonist of AhR ligands including TCDD and BNF (Navas and Segner, 2000; Jos *et al.*, 2007) and is also a potent inhibitor of CYP1A-related activity. Miranda *et al.* (1998) examined the ability of several proven inhibitors of mammalian cytochrome P450s in rainbow trout hepatic microsomes (*in vitro*) and demonstrated that 100 μM ANF potently inhibited (more than 80%) CYP1A1 activity measured as dimethylbenz[*a*]anthracene hydroxylase (DMBA-OH). However, ANF was also shown to behave like an AhR agonist in fish (Aluru *et al.*, 2005). Aluru and colleagues showed that ANF induced CYP1A1 gene and protein expression in rainbow trout. Dose-dependent studies with trout hepatocytes resulted in significant CYP1A1 gene and protein expression at high ANF doses (1-10 μM) (Aluru *et al.*, 2005). However, this induction was less potent than the agonist BNF. ANF was also reported to inhibit TCDD-mediated induction of CYP1A1 gene and protein expression in rat hepatoma cells by competing for the AhR receptor binding sites therefore acting as a weak agonist (Timme-Laragy *et al.*, 2007).

Establishing a reliable chemical inhibitor of CYP1A activity was a main prerequisite to move forward with my overall project. Resveratrol (RVT), an established AhR antagonist and inhibitor of CYP-related activities in mammalian systems, was recently identified as a

reliable AhR antagonist in fish (Aluru and Vijayan, 2006). However, no studies are reported to ascertain direct inhibitory effects of RVT on CYP1A1 activity in rainbow trout or any piscine system. Therefore, the objective of this study was to define the mode of inhibition of RVT in rainbow trout microsomes *in vitro*. This report investigates the direct impact of AhR ligands and antagonists on CYP1A1/EROD activity in the absence of the AhR induction mechanism. Experiments were conducted in hepatic microsomal preparations from non-treated trout. A profile of the inhibition of RVT in hepatic microsomes was also undertaken to determine its effectiveness at an enzyme level, which was then compared against ANF a known inhibitor.

4.1. Effect of PCB-126/BNF on microsomal CYP1A activity

Most AhR ligands/CYP1A inducers (such as those employed in this study; PCB-126 and BNF) are also CYP1A substrates and their binding to the AhR results in induction of CYP1A phase-I enzymes and their subsequent transformation/metabolism into potentially more water-soluble products or carcinogenic metabolites (Vyas *et al.*, 1983; Bastien *et al.*, 1997; Sagredo *et al.*, 2006). Therefore, we investigated the effect of *in vitro* addition of PCB-126 and BNF on hepatic microsomal EROD activity and effects of co-treatments with inhibitors of EROD activity.

The results clearly demonstrate that at concentrations higher than 1 nM (10 – 100 nM), PCB-126 significantly inhibited EROD activity relative to control (Figure 2.3B). This is in agreement with dose-response curves obtained from hepatocyte cultures treated with varying concentrations of PCB-126 (Appendix-A, Figure A.1). Besselink *et al.* (1998) investigated the direct interference of PCB congeners with EROD activity in flounder hepatic microsomes and demonstrated that PCB-126 at concentrations higher than 1 nM exhibited

the same response and that the mode of this inhibition was competitive. High concentrations of typical CYP1A1 inducers (e.g. TCDD, PCB-126, PCB-77) are shown to inhibit EROD activity (Gooch *et al.*, 1989; Kennedy *et al.,* 1995; Hahn and Chandran, 1996; Hahn *et al.,* 1996; Bastien *et al.*, 1997) and this competitive inhibition is explained as the underlying reason behind the typical bell-shaped biphasic dose-response curves (Appendix-A, Figure A.1) of these inducers (Besselink *et al.,* 1998; Petrulis and Bunce, 1999). Incubation with BNF resulted in a more pronounced dose-dependent reduction in EROD activity (Figure 2.3A). BNF is documented to be metabolized by CYP1A1 (Vyas *et al.,* 1983) and therefore competes with the substrate 7-ER for the CYP1A1 binding site, therefore preventing its metabolism to resorufin which is the endpoint measured for the EROD assay.

4.2. Effect of RVT on microsomal CYP1A1 activity in absence/presence of PCB-126/BNF

Data from previous experiments (Appendix-A, Figure A.2) demonstrate the ability of resveratrol (100 μM) to significantly inhibit cellular EROD activity in rainbow trout hepatocyte cultures to approximately 50% in the absence / presence of PCB-126 and hence its ability to permeate through the cell membrane. Observed results are in agreement with the study by Aluru and Vijayan (2006), in which high doses of RVT significantly inhibited BNF-induced CYP1A and AhR protein expression in rainbow trout hepatocytes. This is similar to the CYP1A response reported in mammalian systems treated with RVT (0.1 – 10 μM) (Casper *et al.,* 1999; Lee and Safe, 2001; Revel *et al.,* 2001; Chen *et al.,* 2004). Ciolino *et al.* (1998) also reported that RVT potently inhibited basal and TCDD-induced CYP1A1 expression and activity. In another study by Ciolino and Yeh (1999), RVT in a dose-dependent fashion decreased the metabolism of benzo-*a*-pyrene to water-soluble metabolites in human HepG2 cell cultures by directly inhibiting CYP1A activity. The mode of action of

RVT in inhibiting induced CYP1A1 response in trout hepatocytes is yet to be elucidated. However studies in mammalian systems suggest that RVT acts via two potential mechanisms: (a) by directly inhibiting AhR-induced CYP1A1 enzymatic activity (Chun *et al.,* 1999; Ciolino and Yeh, 1999), or (b) by preventing the binding of the activated receptor (AhR-ligand complex) to the XREs of the CYP1A1 promoter region, hence inhibiting the signal transduction pathway without binding to the AhR itself (Ciolino *et al.*, 1998; Ciolino and Yeh, 1999). However, Casper *et al.* (1999) reported that RVT was able to compete with TCDD for AhR binding in the same cell type (human HepG2). These different results were attributed to differences in experimental methodology.

RVT in trout microsomes significantly inhibited (relative to control) EROD activities *in vitro*. At 100 μM, RVT inhibited (IC_{50} = 43.9 ± 4.7 μM) approximately 70% of the EROD activity while 100 μM ANF inhibited about 50% (Figure 2.2). Table 2.1 summarizes IC_{50} values for RVT from various experimental models. An analysis of the kinetics of enzyme inhibition using a double-reciprocal plot (Figure 2.6) suggests that the RVT inhibition was of the mixed type (Chun *et al.,* 1999; Wang *et al.,* 2006). This suggests that RVT competes for substrate binding site with 7-ER and binds to a different site from the substrate.

Table 2.1: Comparison of IC_{50} values (RVT concentration resulting in 50% inhibition of CYP activity) reported for mammalian cell types and the present study. Values are in μM.

Source	IC_{50}	Reference	Mode of Inhibition
Human HepG2 (CYP1A)	< 1	Ciolino *et al.*, 1998	Not determined
Human Recombinant CYP1A	23	Chun *et al.*, 1999	Mixed
Human HepG2 (CYP1A)	1	Ciolino and Yeh, 1999	Competitive
Human Recombinant CYP1A	-	Chang *et al.*, 2001	Mixed
Human MCF-10A (CYP1A)	2	Chen *et al.*, 2004	Not determined
Human MCF-7 cell line (CYP19)	25	Wang *et al.*, 2006	Mixed
Rainbow Trout Microsomes	43.9 ± 4.7	Present Study	Mixed

There are a number of scenarios to explain the above differences including: (1) responses were dependent on the cell context or experimental system used, (2) different concentrations of the substrate, 7-ER, influenced displacement by RVT.

RVT also inhibited EROD activity in microsomal preparations treated with 1 μM BNF and 1 nM PCB-126 (Figure 2.4B, 2.5B); these concentrations resulted in maximal EROD activities in trout hepatocyte cultures (Appendix A, Figure A.1; Aluru and Vijayan, 2004). In comparison, ANF exposure caused a concentration-dependent decrease in PCB-126 treated preparations resulting in the same inhibition profile of ANF treatments alone (Figure 2.2A). This further supports that at lower concentrations PCB-126 is not a competitive inhibitor of EROD activity and does not compete with either ANF or 7-ER for the substrate

binding site. Co-treatments with BNF resulted in no changes in EROD activity relative to control. This indicates that both ANF and BNF compete for the same binding site on the enzyme preventing the binding and subsequent metabolism of the substrate 7-ER.

RVT is reported as a selective human P450 1A1 inhibitor (Chun *et al.,* 1999). The extent of specificity in piscine models needs further experimental investigation. This work was conducted primarily to evaluate the ability of RVT to inhibit CYP1A activity and gain insight on the effects of co-treatments with established substrates of CYP1A. Results of this study in combination with other work (Aluru and Vijayan 2004; 2006) support the use of RVT as an AhR antagonist/CYP1A inhibitor for the main objective of this thesis.

CHAPTER 3 - Energetic Costs of Toxicant Exposure in Rainbow Trout (*Oncorhynchus mykiss*): An *In Vivo* Study

1. Introduction

Persistent contaminants in the environment include a class of pollutants called persistent organic pollutants or POPs. These include a range of by-products of anthropogenic activities including polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) and polychlorinated biphenyls (PCBs). Their persistence in most environmental compartments and living tissues and their resistance to degradation or biotransformation is mostly attributed to their chemical structures and hydrophobic nature (van der Oost *et al.*, 2003; Evans *et al.*, 2005). It is established that dioxins and dioxin-like compounds share a common mechanism of toxic action which is mediated by their binding to the aryl hydrocarbon receptor (AhR) and in turn initiating subsequent toxic responses (Nebert *et al.*, 1993; Safe, 2001; Van den Berg *et al.*, 2006). The presence of these contaminants triggers specific physiological responses that have the potential of influencing the behavior and physiology of individuals in an ecosystem and ultimately their survival (Jobling 1994; Fent, 2003; Scott and Sloman, 2004).

Stress induced by prolonged chemical exposure initiates a hierarchy of responses that are characterized by the 'General Adaptation Syndrome' (GAS) (Beyers *et al.*, 1999a). This model considers the sequence of responses or changes that occur post-exposure, starting with physiological changes to re-establish homeostasis and to compensate for the chemical burden, and escalating with time and severity beyond tolerance limits (physiological and biological) to become injurious (Jobling, 1994; Wendelaar Bonga, 1997; Beyers *et al.*,

1999a; Francis-Floyd, 2002; Scott and Sloman, 2004). These changes are associated with: (1) changes in the endocrine system leading to release of catecholamines and cortisol into the blood stream; (2) mobilization of energy reserves induced by endocrine changes; and, (3) changes in pathology, reduced reproduction success and growth (Mayer *et al.,* 1992; Jobling, 1994; Wendelaar Bonga, 1997; DuRant *et al.*, 2007).

As outlined in Chapter 1 and as suggested by trends in contaminated systems (Beyers *et al.,* 1999b; Sherwood *et al.*, 2000; Marchand *et al.*, 2004), pollutant exposure and the resultant physiological responses/detoxification mechanisms could imply an energetic cost on the system and depletion of energy reserves (Mayer *et al.*, 1992; Berntssen and Lundebye, 2001). This could result in reallocation of available and limited energy resources from processes such as maintenance and reproduction (Brett and Groves, 1979; Handy *et al.*, 1999; DuRant *et al.*, 2007) to compensate for the increased metabolic cost of detoxification (Odum, 1985; Wilson, 1988; Calow, 1991). This implies that an adaptive response is directly reciprocated by a loss in another variable (Calow, 1991; Jobling, 1994; Beyers *et al.*, 1999a; Handy *et* al., 1999; Lochmiller and Deerenberg, 2000; DuRant *et al.*, 2007). However, quantifying the extent of loss or its significance to the overall survival of the animal in a chemically challenging environment has not yet been clearly established (Knops *et al.*, 2001; Bains and Kennedy, 2004).

Several studies report an alteration in animal and cellular energetics in response to chemical exposure. Rissanen *et al.* (2003) studied energetic changes in rainbow trout hepatocytes in the presence of dehydroabeitic acid (DHAA). They showed that the presence of DHAA resulted in an imbalance in the amount of ATP produced to that consumed in addition to increased glycolytic activity and a concentration-dependent increase in cellular

respiration. Studies on rainbow trout, European eel and Atlantic salmon also showed that chemical-induced stress led to changes in oxygen consumption and disrupted the mobilization and storage of glycogen, lipid and protein (Holmberg *et al.*, 1972; Gimeno *et al.,* 1994; Sancho *et al.*, 1998; Handy *et al.*, 1999; Berntssen and Lundebye, 2001; Brodeur *et al.*, 2001; Fabbri *et al.*, 2003). Chemicals used ranged from metals such as cadmium and copper to biocides such as dieldrin and endosulfan. The physiological mechanisms by which these toxicants affect metabolism are not fully understood. However, a number of studies suggest that these changes are a result of direct toxicant interference with activities of metabolic enzymes (Scott and Sloman, 2004).

Reduction in growth under pollutant stress is also reported in a number of species including juvenile rainbow trout (Helder, 1981), southern armyworm (Cresswell *et al.*, 1992), yellow perch (Sherwood *et al.*, 2000) and *Daphnia* (Knops *et al.*, 2001). The reduced growth rates reported were not a result of decreased food assimilation but rather attributable to a reduced efficiency in energy allocation or metabolism (Cresswell *et al.*, 1992; Sherwood *et al.*, 2000; Knops *et al.*, 2001).

In cases where exposure does not directly affect metabolic rate, tradeoffs between detoxification processes and essential bodily functions may occur. Handy *et al.* (1999) chronically exposed rainbow trout to dietary copper. The study was conducted to integrate changes in biochemical, morphological and physiological parameters with the responses and compensatory changes in trout to sub-lethal oral exposure to copper. They reported possible trade-offs between the metabolic cost of detoxification and physiological process essential to the organism's survival.

A broad range of studies exist demonstrating energetic costs in fish associated with swimming, immune responses, reproduction and growth. However, limited information exists on energetic costs of detoxification, or initiation of defense mechanisms, in fish. Studies done to determine the metabolic costs of detoxification are inconclusive since the presence of the xenobiotic may inhibit feeding and consequently decrease the amount of energy assimilated (Bains and Kennedy, 2004).

This study examines the effects of two CYP1A1 inducers, 3,3',4,4',5-pentachlorobiphenyl (PCB-126) and β-naphthaflavone (BNF) on CYP1A1 activity and mRNA expression in rainbow trout, *Oncorhynchus mykiss*. The objective of the study is to determine whether an increase in detoxification processes will change the metabolism of energy reserves. An *in vivo* approach was adopted to examine the following endpoints: 1) hepatic CYP1A activity estimated as ethoxyresorufin-*O*-deethylase (EROD) in microsomal preparations; 2) whole animal oxygen consumption rates; and, 3) glycogen content in hepatic tissue and glucose and triglyceride concentrations in plasma. In addition, I examine whether changes in CYP1A1 induction are correlated with these metabolic changes. This will be achieved by employing a known mammalian AhR antagonist, resveratrol (RVT). Recent studies by Aluru and Vijayan (2006) reported that RVT is also an AhR antagonist in rainbow trout. Results from Chapter 2 indicate that RVT is also a potential inhibitor of CYP1A1 activity.

2. Materials and Methods

2.1. Chemicals

For a list of materials used refer to Chapter 2. 3,3',4,4',5-pentachlorobiphenyl (PCB-126) was donated by Dr. Sean Kennedy at the National Wildlife Research Centre (NWRC, Ottawa, ON).

2.2. Fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada) and held at the University of Ottawa Aquatic Care Facility. All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Committee and adhere to the guidelines established by the Canadian Council on Animal Care for the use of animals in research and teaching.

2.3. Experimental design

Fish were acclimated to laboratory conditions for a minimum of 1 month before the start of the experiment. Fish were then randomly separated into groups of 10-12 fish per tank (115 L capacity) for two days prior to exposure. The design is depicted in Figure 3.1.

Fish were lightly anaesthetized with benzocaine (0.005 mg/L or 2 ml of 10 mg/L stock in 4 L water). Control groups all received intraperitoneal injections (IP) of the vehicle alone (coconut oil; Sigma). Exposed groups were injected IP with: i) PCB-126 in coconut oil at 50 μg PCB per kg fish; ii) BNF in coconut oil at 50 mg BNF per kg fish; iii) RVT in coconut oil at 100 mg RVT per kg fish; iv) PCB-126 and RVT; or, iv) BNF and RVT. Experiments with fish injected with BNF alone or BNF+RVT were undertaken 3 days after injection as previous studies with trout showed maximal P450 induction at day three of

exposure (Wilson *et al.*, 1998). Fish injected with PCB-126 alone or PCB-126+RVT were sampled ten days after injections. Each experimental period (3d and 10d) contained an RVT control group.

Previous studies demonstrated that with continued exposure to toxicants, fish eat less (Mathers *et al.*, 1985; Palanivelu *et al.*, 2005). Therefore, the control and treated fish were pair-fed to ensure that both groups were provided with the same ration. Exposed fish were fed at approximately 1% body weight daily for the duration of exposure; depending upon the mass of food consumed, an equal mass of food was then fed to the control group.

2.4. Tissue and plasma collection

At the end of the exposure periods, fish were anaesthetized using benzocaine (concentration as detailed above). Blood was sampled immediately by caudal puncture using a heparinized 1 ml syringe fitted with a 26-gauge needle. Fish were then euthanized by a sharp blow to the head. The excised liver was divided into 4 portions for subsequent assays, frozen in liquid N_2 and kept at -80°C until analyzed.

Blood samples were centrifuged at 12,000 x g for 10 min to separate plasma from red blood cells. Plasma was stored at -80°C in aliquots until analyzed for glucose and triglyceride contents (see below, 2.6).

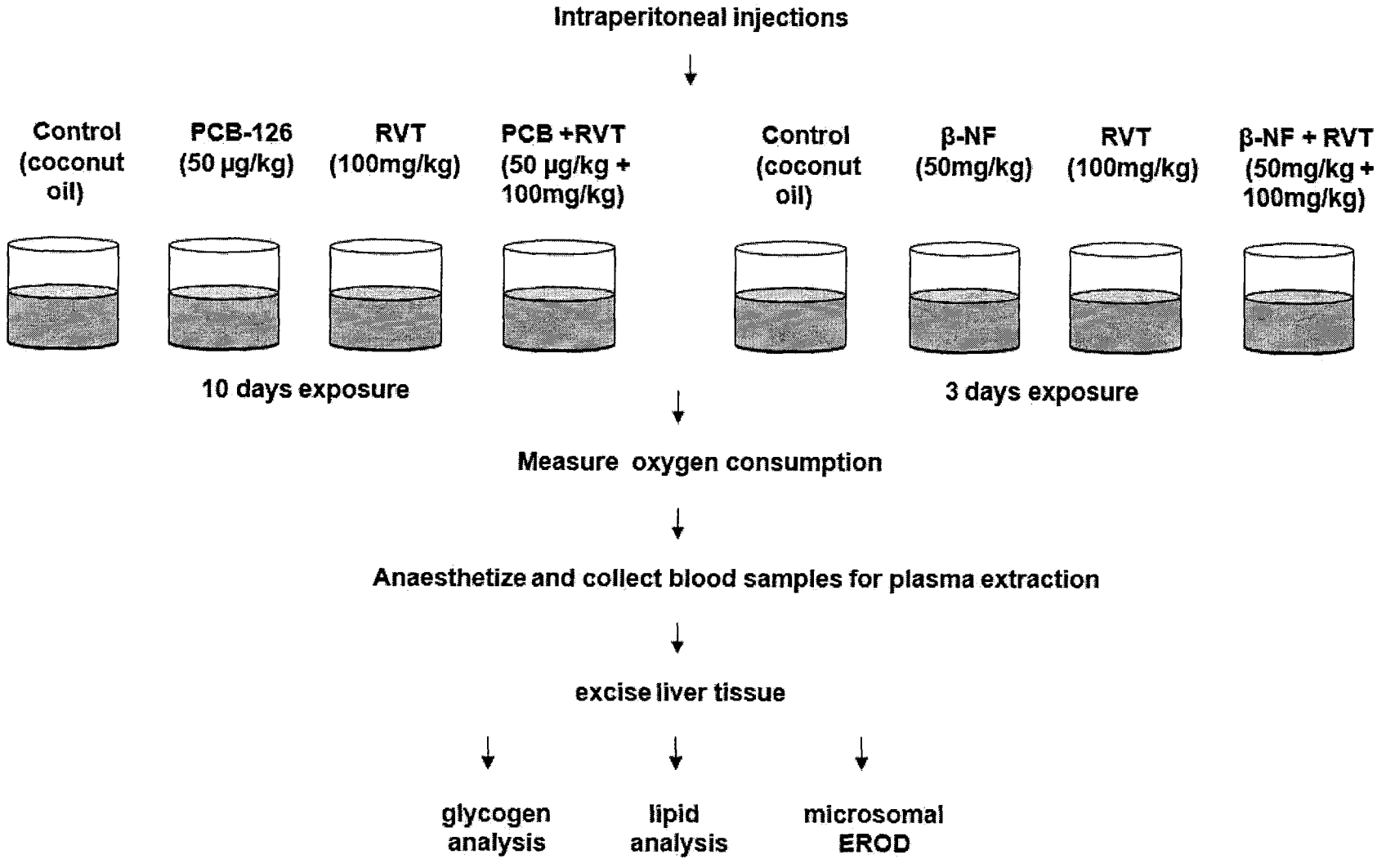


Figure 3.1: Schematic illustrating the experimental design of the *in vivo* study.

2.5. Preparation of microsomal fractions

Liver microsomes were prepared as noted in Chapter 2. Protein concentrations of microsomal preparations were assayed using the BCA assay as stated in Chapter 2. Microsomal fractions were stored at -80°C until analyzed for CYP1A1 activity.

2.6. Metabolic endpoint assays

The following were assessed at room temperature using a SPECTRmax PLUS 384 microplate spectrophotometer (Molecular devices, Sunnyvale, CA).

2.6.1 Glucose

This assay monitors the formation of NADH produced from the coupled reaction of glucose phosphorylation by hexokinase and reduction of glucose-6-phosphate (G6P) by G6P dehydrogenase (G6PDH) and NAD^+. The resulting increase in absorbance at 340 nm is directly proportional to glucose concentration in the sample. Glucose concentrations were quantified against a standard curve of D-glucose. Ten µl samples and standards were loaded into 96-well plates. Two-hundred µl reaction mixture (pH 8.0) consisting of 60 mM Trizma base, 40 mM Tris-HCl, 1 mM $MgSO_4 \cdot 7H_2O$, 2 mM NAD^+, 1 mM ATP and 0.1 units/ml G6PDH (*Leuconostoc mesenteroides*; Sigma), was added to each well. Absorbance at 340 nm was measured to give blank reading followed by the addition of 10 µl hexokinase (HK) solution (0.06 units). The plate was incubated for 30 min at room temperature and a second spectrophotometric reading was taken; the blank reading was subtracted from this second reading before calculating glucose concentrations against the standard curve.

2.6.2 Glycogen

Liver glycogen was assayed by measuring the production of glucose after breakdown of glycogen with amyloglucosidase. The difference between glucose content before amyloglucosidase hydrolysis and after was determined and glycogen concentrations expressed as glucose equivalents. Frozen liver tissue (approximately 150 mg) was ground on dry ice and homogenized in ice-cold 6% perchloric acid (PCA) at 4:1 (vol/wt) using a Kontes Micro Ultrasonic Cell Disrupter (Mandel Scientific). Homogenates were then centrifuged at 10,000 x g for 5 min. One-hundred µl of the resulting supernatant was incubated for 2 h at 37°C with 50 µl 1 M $NaHCO_3$ and 1 ml of 1 mg/ml amyloglucosidase in acetate buffer (pH 4.8). The reaction was terminated by the addition of 25 µl 70% PCA. The reaction mixture was centrifuged at 7000 x g for 5 min and the supernatant was used for the glucose assay as described above.

2.6.3 Triglycerides

Determination of total plasma triglycerides was done using the 'Serum Triglyceride Determination Kit' (Sigma) according to the manufacturer's instructions.

2.7. Flow-through respirometry

Oxygen consumption measurements were undertaken using the method established by Metcalfe *et al.* (1999). The flow-through respirometer (Fig. 3.2) comprised clear Perspex tubes (respiration chambers), closed at either end, 30.5 cm in length by 10 cm in diameter. Openings were placed at the closed ends for water inflow and outflow. Each chamber was supplied with 13°C dechloraminated water from a main tank that was circulated through an

air column to ensure complete O_2 saturation. Flow rates were regulated by screw-clamps placed on the inflow tubes going into each respiration chamber.

Three fish were randomly removed from the exposure tanks and placed into individual flow-through respirometer units. The fish were allowed to acclimate overnight with black plastic sheeting placed over the chambers to reduce any added stress or disturbance to the fish during this period. O_2 consumption measurements were conducted after approximately 24 h in the units.

During each measurement period and for each respirator chamber, the partial pressure of oxygen (pO_2) was measured in the inflowing water and then in the out- flowing water. For every measurement of water pO_2, the flow rate through the chamber was kept constant and measured by weighing the amount of water from the outflow of each tube during that interval (15 sec). pO_2 measurements were made by drawing water across an oxygen electrode connected to an OM-200 oxygen meter (Cameron Instruments, Port Aransas, Texas, USA).

The O_2-meter output was then fed into the analog input of a Biopac MP150WSW Data Acquisition System (Biopac Systems, Inc. California, USA) that was connected to a computer running AcqKnowledge v.3.7.3 data acquisition software (Biopac Systems, Inc.). The above pO_2 measurements were repeated three times and averaged. This allowed for a more accurate estimation of metabolic rate by accounting for any possible activity during the measurement interval. The flow rates were adjusted so as to result in a pO_2 drop of 10-20 torr while ensuring that the fish were inactive. Since the MO_2 of each fish was measured while they were in a quiescent state in the respiration chamber, the MO_2 obtained is a reflection of the fish's resting metabolic rate.

O_2 data analysis

O_2 consumption of each fish was calculated by measuring the reduction in O_2 concentration of fully aerated water flowing past a stationary fish. To obtain the drop of pO_2 in the water and thus the oxygen consumed by the fish, the average outflow pO_2 was subtracted from the inflow pO_2.The formula used to calculate mean O_2 consumption (in mmol O_2/h) is:

$$MO_2 = (\Delta pO_2)\ (\alpha_{O2})\ (\text{Flow Rate})$$

where,

α = Solubility coefficient of O_2 in water @ 13°C (0.002907 mmol/L/torr)

ΔpO_2 = (Average ouflow pO_2 – inflow pO_2) torr

Flow rate = Volume of water collected (L) / 15 s

2.8. EROD assay

Fifty µg microsomal protein from livers of the different treatment groups were added to 48-well plates. EROD activity was measured as stated in Chapter 2. Briefly, 150 µl sodium phosphate buffer (50 mM, pH 8.0) was added to each well. Ten µl 7-ethoxyresorufin (2 µM final concentration) was added and the plate incubated at room temperature for 10 min, followed by the addition of 40 µl NADPH (0.5 mM, final concentration) to initiate the reaction; the plate was left for 15 min. Reactions were terminated by addition of 100 µl cold acetonitrile and resorufin was measured as noted in Chapter 2.

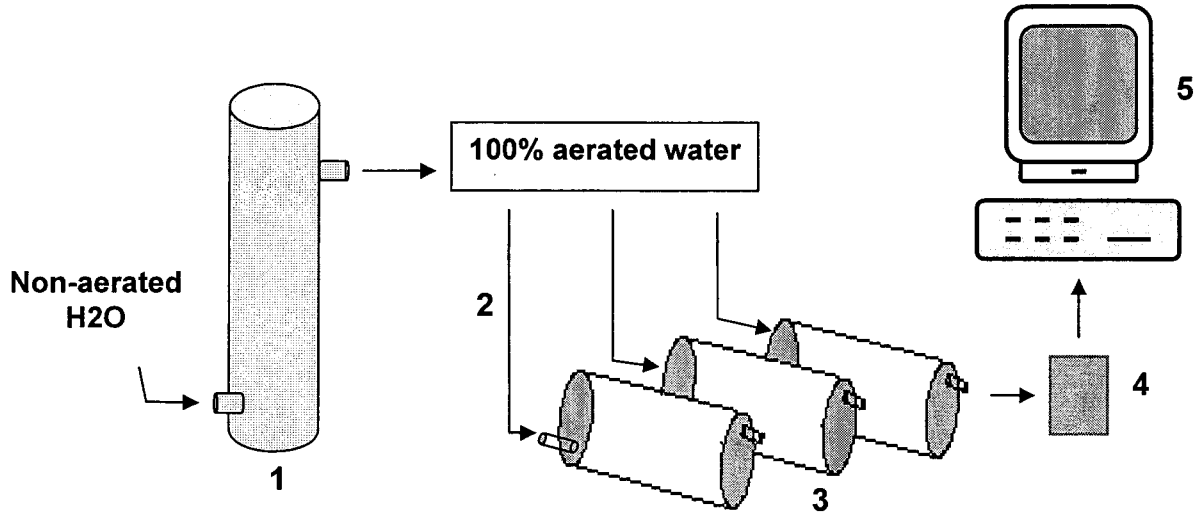


Figure 3.2: A schematic diagram of the flow-through respirometry system used to measure oxygen consumption of exposed fish groups (PCB-126, BNF, RVT, PCB / BNF + RVT) and their respective controls. (1) Air column, (2) fully aerated water going in through inflow tubes, (3) respiration chambers housing fish, (4) O_2-measurement assembly: O_2 probe and O_2 meter; and (5) acquisition software displaying pO_2 data.

2.9. CYP1A1 mRNA expression

2.9.1 RNA isolation and DNase treatment

Total RNA was isolated from exposed fresh trout livers using TRIzol (Invitrogen, Life Technologies, Burlington, ON, Canada) as per the manufacturer's instructions. Concentration and quality of RNA was determined spectrophotometrically at 260 nm and RNA gel electrophoresis, respectively. To avoid DNA contamination, isolated RNA was DNase treated following the manufacturer's instructions using amplification grade DNase I (Invitrogen).

2.9.2 cDNA synthesis

Equal amounts of total RNA (1 μg) were used for all samples. Treated RNA was reverse transcribed to cDNA using a first-strand cDNA synthesis kit according to the manufacture's instruction (Invitrogen). Random primers were used and reverse transcription was done using M-MLV RT.

2.9.3 Primers

An optimized rainbow trout CYP1A1 primer (Aluru and Vijayan, 2006) was synthesized (Invitrogen) to amplify ~104 bp products. A β-actin (GenBank accession no. AF157514) primer was designed and synthesized (Invitrogen) to amplify an ~200 bp product. Primer sequences used were:

CYP1A1	(F) 5'-GATGTCAGTGGCAGCTTTGA-3'
	(R) 5'-TCCTGGTCATCATGGCTGTA-3'
β – actin	(F) 5'-CGTCCCAGGCATCAGGGAGT-3'
	(R) 5'-TCTCCATGTCGTCCCAGTTG -3'

2.9.4 Real-time PCR

Real-time PCR was undertaken using a Stratagene MX3100 using the following conditions: 95°C for 15 min; 40 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 30 s (CYP1A1 and β-actin) and final extension at 72°C for 30 s followed by a 4°C hold.

2.9.5. mRNA data analysis

The relative mRNA expression of the gene of interest (CYP1A1) was determined by comparing the different Ct (cycle number) values between the gene of interest and that of the internal control. Using β-actin as the reference gene, PCR results are reported as **2^(-ΔCt)**, where ΔCt = difference between cycle numbers (Ct) for CYP1A1 and β-actin.

2.10. Statistical Analysis

All experimental results are presented as means ± standard error of the mean (SEM). A value of $p < 0.05$ was accepted as significant. SigmaStat™ 3.1 (SPSS Corp., Chicago, IL, USA) was used to evaluate statistical significance using a One-way ANOVA. An appropriate post ad-hoc test was used where statistical differences were detected.

3. Results

The effects of PCB-126 and BNF on CYP1A activity and energy metabolism were determined from excised livers of toxicant-treated juvenile rainbow trout. The two toxicants were used for comparative purposes under the same experimental conditions. Investigated parameters in the study are also examined with co-treatments with 100 mg/kg of RVT, an AhR antagonist and inhibitor of CYP1A activity.

Since assays of the experimental treatments were run on separate days, each exposed group was run in parallel with a control group (IP injection with vehicle; coconut oil) for the same exposure duration; i.e., 3 and 10 days for BNF and PCB exposures, respectively. As no statistical differences were identified between means of controls within each exposure period, the data means from the respective control tanks were pooled for graphical presentation and statistical analysis where applicable (i.e., this was not done in all cases since I had enough control data from one set); n-values represent the number of control tanks used.

3.1. Relative CYP1A1 mRNA Expression

The relative expression of CYP1A1 was examined in liver from trout injected with vehicle, RVT, PCB-126 and BNF. Statsicial analysis indicated a significant effect of treatments on relative mRNA expression (Fig. 3.3). Relative mRNA expression in livers from PCB-126 treated trout was significantly higher when compared with control and RVT treatments (Fig. 3.3).

3.2. Effects of AhR agonists on CYP1A1 Enzyme Activity (EROD)

3.2.1. Effect of PCB-126 and BNF on EROD activities

Intraperitoneal treatment of rainbow trout with 50 μg PCB-126 per kg elicited a significant increase in hepatic EROD activity (Fig. 3.4A). CYP1A activity was about 8-fold above the control level 10 days after treatment. Administration of 50 mg BNF per kg similarly significantly increased hepatic EROD compared with the control fish (Fig. 3.4B).

3.2.2. Co-treatments with 100 mg/kg RVT

In vivo exposures to the AhR agonists PCB-126 and BNF (Figure 3.4) were run in parallel with exposures to RVT at the two incubation periods for PCB-126 and BNF of 10 days and 3 days, respectively. This was done to examine the effect of RVT on the experimental parameters in the absence of the agonist. No difference in EROD enzyme activity was observed between RVT-treated and control groups after the treatment period (Figure 3.4A, B). RVT co-treatment with PCB-126 did not reduce EROD activities compared with PCB-126 alone (Fig. 3.4A). However co-treatment with BNF resulted in a significant reduction of activity compared with the BNF treatment alone (Fig. 3.4B).

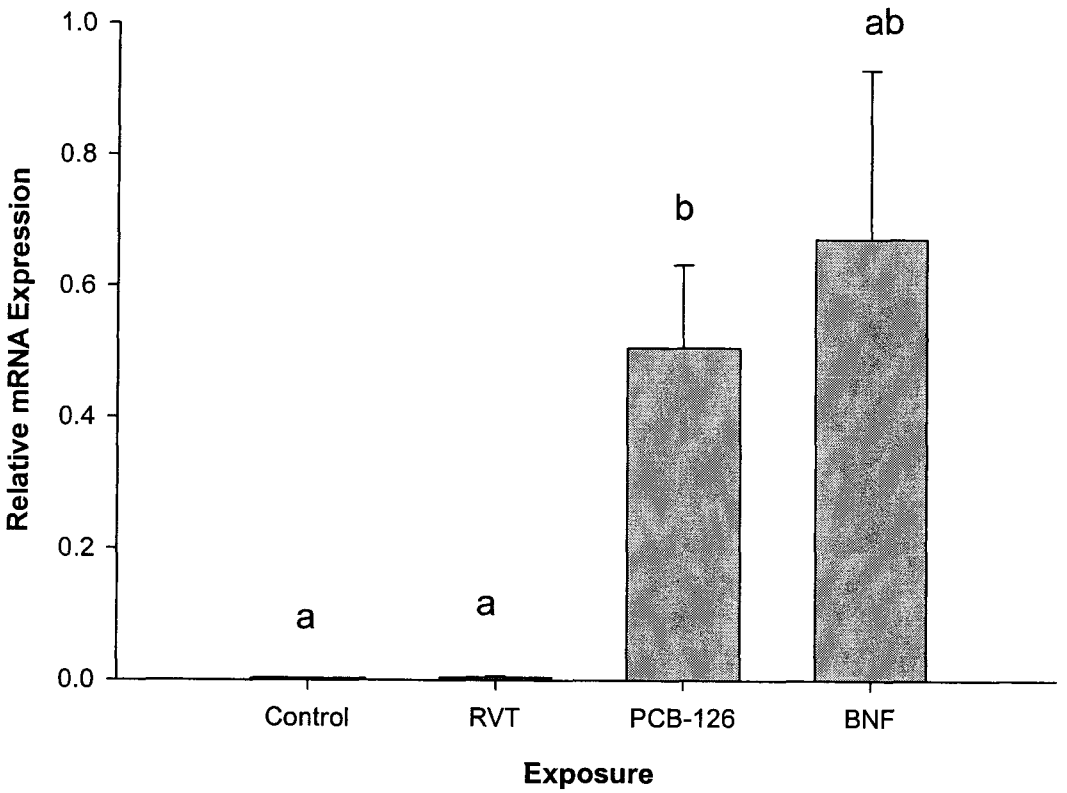


Figure 3.3: CYP1A1 relative mRNA expression (compared with β-actin) in livers taken from control groups and rainbow trout injected IP with 100 mg/kg RVT or 50 μg/kg PCB-126 or 50 mg/kg BNF. One-way ANOVA on ranks followed by Tukey's pair-wise multiple comparison test show a significant effect of treatment ($p = 0.04$). All incubations and experimental conditions are as indicated in Materials and Methods. Graphical data represent means + SEM ($n = 3$). Different letters indicate significant differences between treatments.

3.2.3. Effect of RVT administration in vitro on hepatic EROD activities

To determine the impact of RVT on EROD activities in hepatic tissue isolated from exposed trout, 100 μM RVT was incubated with hepatic microsomes extracted from exposed fish. RVT resulted in a significant reduction of hepatic EROD activities to the control level in PCB-126 and PCB-126 + RVT exposed fish (Fig. 3.5A, B). The same was observed with treatments of BNF and BNF + RVT treated fish (Fig. 3.6A, B).

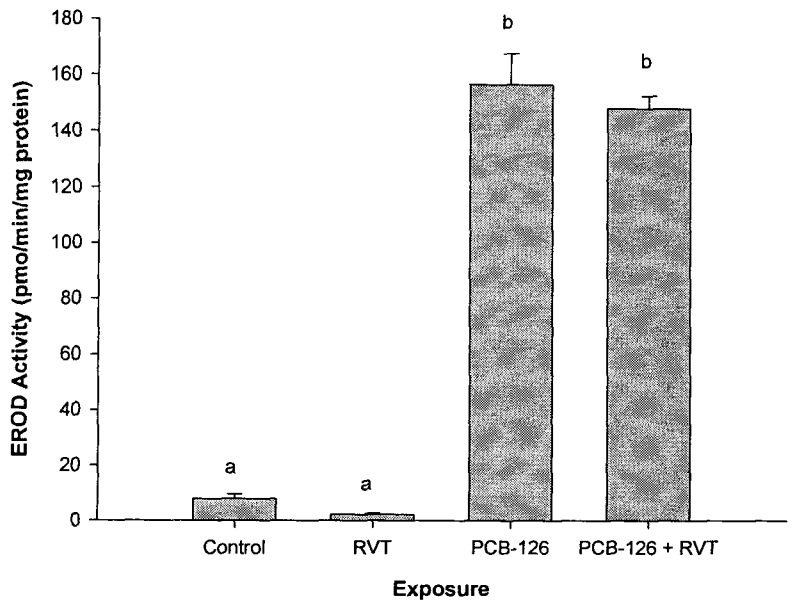
3.3. O_2 consumption

Indirect calorimetry using flow-through respirometry was used to estimate O_2 consumption (MO_2) and thus aerobic metabolic rates. Resting metabolic rate was calculated as detailed in Materials and Methods (see section 2.7). Figure 3.7 presents changes in mass specific O_2 consumption as a function of weight of all test animals. Linear regression analysis indicates that any observed changes in O_2 consumption are presumably a result of treatment rather than fish mass.

Although there was a general increase in O_2 consumption with treatment, there was no statistically significant change in O_2 consumption with PCB-126 treatments compared with the control (IP injection with control vehicle; coconut oil) (Fig. 3.8A).

BNF and BNF + RVT exposure significantly increased O_2 consumption compared with the control group ($p = 0.007$). No change was observed between BNF and BNF + RVT treated groups. Both treatments resulted in an O_2 consumption rate of 2.7 ± 0.26 and 2.7 ± 0.12 mmol O_2/kg/h, respectively.

A)



B)

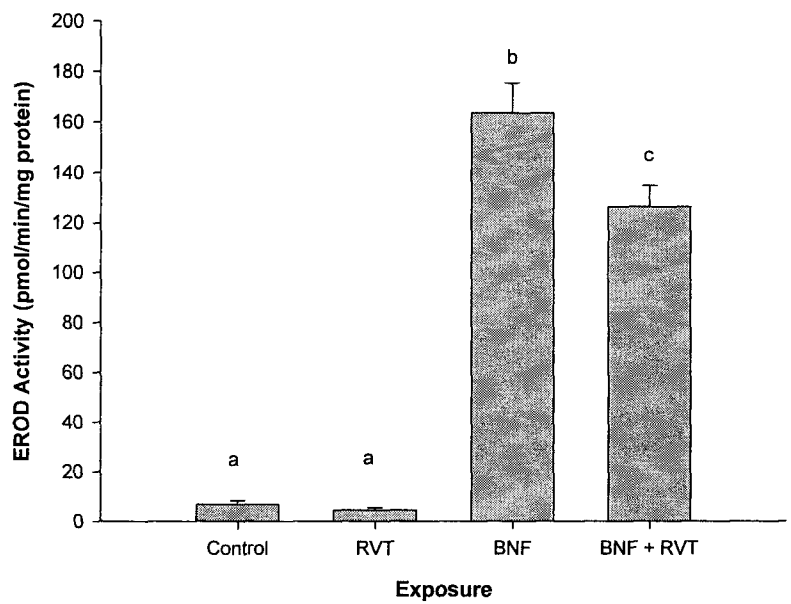
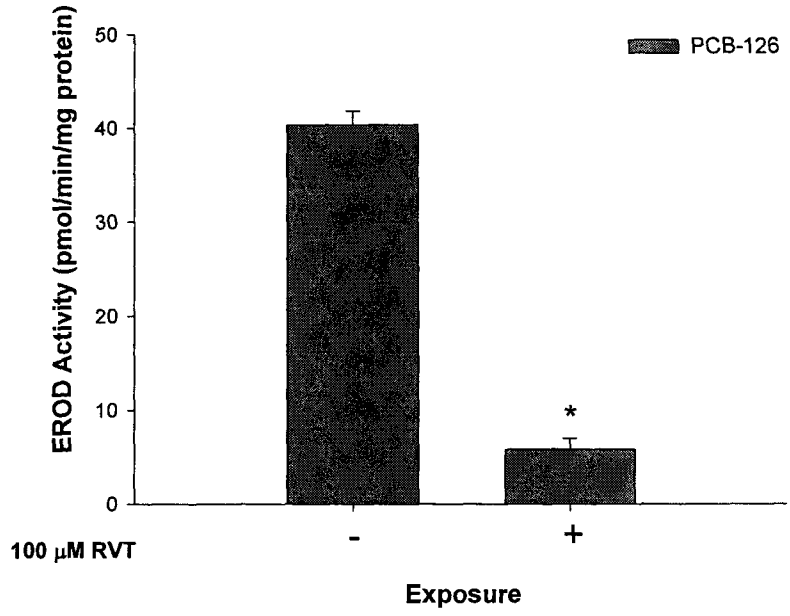


Figure 3.4: (A) Hepatic EROD activity in juvenile rainbow trout from control groups and fish injected IP with 50 μg/kg PCB-126 alone or 100 mg/kg RVT alone or 50 μg/kg PCB-126+100 mg/kg RVT. One-way ANOVA on ranks followed by a Dunn's pair-wise multiple comparison test show a significant effect ($p \leq 0.001$). (B) Hepatic EROD activity following treatment with treated with 50 mg/kg BNF alone or 100 mg/kg RVT alone or 50 mg/kg BNF + 100mg/kg RVT. One-way ANOVA analysis followed by Tukey's pair-wise multiple comparison test show a significant effect of treatment ($p \leq 0.001$). All incubations and experimental conditions are as indicated in Materials and Methods. Graphical data represent means + SEM (n = 3-7). Different letters indicate significant effect between different treatments.

(A)



(B)

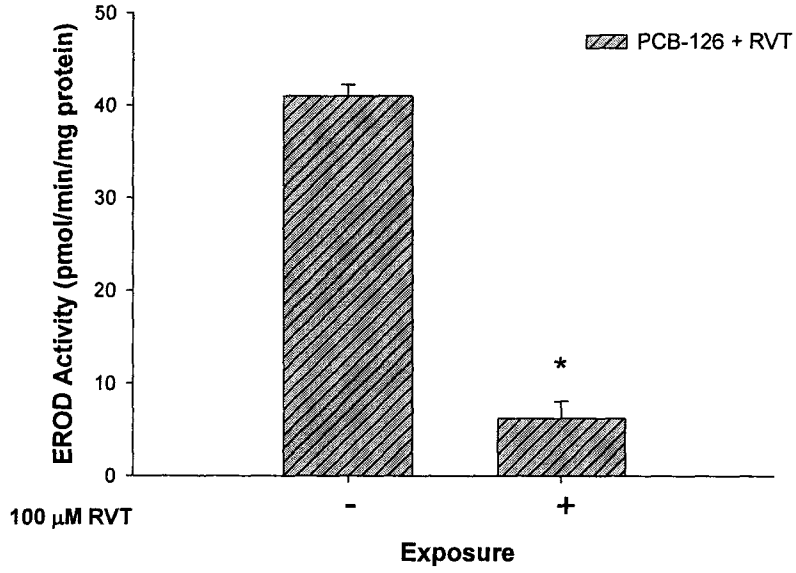
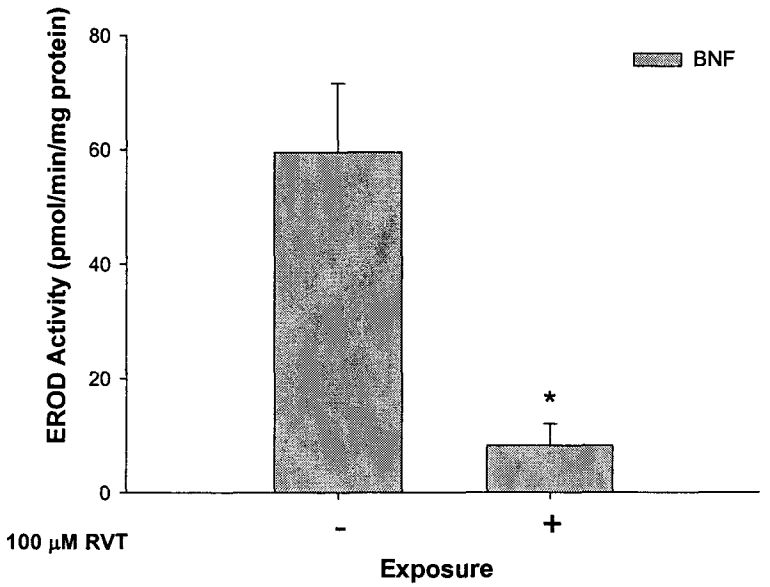


Figure 3.5: *In vitro* effects of incubating 100 μM RVT (+) on hepatic EROD activities of microsomes isolated from (A) PCB-126 and (B) PCB-126 + RVT exposed juvenile rainbow trout. Incubations and experimental conditions are as indicated in Materials and Methods. Data represent means + SEM (n = 5-6). One-way ANOVA analysis indicates a significant effect of treatment ($p \leq 0.001$); asterisks indicate a significant effect compared with the absence of 100 μM RVT.

(A)



(B)

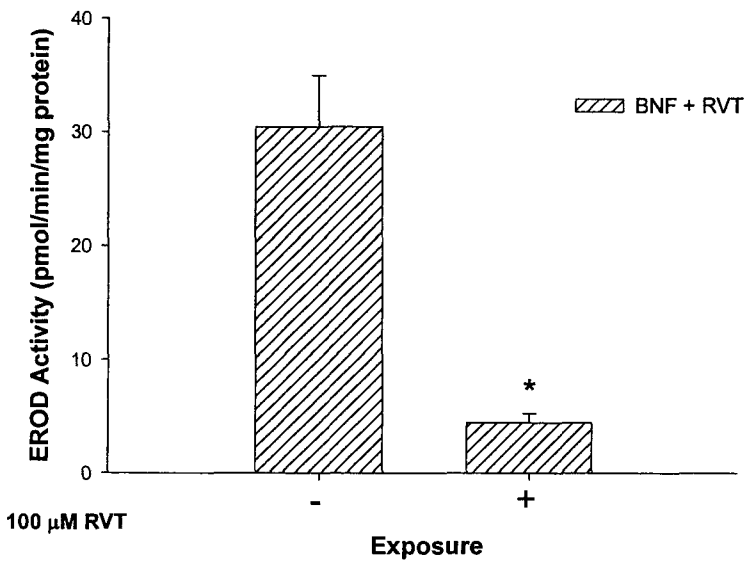


Figure 3.6: *In vitro* effects of incubating 100 µM of RVT (+) on hepatic EROD activities of microsomes isolated from (A) BNF and (B) BNF + RVT exposed juvenile rainbow trout. Data represent mean + SEM (n = 3-4). One-way ANOVA analysis indicates a significant effect of treatment (A: $p = 0.016$; B: $p = 0.029$); asterisks indicate a significant effect compared to absence of 100 µM RVT.

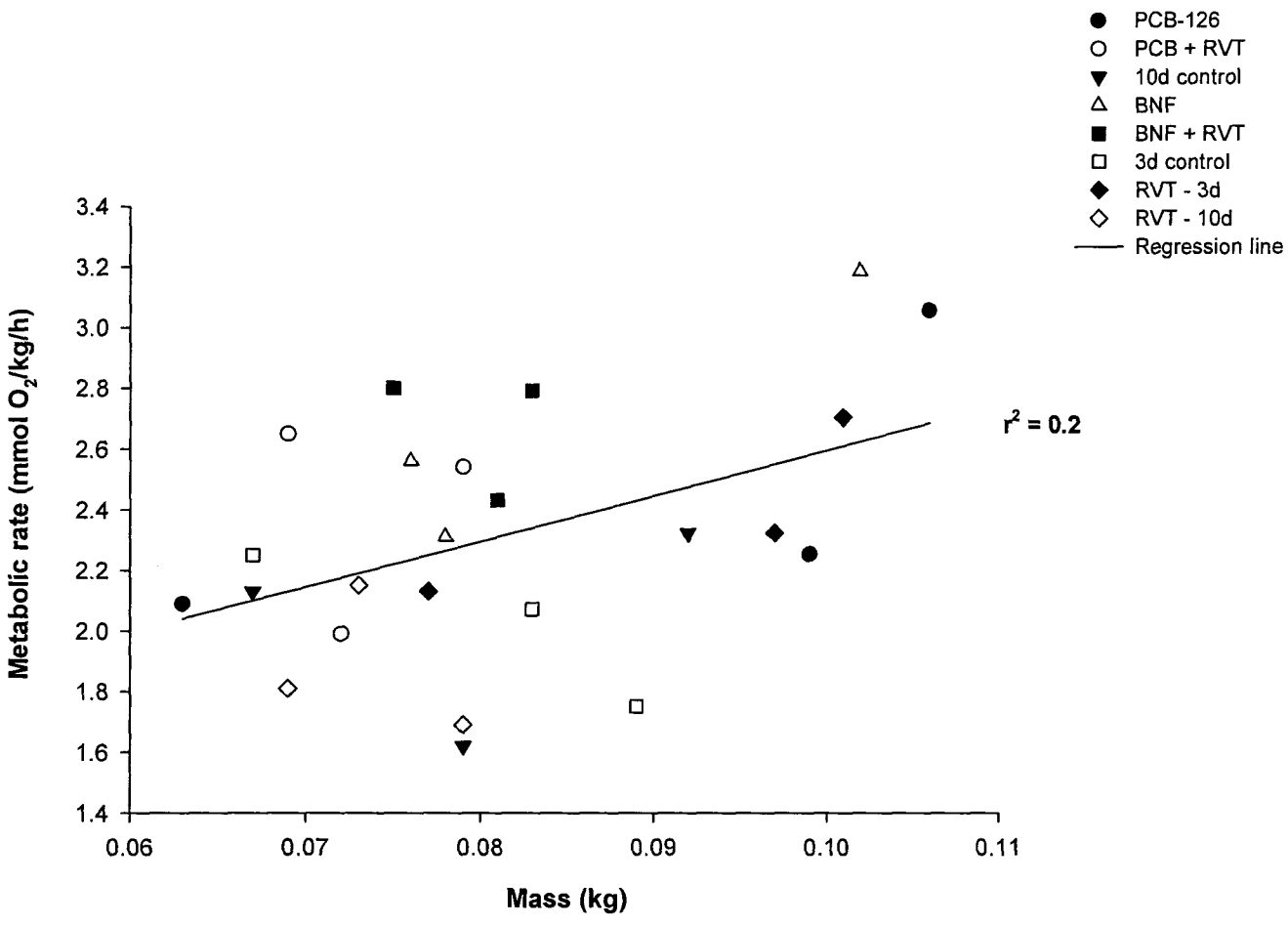
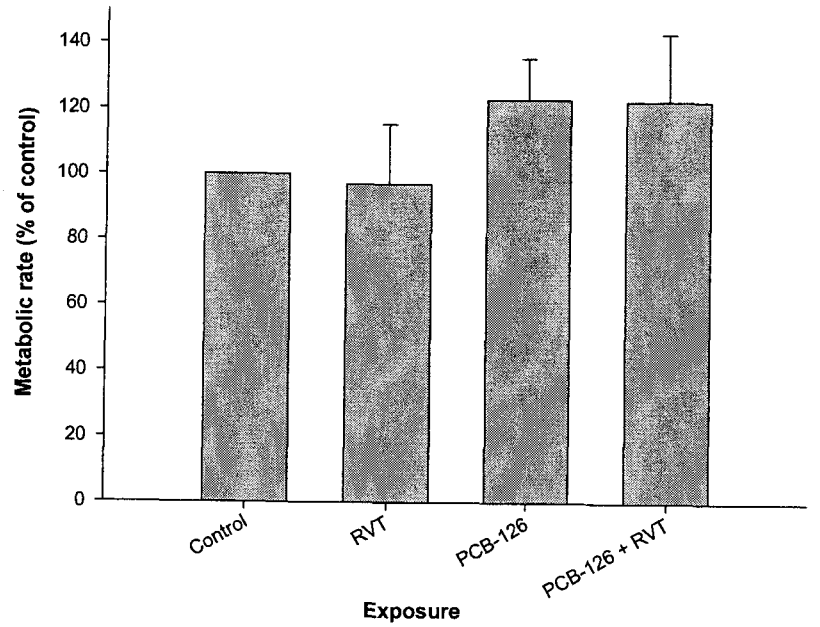


Figure 3.7: Plot of metabolic rate (mmol O_2/kg/h) vs mass (kg) of all fish used for experimental exposures in this study. Linear regression analysis (r^2 = 0.2) indicates no significant differences in O_2 consumption as a function of fish mass.

(A)



(B)

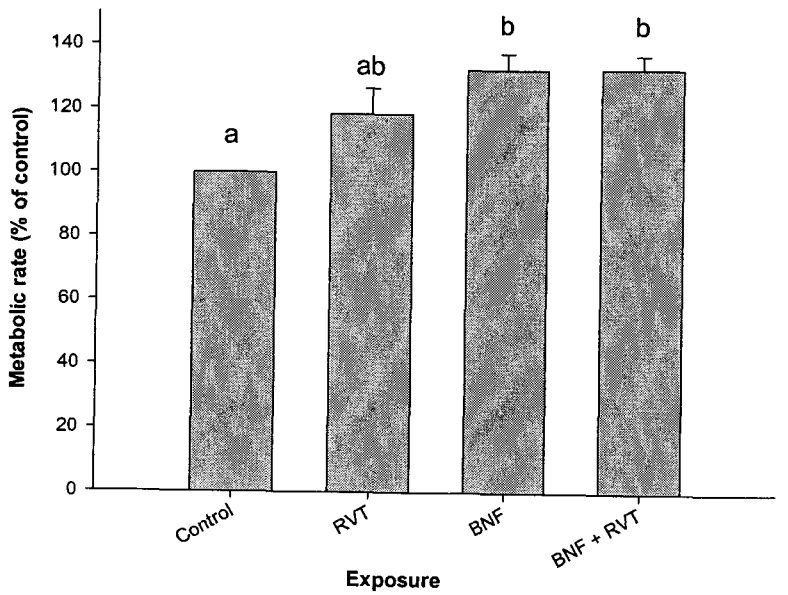


Figure 3.8: O_2 consumption of rainbow trout exposed to PCB-126 (A) or BNF (B). See Figure 3.4 legend for details of exposure. Data (n = 3) are shown relative to control treatment (IP injection with coconut oil; 100% control = 2.0 ± 0.21 mmol O_2/kg/h). (A) No statistical difference was found with PCB-126 related exposures. (B) A significant effect of BNF-treatments was determined with one-way ANOVA ($p = 0.007$); different letters indicate significant effect between different treatments.

3.4. Plasma Analysis

3.4.1. Glucose

In vivo exposure of rainbow trout to PCB-126 resulted in a significantly higher plasma glucose level compared with the control group. RVT exposure alone did not illicit any changes in glucose levels although trout co-treated with PCB-126 had glucose levels not statistically different from the values found in the control group (Fig. 3.9A).

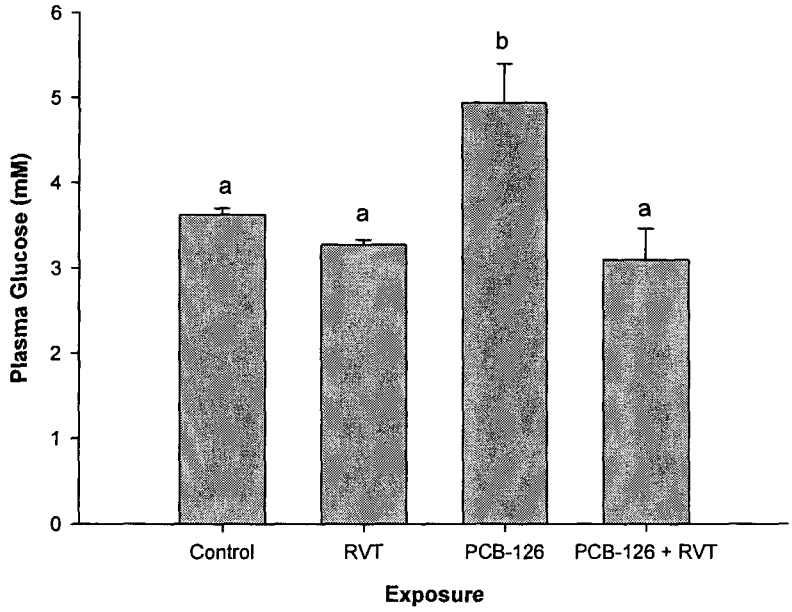
There was no significant effect of *in vivo* BNF treatment on plasma glucose concentration whereas co-treatment with RVT significantly increased glucose levels compared with the other treatments (Fig. 3.9B).

3.4.2. Triglycerides

Exposure to PCB-126 resulted in significant increases in the plasma triglyceride concentration (Fig. 3.10A) relative to the control group. Co-treatment with RVT resulted in a significant decrease in triglyceride concentrations compared to PCB-126 treatment alone that was significantly different from the control or RVT groups.

BNF related treatments resulted in a significant increase in the plasma triglyceride concentrations (Fig. 3.10B). However, RVT related treatments did not change triglyceride concentrations compared with the other treatments.

(A)



(B)

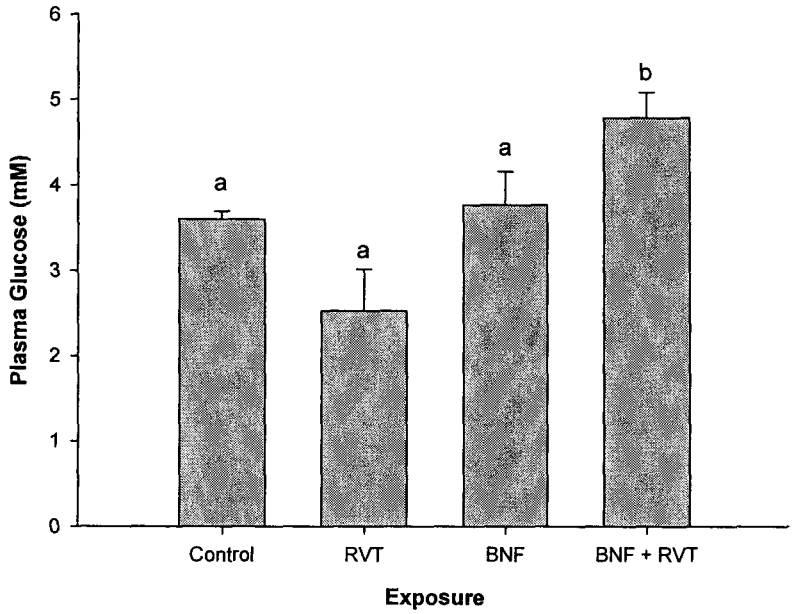
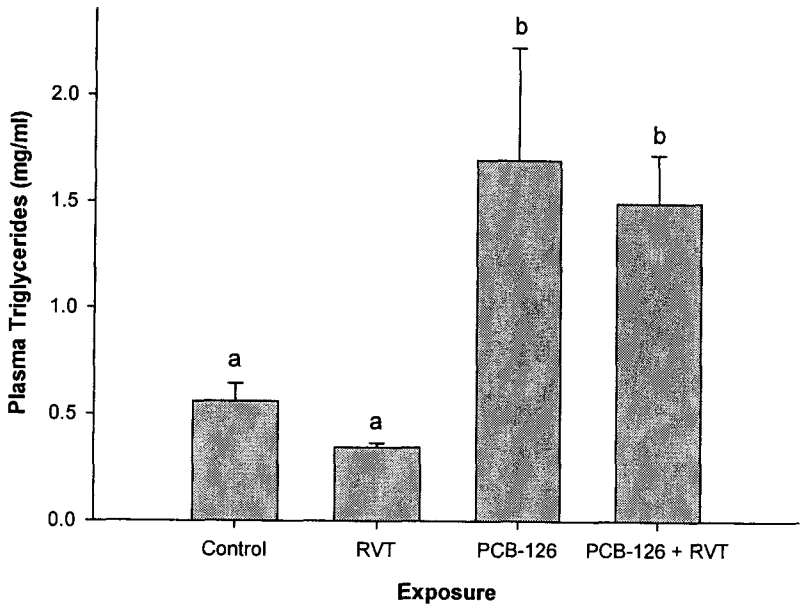


Figure 3.9: Plasma glucose levels in rainbow trout exposed to PCB-126 (A) and BNF (B). See Figure 3.4 legend for details of exposure. Data represent mean + SEM (n = 3-7). One-way ANOVA analysis shows a significant effect of treatment (A: $p = 0.001$; B: $p \leq 0.001$); different letters indicate significant effect between different treatments.

(A)



(B)

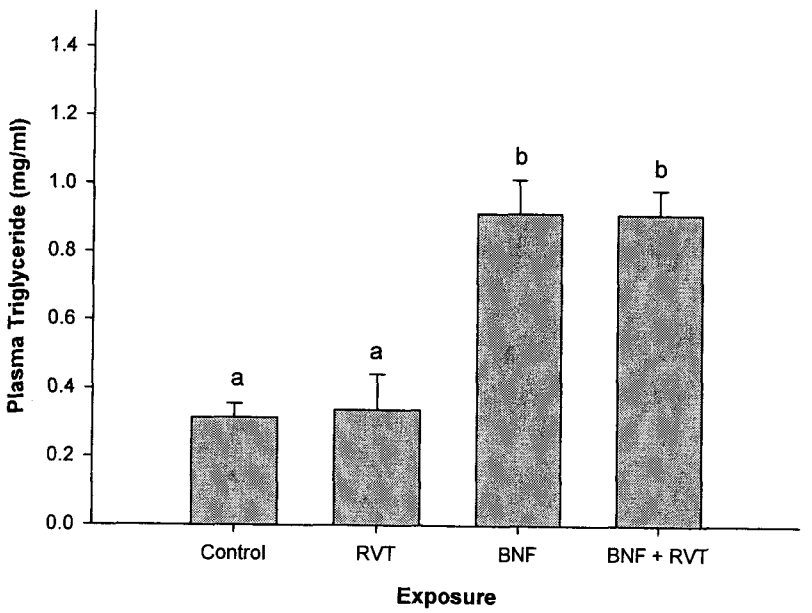


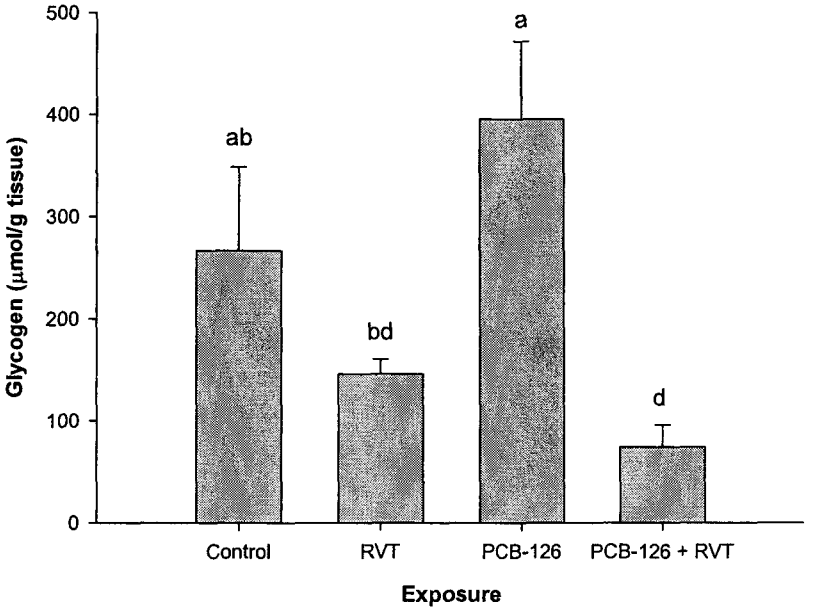
Figure 3.10: Plasma triglyceride levels in rainbow trout exposed to PCB-126 (A) and BNF (B). See Figure 3.4 legend for details of exposure. Data represent mean + SEM (n = 4-5). One-way ANOVA analysis shows a significant effect of treatment (A: $p = 0.005$; B: $p \leq 0.001$); different letters indicate significant effect between different treatments.

3.5. Hepatic Glycogen Analysis

PCB-126 treatment of rainbow trout alone did not affect trout liver glycogen content relative to the control group. Co-treatment with RVT resulted in a significant decrease in glycogen compared with the control; this decrease is significantly lower than the PCB-126 treatment (Fig. 3.11A).

In vivo treatment with BNF significantly lowered liver glycogen with respect to control whereas co-treatment with RVT resulted in a highly significant increase in glycogen with respect to control. Multi-group comparison also shows a significant difference in treatment with BNF and BNF + RVT (Fig. 3.11B).

(A)



(B)

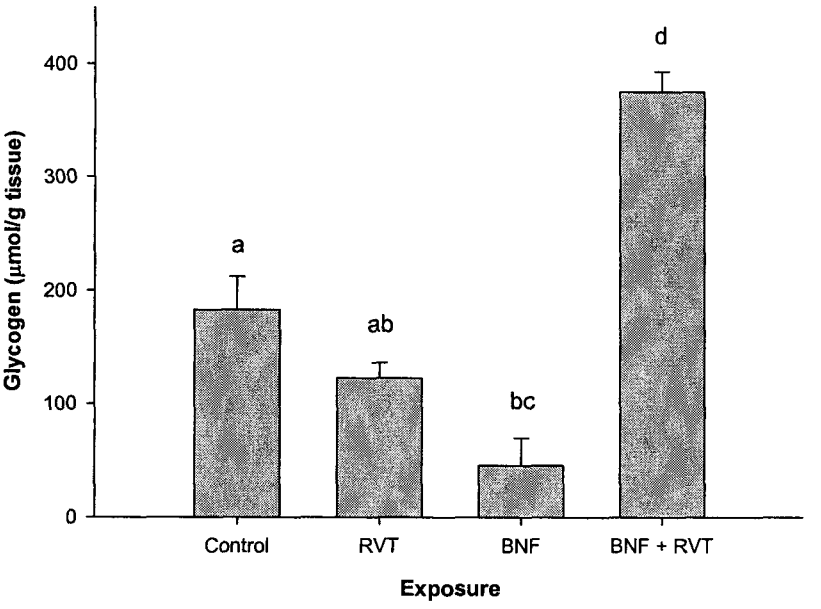


Figure 3.11: Hepatic glycogen content of juvenile rainbow trout exposed to PCB-126 (A) or BNF (B). See Figure 3.4 legend for details of exposure. Data represent mean + SEM (n = 3-5). One-way ANOVA analysis shows a significant effect of treatment (A: $p = 0.019$; B: $p = \leq 0.001$); different letters indicate significant effects between different treatments.

4. Discussion

Alterations in tissue energy metabolism can occur as a result of the initiation of compensatory mechanisms to cope with chemical toxicity, resist/repair damaging effects of exposure and to maintain overall homeostasis. Some studies report changes in energy metabolism in response to toxicant-induced stress, while others report a correlation between increases in detoxifying enzymes and increased energy expenses (Handy *et al.*, 1999). Stress resulting from chronic toxicant exposure is suggested to initiate physiological adjustments to compensate for any required changes in energy metabolism in order to maintain normal functions such as growth, maintenance and reproduction (Wendelaar Bonga, 1997; Beyer *et al.*, 1999a; Sherwood *et al.*, 2000; Bains and Kennedy, 2004). The potential toxicological effects of toxicant exposure can confound measurements of energetic costs of detoxification. No observable toxic effects (detrimental injury or death) of the chemicals at the concentrations used in this study were detected. However, other non-visible effects may have occurred including disruption of animal physiologies.

The premise of this project was based on the successful induction of the detoxification system in the exposed fish as established through AhR-activities as measured as increased EROD activities. Results demonstrate higher hepatic EROD activity (Fig. 3.4) and CYP1A1 mRNA expression (Fig. 3.3) in fish exposed to two model AhR agonists, PCB-126 (50 μg/kg) and BNF (50 mg/kg). EROD activities measured for the two exposures were not significantly different from one another (Table 3.1) although PCB-126 is documented as a more potent inducer of CYP1A induction. However this difference may be attributed to the different concentrations of chemicals used where PCB-126 was one-thousand times less than BNF hence confirming its specificity and potency of induction. The underlying logic for

using two test compounds was to investigate the differences between using different classes of inducers of the P450 system, PCB-126 being a more potent inducer than BNF both *in vivo* and *in vitro* (Zhang *et al.,* 1990; Newsted *et al.*, 1995; Hahn *et al.,* 1996; Navas and Segner, 2000; Gravato and Santos, 2002). Table 3.1 summarizes the statistical differences between the effects of the two on the reported parameters in this study.

Table 3.1: Comparison of PCB-126 and BNF exposure effects on EROD activity, O_2 consumption, plasma glucose and triglycerides, hepatic glycogen and lipid contents. Values represent means ± SEM.

Experimental Endpoints	**PCB-126**	**BNF**
EROD Activity (pmol/min/mg protein)	157 ± 11	164 ± 12
O_2 Consumption (mmol O_2/kg/hr)	2.5 ± 0.3	2.7 ± 0.3
Plasma Glucose (mM)	4.9 ± 0.5	3.8 ± 0.4 *
Plasma Triglyceride (mg/ml)	1.7 ± 0.5	0.9 ± 0.1
Liver Glycogen Content (µmol/g tissue)	395 ± 77	45 ± 24 *

* Significantly different from PCB-126 ($p<0.05$)

The main objective of this study was to establish a correlation between induction of detoxification mechanisms upon toxicant exposure and any observed changes in metabolic/energetic parameters resulting from this induction. This required employing an AhR antagonist that would inhibit the induction of the detoxification system and hence any associated changes. To this end, RVT an established AhR antagonist in mammalian systems and an inhibitor of CYP1A activity (Aluru and Vijayan, 2006; see Chapter 2) was employed.

The concentration of RVT (100 mg/kg of fish) used in this study failed to block PCB-126 mediated induction of EROD suggesting lack of AhR inhibition (relative mRNA expression in samples co-treated with RVT and PCB-126/BNF were not presented as a result of high variation in acquired data). On the other hand, co-treatment with BNF resulted in a slight but significant reduction in CYP1A activity. *In vitro* experiments demonstrate that RVT is capable of inhibiting CYP1A activity in rainbow trout liver cells and microsomal preparations (Appendix A; and Chapter 2). Figures 3.5 and 3.6 support the ability of RVT to inhibit EROD activities and suggest the following: 1) the *in vivo* concentration used for RVT co-exposures was not sufficient to effectively inhibit CYP1A induction and in particular for the more potent inducer PCB-126, and 2) RVT is being metabolized faster than it is displacing either PCB-126 or BNF.

Another objective of this work was to quantify the energetic costs of toxicant exposure in juvenile rainbow trout by measuring oxygen consumption rates in exposed fish. In a number of studies relating O_2 consumption to fish weight, it was shown that O_2 consumption increased with fish weight. Therefore in this study, similar sized fish were used. Figure 3.6 plots O_2 consumption and body weight (per fish) of the test animals used. As shown by linear regression analysis, a linear relation exists with a slope of less than 1 (r^2 = 0.2). As much as possible, fish of the same size class (75 g) were randomly distributed into each exposure tank to avoid the confounding effect of weight variability on metabolic rate. Handling stress can contribute to increases in oxygen consumption rates. Therefore, fish were allowed to acclimate for a 24 h period in order to reduce effects on respiration contributed by activity or handling stress. Energy used for metabolism is typically measured as oxygen consumption rates in fish biology. Several studies have reported the effects of toxicant

exposure on oxygen consumption in fish (Beyers *et al.,* 1999a; Brodeur *et al.,* 2001; Palanivelu *et al.,* 2005). Kim *et al.* (1996) reported that oxygen consumption rates were significantly increased by exposing fish to various concentrations of pentachlorophenol (PCP). This was attributed to the possibility that this was caused as a result of detoxification costs and the toxic effects of the PCP. The chemicals used in similar studies have known toxicological effects in fish making it difficult to separate out the energy allocated to detoxification from any direct toxic effects. Palanivelu *et al.* (2005) found that fish exposed to different sublethal concentrations of urea experienced reduction in growth rates which the authors suggested was due to poor energy consumption and/or increased metabolism in response to toxic stress. In the current study, slight increases in O_2 consumption rates were measured but these were not significant in co-treatments with PCB-126 and were only marginally significant in the BNF group. These small increases in oxygen consumption suggest a minimal cost associated with the detoxification of PCB-126 and BNF. For the purpose of bioenergetic studies, oxygen consumption rate is an accurate measure of metabolism; however it might not be a sensitive measure for detecting small changes in energetic costs.

Estimates of the measured metabolic parameters in this study suggest that the treatments with the inducers of the CYP system result in an altered metabolic status of the liver; however, the mechanisms governing these changes are not clear. BNF treatment in this study had no significant effect on plasma glucose. This is supported by the findings of Vijayan *et al.*, (1997) where the same concentrations of BNF and exposure periods were used. However, they found that BNF significantly reduced metabolic enzymes suggesting that BNF affects intermediary metabolism in rainbow trout liver. They suggested that the

subsequent decrease in metabolic enzyme activities may cause impairment in eliciting a metabolic response to stress. This could be an indication that the ability of the liver for gluconeogenesis is being affected by the chemical exposures.

On the other hand, Sancho *et al.* (1998) demonstrated a significant decrease in liver glycogen and lipid levels following water borne exposure to fenitrothion in the European eel. This was accompanied by a significant increase in blood glucose levels. Therefore, the concentration used in their experiment elicited an increase in liver glycogenolysis. This is true of the PCB-126 exposure in the current study where plasma glucose levels were significantly higher compared with the control and BNF groups. The lower glycogen content in the BNF group (Table 3.1) was associated with lower plasma glucose when compared with the PCB-126 group. This implies a disruption in the capacity of the BNF-treated fish to increase their plasma glucose concentrations possibly because of the low liver glycogen reserves. Under conditions of acute stress, fish utilize other energy stores in order to maintain their liver glycogen reserves (Vijayan and Moon, 1992; Levesque *et al.*, 2002). This is demonstrated by the higher concentrations of plasma triglycerides in both BNF and PCB-126 treated groups. Measurement of gluconeogenic and glycolytic enzymes would provide more evidence of the changes in metabolic demands as a result of toxicant exposure. Encomio and Chu (2000) demonstrated that with increasing PCB exposure there is a simultaneous and significant reduction in glycogen reserves in the adductor muscle of the eastern oyster (*Crassostrea virginica*); however, this effect was not observed in other tissues. It was suggested that under the study's exposure conditions, the muscle glycogen was the initial energy store utilized during stress.

A number of studies have concluded that in addition to changes in tissue metabolism and metabolic rates, energetic trade-offs may occur as a consequence of environmental stressors (Handy *et al.*, 1999; DuRant *et al.*, 2007). These are defined as adaptive response to meet the additional metabolic demands of detoxification. Although these trade-offs strongly support the concept of energetic balance, their occurrence makes the detection of changes in overall energetics difficult and may potentially mask smaller effects of interest. Therefore, to gain a comprehensive and holistic view of these effects, other portions of an animal's energy budget need to be examined simultaneously. All studies done to date indicate a variety of energetic responses to stress or chemical exposure that could be a function of the species studied and the specific effects that these stressors may have on biological processes.

Conclusions of all studies conducted to date can be summarized as follows: 1) energy utilization parameters such as feeding, growth and metabolic rate were significantly reduced following exposure; 2) based upon the energy budget equation (reviewed in Chapter 1), decreased efficiency of food assimilation would effectively reduce the amount of energy available for growth and maintenance; 3) energetic trade-offs occur as a result of exposure, e.g. decreased locomotory activity or increased metabolic rates; 4) changes in oxygen consumption are either subject to the impact of the chemical on gill physiology resulting in decreased oxygen consumption as a response to gill damage, or, increased consumption as a result of increased metabolic/energy demand; and, 5) metabolic and energetic responses vary with species and type of exposure.

The responses observed in this study illustrate the importance of further investigations into factors that regulate the allocation of energy between different physiological processes. In conclusion, the results of this study show that exposure of trout to

non-lethal concentrations of PCB-126 and BNF over the exposure duration used does not cause measurable additional metabolic costs at the organismal level. Further investigations are required to decide whether energetic demands due to toxicant-stress responses are marginal and thus difficult to assess at the whole fish level or whether possible additional costs are masked by other effects (Knops *et al.*, 2001).

CHAPTER 4 - Conclusion and Future Work

This study investigates the correlation between chemical detoxification and its impact on fish energetics. The results demonstrate that there are possible metabolic costs associated with the induction of the CYP detoxifying enzymes system both *in vivo* (Chapter 3) and *in vitro* (Appendix A). These were demonstrated by increases in oxygen consumption rates following exposures to the two AhR agonists employed in this work, PCB-126 and BNF. *In vivo*, these two agonists induced CYP activity measured as EROD in agreement with literature.

An attempt to attribute these metabolic changes to the induction of the detoxification mechanism used RVT, a proven AhR antagonist and CYP activity inhibitor in mammalian systems and fish models (Chapters 2 and 3; Appendix A). *In vitro* (microsomal and hepatocyte preparations) treatments with RVT significantly reduced EROD activities to at least 50% of the control groups. However, this inhibition was not as significant in *in vivo* co-exposures with PCB-126 and BNF. This may indicate that RVT, as an antagonist, is not as potent as the two AhR agonists suggesting that a higher concentration of RVT may be needed to achieve an effective inhibition of the CYP system. Another possibility would be to utilize an alternative administration method including lacing the feed with the compound and investigating the minimum concentration required to achieve 50% inhibition. The ineffective inhibition by RVT makes it difficult to ascertain whether the observed changes were in all cases a result of RVT action countering metabolic changes as result of exposure or induction of detoxification.

The major conclusions that I can make from these studies are: 1) there are some indications of some costs associated with the administered exposures; 2) these are variable but still support the interference of chemical exposure with the metabolic status of an animal; 3) the inhibition tool (RVT) utilized to achieve that objective was effective in an *in vitro* system but did not result in the expected inhibition in vivo at the dosage used; 4) the concept and approach to this study is sound therefore, future studies can target the usage of a different inhibition tool or continue with RVT following investigations into the required concentration and exposure method that will result in an effective inhibition of EROD *in vivo*.

There are a number of explanations for not observing the expected metabolic changes under exposure. These include: 1) energetic demands of detoxification are too small to be evident when compared to whole-metabolic costs; 2) direct toxicant effects are masking any additional costs associated with exposure; 3) the investigated exposure duration did not elicit any additional changes due to toxicant stress; and, 4) the experimental exposure setup (e.g. IP injection as opposed to water borne exposure) did not result in a measurable change in overall animal energetics.

Despite the growing body of evidence of tangible energetic costs of exposure, it is difficult to extrapolate these results to predict energetic consequences and trade-offs. Therefore there is a need to look at a holistic view of toxic responses and compensations by making biochemical and behavioral measurements over an extended period of exposure to determine if there are any compensatory changes in daily activities to off-set costs of exposure. Some studies examine both sides of the equation under chemical stress by measuring energy acquisition and multiple metabolic parameters. Additional studies must be undertaken to enhance the groundwork that has been accomplished to date. These could

include identifying indicators that are sensitive to specific kinds of exposure. Handy *et al.* (1999) reported swimming behavior in trout as a more sensitive indicator to Cu exposure than nutritional or survival parameters. Other suggestions would be the selection of a toxicant load that is not overtly toxic and test different exposure routes (such as waterborne or dietary) in order to observe any adaptive or compensatory biological responses.

Toxicant disruption of metabolism covers a range of responses, from whole-animal responses to ones that are tissue specific. In either case, this alteration in metabolism would alter food assimilation processes and in turn the energy available for other physiological processes. This sequence of events has certain implications on general individual behaviors such as metabolic rate and average performance. Outside of laboratory conditions, wildlife are exposed to repeated pulses or continuous exposure to a variety of persistent environmental stressors that could result in energetic trade-offs or an altered energy balance. In order for the animal to survive, it may adapt its behavior and physiology to forego energetically costly processes like reproduction. Although most studies suggest that there is no net change in the energy expenditure, there is however evidence of energy allocation that is associated with exposure/stress. One of these documented trade-offs is a reduction in locomotion or general animal activity as mentioned above. This could be of serious consequence since it makes the individual more susceptible to predation and possibly decrease foraging.

This study in addition to all other energetic studies to date can add to a better understanding of the toxicological impacts on energy budgets which will consequently has a broad ecological application to all organisms.

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APPENDIX A - Energetic Costs of Detoxification in Rainbow Trout (*Oncorhynchus mykiss)* Hepatocyte Cultures

The following section details *in vitro* experiments undertaken to determine the energetic costs at a cellular level of intoxication by a PCB in rainbow trout hepatocytes. Preliminary work was also done (not shown) to optimize assays for glucose determination from exposed hepatocytes that were cultured in 48-well plates over a 24 or 48 h period since no documented methodology was available. Work was discontinued due to difficulties in obtaining isolated hepatocytes with a viability >80-90%.

1. Introduction

This study proposes that stress induced by the presence of a toxicant and specifically the required metabolism to overcome its presence, can cause changes in the concentration of energy reserves and partitioning of these reserves in a fish. Detoxification is an essential cellular process to ensure an organism's survival. The question addressed is what costs are associated with detoxification. Organisms have a fixed energy budget that they obtain through ingested food that is then allocated to maintenance, growth and reproduction (Brett and Groves, 1979). Detoxification requires energy and the question is whether with a fixed energy budget, is there energy reallocation from other processes to compensate for the energy demands of detoxification?

1.1. Hypotheses

Energetic costs associated with detoxification of PCB-126 are significant at a cellular level in rainbow trout hepatocytes. In addition, induction of detoxifying enzymes results in an increased metabolism of energy reserves.

1.2. Objectives

The main objective of this study was to investigate the costs of detoxification in rainbow trout hepatocytes, by quantitatively determining if the energy spent on the process elicits any significant additional metabolic demands. The specific objectives were as follows:

1. To determine cytochrome P450 (CYP1A) activity in hepatocytes exposed to PCB-126;
2. To determine the impact of toxicant exposure (PCB-126) on oxygen consumption rates in hepatocytes;
3. To determine changes in glucose, lipid and protein metabolism following exposure to PCB-126 in hepatocyte cultures; and,
4. To establish whether changes in CYP1A are correlated with the metabolic changes. Cycloheximide (CHX), an inhibitor of translation and resveratrol (RVT) are used in this study. Resveratrol is a phytoalexin compound found in grape juice and wine and is characterized as an AhR antagonist in mammals; Aluru and Vijayan (2006) reported that RVT is also an AhR antagonist in rainbow trout hepatocytes.

2. Materials and Methods

2.1. Fish

Refer to Chapters 2 and 3 for details on experimental animals. The only modification is that for the purpose of this study 250 g fish were used instead of 75 g.

2.2. Chemicals and solutions

Essential (11130-051) and non-essential (11140-050) amino acids and antibiotic-antimycotic (15240-096) solutions were purchased from Gibco Canada Inc. (Burlington, ON, Canada). BD matrigel matrix (356234) was obtained from VWR scientific. 3, 3', 4, 4', 5 Pentachlorobiphenyl (PCB-126) was provided by Dr. Sean Kennedy at NWRC. Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Hepatocyte isolation

Glassware and instruments were autoclaved prior to use. All solutions were sterilized by filtration (0.22 μm pore size) using syringe driven filters for volumes less than 100 ml; vacuum driven bottle top filters were used for larger volumes. Hepatocytes were isolated using a two-step collagenase perfusion according to Mommsen *et al.* (1994). Fish were anaesthetized with benzocaine (0.005 mg/L or 2 ml of 10 mg/L stock in 4 L water) and euthanized by a sharp blow to the head. A ventral cut was made to expose the liver and intestines and the hepatic portal vein was cannulated. The liver was perfused clear of blood with Hanks' solution (0.33 mM $Na_2HPO_4.7H_2O$, 0.44 mM KH_2PO_4, 0.8 mM $MgSO_4.7H_2O$, 136.9 mM NaCl, 5.4 mM KCl, 5 mM HEPES, 5 mM HEPES.Na, 5 mM $NaHCO_3$, pH 7.63) containing 1 mM EGTA using a peristaltic pump at a perfusion rate of about 2 ml/min per g of liver. Following liver blanching (approximately 5 min), the rinsing media was replaced

with Hanks' EGTA-free solution containing collagenase (from *Clostridium histolyticum*, Type IV, 369 units/mg, 7.5 mg/50 ml). Perfusion was continued for 20-25 min with gentle massaging to increase cell dissociation. The liver was then removed from the trout and diced in Hanks' solution. The cell suspension was then filtered through 250 and 75 μm nylon mesh screens and centrifuged at 1000 rpm for 4 min. The supernatant was discarded and replaced by 25 ml fresh Hanks' solution. The cells were centrifuged twice again, once with 1:1 Hanks' solution to Hanks' solution supplemented with 1.5 mM $CaCl_2$ and 1.5% BSA; a final centrifugation contained only Hanks'-BSA. During the final wash, the cells were resuspended in Hanks' culture media (Hanks' solution with 1.5 mM $CaCl_2$, 5 mM D-glucose, essential and non-essential amino acids, antibiotic-antimycotic solution). Cells were counted using a hemocytometer and viability determined using Trypan Blue exclusion. Preparations that had greater than 90% viability were used for exposure experiments.

2.4. Primary cultures and experimental treatments

Prior to plating the cells, 48-well Costar (BD Falcon) culture plates were coated with BD membrane matrigel matrix to enhance cell attachment. Each well was treated with 100 μl of culture media containing 0.1 mg protein matrigel per ml. Plates were allowed to incubate for a few hours. Unbound material was aspirated and wells were rinsed with culture media. All of the above was done in a laminar flow hood to maintain sterility of manipulations and materials. Isolated cells were plated in the coated wells at concentrations of 0.5 x 10^6 cells / well in 0.5 ml media. Eighteen wells were kept free of cells to use for resorufin and BSA standards. Plates were then maintained in an incubator for 24 h at 15 ± 1°C before exposures.

Following the incubation period, hepatocytes were exposed to DMSO solutions containing varying concentrations of PCB-126 (10^{-3} to 10^3 nM), CHX (10^{-10} to 10^{-3} M) and

RVT (10^{-11} to 10^{-4} M). Co-treatments involved PCB-126 (1 nM; concentration corresponding to maximal EROD activity, Figure 1) and RVT (10^{-11} to 10^{-4} M). The same treatment was done replacing RVT with CHX (10^{-10} to 10^{-3} M). These exposures were conducted to establish dose-response curves. Final DMSO concentration in the well was 0.3%. Control wells received 0.3% DMSO alone. Cells were incubated for 24-48 h for PCB-126 and 24 h for the other exposures. Following the exposure period, the media was aspirated from the wells and each well was rinsed with phosphate-buffered saline (PBS; 250 µl per well). Plates were then stored at -80°C until EROD activity was analyzed.

2.5. Cytotoxicity

Lactate dehydrogenase (LDH) release into the culture media was used as a measure of cell membrane integrity and cytotoxic damage that may have occurred due to different exposure treatments. The principle of the assay is the kinetic determination of the activity of cytoplasmic LDH by measuring the rate of oxidation of NADH to NAD^+ in the presence of pyruvate. Culture media was removed from the wells and LDH activity in these samples was measured. Cells in aspirated wells were lysed with dH_2O and sonicated and these samples were assayed for LDH activity. This was done to compare the amount of LDH released to the total amount of LDH in the cells. LDH assays were conducted in 96-well plates; 20 µl samples (in triplicates) were loaded using an appropriate dilution factor. 100 µl imidazole buffer (50 mM) and 10 µl pyruvate (final concentration, 5 mM) were added to each well. Reactions were initiated by adding 20 µl NADH (final concentration, 0.2 mM). Absorption was measured at 340 nm using a SPECTRAmax PLUS 384 microplate spectrophotomer (Molecular Devices, Sunnyvale, CA). Total enzymatic activity was assessed using the following formula

$$\frac{[(OD/\min)_{sample} - (OD/\min)_{blank}] * total\ volume\ of\ assay\ in\ ml * df}{6.22\ mM^{-1}\ .\ cm^{-1} * volume\ of\ enzyme\ used\ in\ ml}$$

2.6. EROD assay

Prior to assay, plates were removed from the freezer and thawed for 10 min at room temperature. The EROD assay and cell protein determinations were simultaneously conducted in the 48-well plates following the method of Kennedy *et al.* (1995).

Resorufin and protein concentrations were fluorimetically determined using a SPECTRAmax GEMINI XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) and compared against resorufin and BSA standard curves, respectively. Briefly, 125 µl sodium phosphate buffer (50 mM, pH 8.0) was added to each well followed by 25 µl 7-ethoxyresorufin (final concentration, 8 µM); the plates were allowed to incubate for 15 min at room temperature. Reactions were initiated by adding 25 µl NADPH (final concentration, 0.1 mM) and left to stand for 20 min (preliminary experiments show linear reactions for at least 30 min). Reactions were terminated by addition of 150 µl cold acetonitrile containing fluorescamine at a concentration of 0.15 mg/ml. After 10 min, the reaction product, resorufin, was measured at 530 nm excitation and 590 nm emission; total protein determination used 400 nm excitation and 460 nm emission wavelengths.

2.7. O_2 consumption measurements

Oxygen consumption of hepatocyte preparations were measured in a sealed chamber (3 ml volume) equipped with a Clark-type polarographic oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The experimental setup included an oxygen monitor and a chart recorder that displays oxygen content as a function of time. During measurements, medium solutions or cell suspensions are thoroughly stirred using a magnetic

stir bar to ensure homogeneity and sufficient oxygen diffusion to the electrode. All measurements were done at room temperature. Before measuring O_2 consumption in cell suspensions, the probe was caliberated with an air-saturated medium solution. O_2 measurements with air-saturated water and culture medium were initially done to determine oxygen saturation levels in both solutions. The culture medium and water had similar O_2 profiles, therefore, oxygen solubility in the media was assumed to be the same as water. Subsequent measurements were done using various concentrations of cell suspension to optimize cell concentrations to use for PCB-126 (1 nM) exposure (Figure 4). A concentration of 40 mg cells per ml was found to coincide with the linear part of the activity line so it was used in the assays. Cells were exposed to the vehicle, 0.3% DMSO (control group) and 1 nM PCB-126 (experimental group) and O_2 consumption was measured at 2 and 24 h after exposure (Table A.1). Oxygen consumption rates (ng O_2/min/mg) of hepatocytes were calculated using the average of triplicate determinations of slopes from the recorded O_2 chart.

2.8. Statistical Analysis

Experimental results are presented as means ± standard error of the mean (SEM). A value of $p<0.05$ was considered significant. Graphs were plotted using SigmaPlot 10.0 Systat Software, Inc.). SigmaStat 3.1 (SPSS Corp., Chicago, IL, U.S.A.) was used to evaluate statistical differences.

3. Results and Discussion

3.1. Effect of PCB-126 on CYP1A1 activity

Hepatocytes were exposed to varying concentrations (10^{-3} to 10^{3} nM; DMSO control) of PCB-126 for 24 and 48 h. The purpose of this exposure was to determine an optimal concentration of PCB-126 that induced maximal EROD activity (Figure A.1); this concentration was determined to be 1 nM for both time points. The peak EROD induction was significantly different from the control and the extent of EROD activation increased between 24 and 48 h.

3.2. Effect of inhibitors, RVT and CHX, on CYP1A1 activity:

Hepatocyte exposures were also undertaken with increasing concentrations of resveratrol, RVT (10^{-11} to 10^{-4} M; DMSO control) and cycloheximide, CHX (10^{-10} to 10^{-3} M; DMSO control) on basal (non-stimulated) EROD activities. Results suggest a significant difference in EROD activity with various treatments (Figure A.2A, A.3A).

EROD activities of PCB-126 (1 nM) induced hepatocytes in the presence of RVT and CHX were also measured (Figure A.2B, A.3B). The inhibition trend described above was also observed with RVT exposures. With CHX, EROD activities were inhibited to basal levels at a concentration of 10^{-4} M with an IC_{50} of 0.24 μM. RVT inhibited EROD activity by 50% relative to control (1 nM of PCB-126) at a concentration of 10^{-5} M.

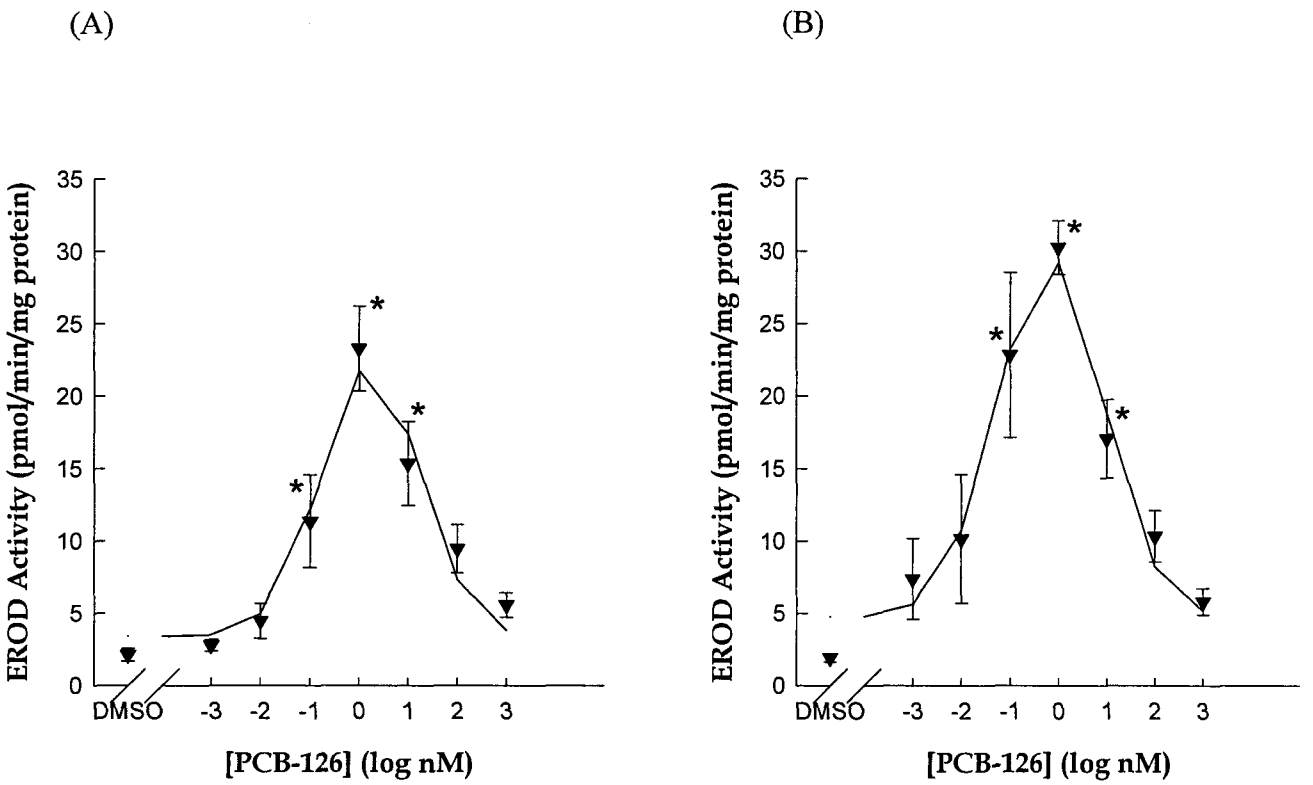

Figure A.1: Induction of EROD activity in rainbow trout hepatocytes at increasing concentrations of PCB-126 after (A) 24 h and (B) 48 h exposures. Points represent means ± SEM of 4-5 individual preparations. Data were empirically fitted to a modified Gaussian curve. One-way ANOVA analysis demonstrated a significant effect of exposure ($p<0.001$) for both exposure times; stars indicate a significant difference compared to DMSO (control).

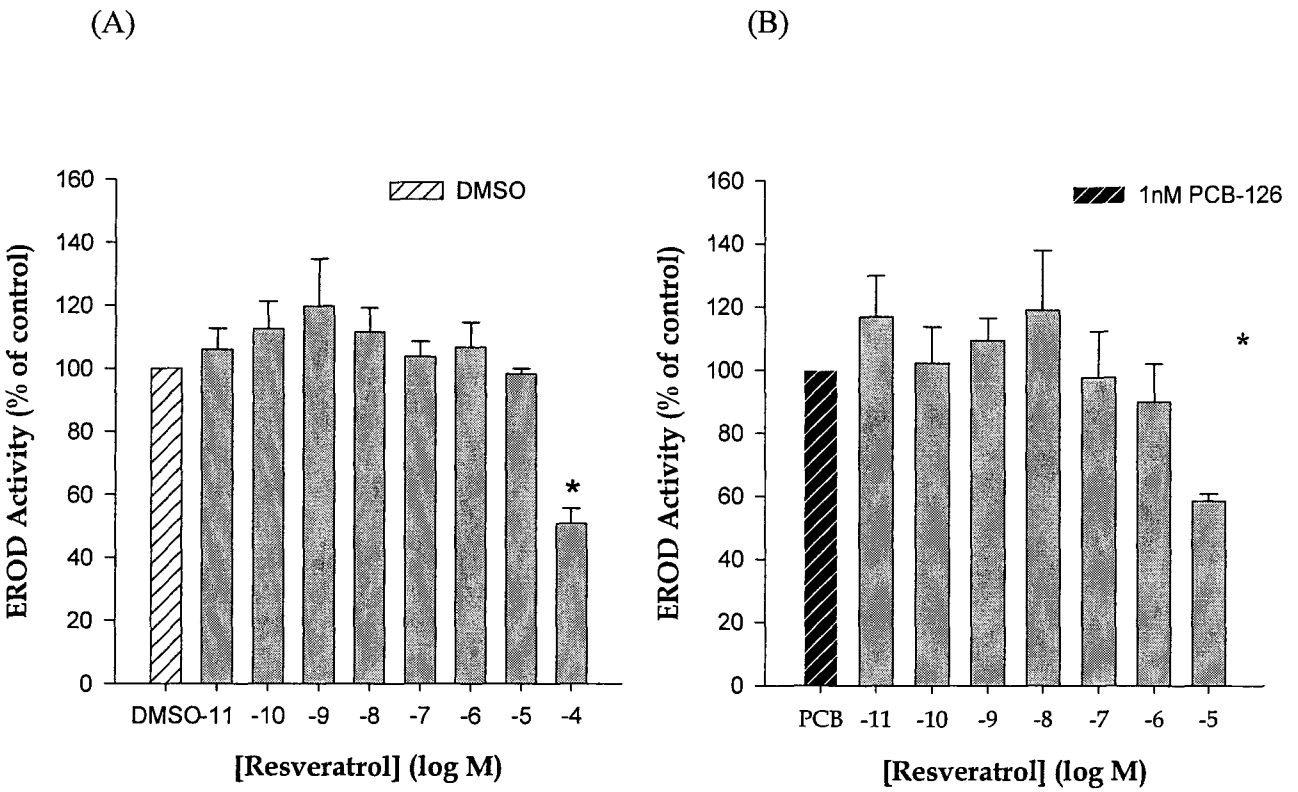

Figure A.2: (A) Inhibition of EROD activity in the presence of different concentrations of RVT relative to DMSO (100% control activity = 2.4 pmol/min/mg protein). Data represents means + SEM (n = 3-5). One-way ANOVA revealed a significant effect of treatments ($p<0.001$); stars indicate a significant effect compared with DMSO control. (B) RVT inhibited PCB-126 (1 nM) induced EROD activity (n = 3). Activities are presented as % of control (1 nM PCB-126; activity = 21.5 pmol/min/mg protein). One-way ANOVA show significant effect of treatments ($p = 0.045$).

(A) (B)

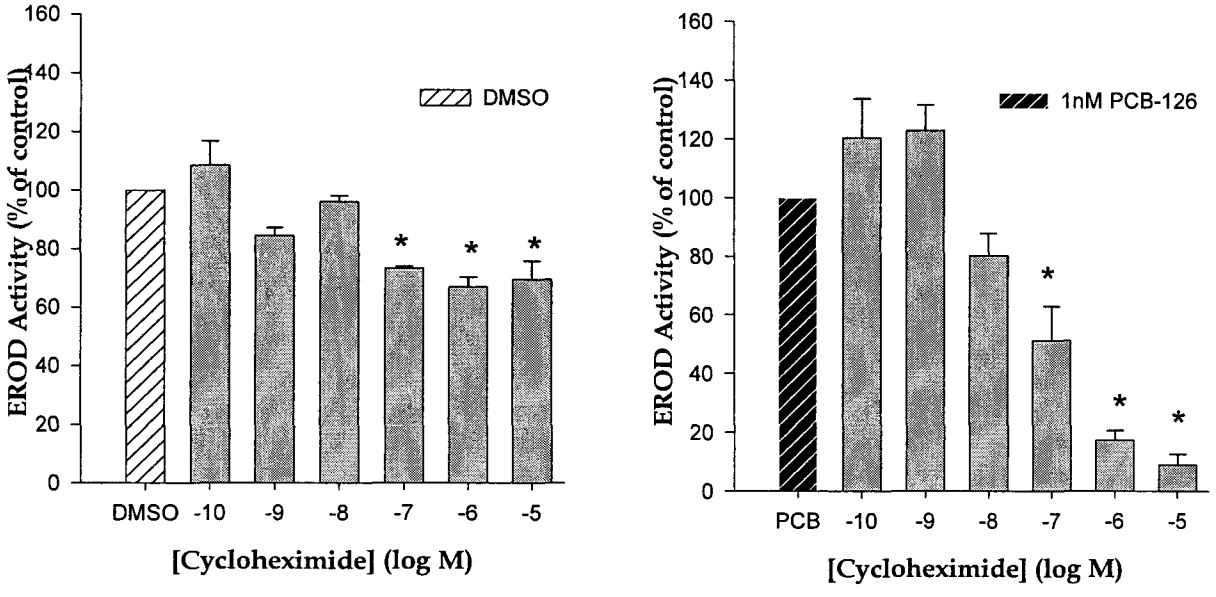

Figure A.3: Different concentrations of CHX inhibit EROD activity in (A) non-treated hepatocytes and (B) PCB-126 (1 nM) exposed hepatocytes. EROD activities are presented relative to DMSO (100% control activity = 2.5 pmol/min/mg protein) or PCB (100% control activity = 27.9 pmol/min/mg protein). Data represents means + SEM (n = 3-5). One-way ANOVA analysis revealed a significant effect of treatment in non-treated cells and exposed cells ($p<0.001$); stars indicate a significant effect of treatment compared to DMSO control.

3.3. Cytotoxicity:

Cytotoxicity as indicated by Trypan Blue and LDH was not affected by the chemical exposures used. Figure A.4 demonstrates results of LDH activities from preparations treated with the highest concentration of each chemical. No significant differences in activities were observed.

3.4. O_2 consumption:

Subsequent measurements were done using various concentrations of cell suspension to optimize cell concentrations to use for PCB-126 (1 nM) exposure (Figure A.5). A concentration of 40 mg cells per ml was found to coincide with the linear phase of the O_2 uptake line so this concentration was used in the assay. Cells were exposed to selected PCB-126 concentrations and oxygen consumption was measured at 2 and 24 h of PCB exposure (Table A.1). Results are from one fish only. This preliminary experiment suggests that PCB exposure increases metabolic costs in hepatocytes.

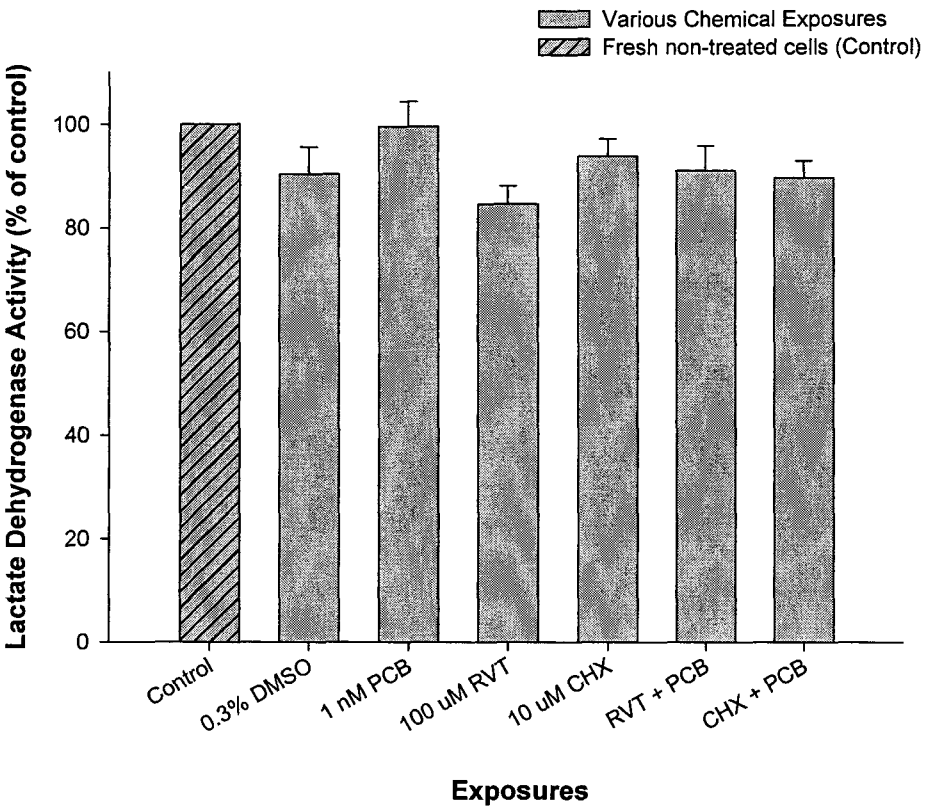

Figure A.4: Measurement of Lactate Dehydrogenase (LDH) activity in the media of cultured rainbow trout hepatocytes as an indicator of cytotoxicity under different chemical treatments. Data represents means + SEM (n = 6). One-way ANOVA analysis revealed no significant effect of treatments on LDH activity relative to control (non-treated cells).

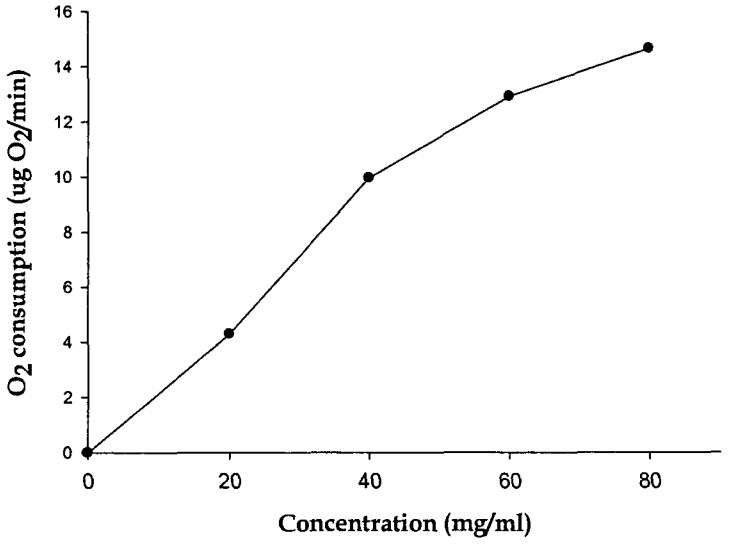

Figure A.5: Measurements of oxygen consumption at various concentrations of cell suspensions of rainbow trout hepatocytes (n = 2). Graph was established as described in Materials and Methods.

Table A.1: O_2 consumption measurements of hepatocytes following 2 and 24 h exposures to 1 nM PCB-126. Data represent a sample size of 1.

Duration of Exposure (hours)	O_2 consumption (ng O_2/min/mg)	
2	Blank	158.4
	PCB (1 nM)	171.8
24	Blank	113.8
	PCB (1 nM)	190