DRUG METABOLISM AND CYP INDUCTION AND INHIBITION IN FISH

CYTOCHROME P450 DRUG METABOLISM AND PROTEIN INDUCTION AND INHIBITION IN FISH LIVER MICROSOMES

By

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ABSTRACT

The emergence of pharmaceuticals in wastewater effluent is of increasing concern to aquatic organisms. Cytochrome P450s (CYPs) are important pharmaceutical and xenobiotic-metabolizing proteins, and their functions are well studied and understood in mammals. However, studies on CYP function and modulation in non-mammalian vertebrate systems are much less defined, and function is often inferred from mammalian data, assuming similar function across vertebrate taxa. In this study, we investigate *in vivo* induction and *in vitro* metabolism and inhibition of CYPs in fish liver microsomes.

In this thesis, a number of fluorometric CYP-catalyzed assays and fluoxetine, a model selective serotonin reuptake inhibitor (SSRI), have been used to assess CYP-mediated metabolism in fish. Overall, we raise questions about extrapolating known mammalian data to fish CYP function. Fluoxetine, a CYP2D6 substrate in humans, was not metabolized well in fish liver. These data suggest that perhaps fish produce a fluoxetine metabolite that is not produced by mammals. We investigate the basal metabolism and induction potential for hepatic CYPs in two fish species, rainbow trout (*Oncorhynchus mykiss*) and killifish (*Fundulus heteroclitus*). Species differences were found in the baseline metabolism of these substrates. Treatment with known mammalian CYP inducers, dexamethasone (DEX), pregnenolone-16α-carbonitrile (PCN), and rifampicin (RIF) did not cause broad, measurable CYP induction in either fish species. β-naphthoflavone (BNF), a prototypical CYP1A inducer, showed significantly induced activity across many substrates, providing evidence that BNF induces more broadly than

expected. We also assess the inhibition potential of potent inhibitors of important drug-metabolizing CYPs in mammals: fluoxetine, ciprofloxacin, gemfibrozil and erythromycin. These compounds produce significant inhibition over most substrates tested, and are not substrate-selective as would be predicted by their inhibitions in mammals.

The experiments carried out within this thesis add to our knowledge of functional characterization of CYP induction, inhibition and metabolic activity in fish. Using a comparative approach, a number of fish species were tested for *in vitro* catalytic function of these contaminant-metabolizing proteins and provide evidence that fish CYPs respond differently to inducing and inhibitory compounds compared to mammals. Extrapolation across taxa should be carefully considered before assuming similar function.

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ABBREVIATIONS

3-MC: 3-methylcholanthrene
7-BR: 7-benzyloxyresorufin
7-ER: 7-ethyoxyresorufin
7-MR: 7-methoxyresorufin
7-PR: 7-pentoxyresorufin
AhR: arylhydrocarbon receptor
ARNT: AhR nuclear translocator
AMMC: 3-[2-(N,Ndiethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin
AROD: alkoxyresorufin-*O*-dealkylase
BFC: 7-benzyloxy-4-trifluoromethylcoumarin
BNF: β-naphthoflavone
BQ: 7-benzyloxyquinoline
BROD: 7-benxyloxyresorufin-*O*-dealkylase
CAR: constitutive androstane receptor
CBZ: carbamazepine
CEC: 3-cyano-7-ethoxycoumarin
CYP (or P450): cytochrome P450
DBF: dibenzylfluorescein
DEX: dexamethasone
EROD: 7-ethoxyresorufin-*O*- dealkylase
HNF-4: hepatocyte nuclear factor 4
K_m: Michaelis-Menten constant
K_i : Inhibition constant
LOD: Limit of detection
LOQ Limit of quantitation
MAMC: 7-methoxy-4-(aminomethyl)coumarin
MFC: 7-methoxy-4-trifluoromethylcoumarin
MROD: 7-methoxyresorufin-*O*-dealkylase
NR: nuclear receptor
P450 (or CYP): cytochrome P450
PAH: polyaromatic hydrocarbon
PB: phenobarbital
PCN: pregnenolone-16α-carbonitrile
PROD: 7-pentoxyresorufin-*O*-dealkylase
PXR: pregnane X receptor
RAR: retinoic acid receptor
RIF: rifampicin
SSRI: selective serotonin reuptake inhibitor
V_{max}: Maximum reaction velocity
XRE: Xenobiotic response element

CHAPTER 1:

GENERAL INTRODUCTION

1.1 Cytochrome P450 enzymes

1.1.1 Nomenclature and evolution

Cytochrome P450s (CYPs) are a superfamily of heme-proteins, which aid in the metabolism of both exogenous and endogenous compounds. CYPs are named in order of discovery and are classified based on percent amino acid sequence identity. CYP genes are categorized into families (> 40% identical sequence), subfamilies (>55% identical sequence) and individual genes (Nelson *et al.* 1996). With such large diversity and continuous gene discovery, the CYP nomenclature system and naming committee are essential in allowing the scientific community to communicate findings accurately. CYP families are named numerically and subfamilies alphabetically. For example, CYP2C9 is the 9th gene named in the CYP '2' family, 'C' subfamily. One exception to this nomenclature is CYP1A. In fish, CYP1A is an individual protein that has not been given a specific gene number.

CYPs are greatly conserved across vertebrate lineages. CYP families and subfamilies, with the exception of subfamilies within CYP2s, are nearly identical in vertebrate taxa and the total numbers of CYP genes in vertebrate species is similar. For example, fish and mammalian lineages share all families except CYP39, which has arisen

within mammals (Nelson 2003). All other 17 CYP families have clear orthologs in both fish and mammals and intron-exon boundaries are conserved within CYP families (Nelson 2003). CYP2s are the least conserved family with extensive subfamiliy diversification (Nelson 2003). Fish and mammals have 13 and 10 CYP2 subfamilies, respectively; of these, only CYP2R and 2U are shared between the two taxa. CYP evolution appears to reflect the divergence of fish and tetrapods 420 mya, although the origin of CYPs within deuterosome evolution, 520 mya, is still unclear (Nelson 2003).

1.1.2 Distribution and function

CYPs are membrane-bound monooxygenase proteins involved in the biotransformation of many endogenous and exogenous compounds and have been characterized in both eukaryotic (Andersson and Forlin 1992; Bolwell *et al.* 1994; James and Boyle 1998; Walker 1998; Snyder 2000; Otyepka *et al.* 2007; Seliskar and Rozman 2007) and prokaryotic species (Urlacher and Schmid 2002; Lewis and Wiseman 2005). CYPs have been found in nearly all vertebrate tissues examined but are generally most prevalent in the digestive tract, specifically the liver (Debri *et al.* 1995).

Xenobiotic biotransformation is a multi-phase process, the first of which involves oxidation of the compound by CYPs (Andersson and Forlin 1992; Xu *et al.* 2005). CYPs from families 1 through 4, specifically human CYPs 2D6 and 3A4, are highly associated with pharmaceutical metabolism. CYP3A4 is responsible for 50% of all human drug metabolism followed by 2D6, 2C9 and 1A2 at 25%, 15% and 5%, respectively (Hemeryck and Belpaire 2002). Orthologous genes to CYP 3A4, 2D6, 2C9 and 1A2

have been identified as major enzymes in drug metabolism in other mammalian species. Once outside of mammalian systems, however, the metabolic pathway for many pharmaceuticals and the CYPs involved are still unknown.

Although the full catalytic function of many fish CYPs is still unknown, organ and cell-type distribution of major fish CYPs have been investigated. Immunohistochemistry, PCR, Northern and Western blots are among the common tools used to determine mRNA and protein expression levels of CYP1A and 3A in fish tissues. CYP1A is found in the liver, heart, gill, kidney, and intestinal tract of fish (Husoy *et al.* 1994) and CYP3A is highly expressed in the liver and intestinal tract (Lee *et al.* 2001; Tseng *et al.* 2005). In the liver, CYP3A isoforms are found in higher concentrations in hepatocytes than CYP1A but epithelial and endothelial cells appear to have higher concentrations of CYP1A (Husoy *et al.* 1994). Expression of other CYP1 genes has been investigated in zebrafish; CYP1A appears to have higher expression in the liver compared to CYP1B1, 1C1, 1C2 and 1D1 (Jonsson *et al.* 2007; Goldstone *et al.* 2009).

In contrast to fish CYP1 and CYP3A, identification of expression of the CYP2 family in fish is more problematic. CYP2s are the least conserved CYP family in vertebrates and studies on expression levels in fish are still scarce. Trout CYP2K and CYP2M proteins were found to be highly expressed in the liver (Cok *et al.* 1998). Channel catfish CYP2X mRNA was highly concentrated in the liver (Mosadeghi *et al.* 2007). Studies on CYP2s in fish are increasing but overall expression, distribution and function of CYP2s in fish is not yet clear.

1.1.3 Induction of CYPs via nuclear receptors

A key aspect of CYPs is the induction of expression via nuclear receptors. Modulation of the CYP profile is often used as an indicator of exposure to environmental contaminants. CYP1A induction, specifically, is commonly used to identify the presence of polyaromatic hydrocarbons (PAHs) and other aryl hydrocarbon receptor (AhR) agonists, the specific nuclear receptor responsible for induction of CYP1 genes in all vertebrates (Hahn *et al.* 1998). In addition to PAHs, other contaminants present in aquatic systems have the potential to alter CYP activity. Pharmaceuticals are potent CYP-inducing agents, upregulating transcription of specific CYP genes via a suite of nuclear receptors. The mammalian CYP2 family is regulated through a number of receptors, including the retinoic acid receptor (RAR), the constitutive androstane receptor (CAR) and hepatocyte nuclear factor 4 (HNF-4) (Honkakoski and Negishi 2000; Lewis *et al.* 2002). Pregnane X receptor (PXR) and glucocorticoid receptors are involved in mammalian CYP3 induction (Handschin and Meyer 2003). Similar induction pathways exist across vertebrate classes and with the exception of CAR, all of these nuclear receptors have been identified in fish. PXRs respond to a structurally diverse group of xenobiotics and endogenous compounds in mammals and this characteristic appears to be conserved in fish (Moore *et al.* 2002). Collectively, these data suggest that fish CYPs are likely inducible by compounds that induce CYPs in mammals via the conserved nuclear receptor pathways; the specific CYP isoforms which would be responsive to such induction is still unclear.

Mammalian CYP2 and 3 genes can be induced by pharmaceuticals such as carbamazepine (CBZ), dexamethasone (DEX), phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN) and rifampicin (RIF), although species differences are known to occur (Lu and Li 2001; Luo *et al.* 2002; Lee *et al.* 2006). Discrepancies exist with respect to typical mammalian CYP inducers and their affect in fish and on respective CYP isoforms. PB is a strong inducer of CYP2s and 3s in mammals (Waxman and Azaroff 1992); however, in fish, which lack CAR (the nuclear receptor responsible for PB induction in mammals), CYP2 and 3 isoforms were not induced by PB in several studies (Addison *et al.* 1987; Ankley *et al.* 1987; Kleinow *et al.* 1990; Li *et al.* 2008) although PB induced CYP1A in fish hepatocytes (Sadar *et al.* 1996). β-naphthoflavone (BNF) is considered a prototypical ligand for the AhR and standard CYP1A inducer in both mammals and fish (Kloepper-Sams and Stegeman 1989; Haasch *et al.* 1994; Lemaire *et al.* 1996). With few exceptions (Parente *et al.* 2009), BNF is a potent inducer of the CYP1A associated ethoxyresorufin-*O*-deethylase (EROD) activity in nearly all species. Induction of fish CYPs have been documented by a variety of typical mammalian inducers, however results appear to be conflicting. For instance, DEX and RIF induced zebrafish CYP3A65 in fry (Tseng *et al.* 2005), yet the sea bass CYP3A isoform, CYP3A79, was not affected by DEX (Vaccaro *et al.* 2007). *In vivo* CYP3A induction by RIF in fish appears promising (Li *et al.* 2008) but studies with this compound are limited. It is crucial to understand and identify, specifically, which types of chemicals alter CYP expression and function in fish in order to understand basic fish physiology, the evolution

of CYP function in vertebrates and for environmental concerns with respect to the suite of xenobiotics that are constantly entering the environment.

1.1.4 CYP inhibition

CYPs, like most enzymes, are susceptible to enzyme inhibition. Drugs and other compounds can be selective inhibitors for CYPs. Inhibition of CYP-mediated reactions is well studied in humans and other mammalian model species. An important area of drug discovery involves researching possible drug-drug interactions. Some drugs are designed to inhibit the enzymes responsible for their metabolism, thus increasing the half life of the parent compound within a patient. In other pharmaceuticals, this is a serious side-effect and extreme caution must be taken when using mixtures of pharmaceuticals.

Few studies have looked at CYP inhibition in fish. Without real structural or functional data on fish CYPs it can be hard to determine which CYPs are susceptible to inhibition by pharmaceuticals. Laville *et al.* (2004) provided the first extensive look at the effects of pharmaceuticals on fish cells. Testing a broad range of drug classes *in vitro*, they found that following 24 hours exposure, fibrates and anti-depressants, in particular, were potent inhibitors of EROD activity in fish hepatocytes. Studies are scarce on fish CYP inhibition outside of CYP1A.

1.1.5 Measuring CYP activity in fish

Quantification of mRNA and protein content are useful tools in identifying target pathways of specific compounds; however, these changes may not lead to meaningful

alterations in functional protein activity. Furthermore, antibodies specific to fish CYPs are lacking and the development of relevant antibodies and application of qPCR approaches are expensive. The use of catalytic assays is effective at determining increased functional protein activity and these assays are relatively inexpensive; however, characterization of CYP-mediated substrate metabolism in fish is limited. Alkoxyresorufin-*O*-dealkylase (AROD) substrates have been used in fish studies to determine changes in CYP activity. Ethoxyresorufin- (EROD) and methoxyresorufin-*O*-dealkylase (MROD) are catalyzed by CYP1s in fish (Berndtson and Chen 1994; Gooneratne *et al.* 1997). Benzyloxyresorufin (BROD) and pentoxyresorufin-*O*-dealkylase (PROD) are catalyzed by a variety of CYP proteins including the CYP1s (Hartl *et al.* 2007; Scornaienchi 2009). These substrates can be rather broad and determining more specific changes in CYP activity is needed. A number of fluorometric substrates have been developed for use in mammalian systems but have yet to be widely applied to non-mammalian species. For example, AMMC and MAMC are selective substrates for human CYP2D6 (Onderwater *et al.* 1999; Miller *et al.* 2000). BQ and BFC have been developed as more selective replacements for 7-BR as substrates for human CYP3A4 activity. Both BQ and BFC have been used in fish studies with activity being attributed to CYP3A activity (Haasch *et al.* 1994; Burke *et al.* 1995; Thibaut *et al.* 2006). Whether BFC and BQ are specific substrates of fish CYP3A is not clear as zebrafish CYP1s (particularly CYP1A) metabolize BFC better than CYP3A65 (Scornaienchi 2009). A more extensive investigation of BFC and BQ metabolism in fish has not yet

been explored. DBF and CEC are compounds that can be used as broader mammalian CYP substrates. For example, DBF, a fluorescein-derived substrate, is metabolized by mammalian CYP 3A4 and 2C9 and is the first reported substrate for CYP2C8 in humans (Miller *et al.* 2000). Characterization of such substrates using fish tissues would be extremely useful and a well characterized CYP-mediated functional comparison between mammalian and fish species is needed in order to accurately compare across taxa.

1.2 Pharmaceuticals in the environment

1.2.1 Background

The presence of pharmaceutical contamination was first reported in the mid-1970s (Hignite and Azarnoff 1977) and has since grown to be a major area of concern in environmental toxicology. These emerging contaminants enter the aquatic environment as parent compounds or metabolites, via sewage treatment plants, direct discharge of raw sewage or aquaculture/agricultural runoff. Discharge rates of sewage effluent are estimated to reach kilograms per year (Daughton and Ternes 1999; Lindberg *et al.* 2005). The uniqueness of this class of contaminants is that they enter the environment in large, complex mixtures and the dynamic of these mixtures is not necessarily predictable. Some compounds will undergo degradation while others will be more persistent. Concentrations of different drug classes will also change depending on the demographic of each community. Compounds that are not largely persistent in the environment may elicit similar biological effects as persistent contaminants because of their continuous discharge into the aquatic system. Pharmaceuticals are designed to have an inherent

effect on physiological systems, and even at low concentrations may pose considerable risk to aquatic organisms.

1.2.2 Compounds and concentrations

A variety of pharmaceutical classes have been detected in waste water effluent and surface waters across North America and Europe. Lipid regulators, antidepressants, analgesic/anti-inflammatories, hormone supplements, anti-epileptics, and antibiotics are some of the drug classes which have been detected in the environment at concentrations ranging from ng L^{-1} to μg L^{-1} (Halling-Sorensen *et al.* 1998; Daughton and Ternes 1999; Kolpin *et al.* 2002; Metcalfe *et al.* 2003; Metcalfe *et al.* 2003; Brooks *et al.* 2005; Lindberg *et al.* 2005). Some compounds, like fluoxetine, carbamazepine and gemfibrozil are continuously discharged into the environment at high concentrations. In addition, compounds like caffeine and cotinine (metabolite of nicotine) have also been detected at measureable concentrations in the aquatic environment (Metcalfe *et al.* 2003).

Analytical techniques used in assessing concentrations of pharmaceuticals in the environment dictate the limits of detection and quantitation. Non-detection does not necessarily mean that the compound is not present. Development of analytical methods to detect low dose pharmaceuticals has likely delayed the urgency of research for the effects of these contaminants in aquatic species. More sensitive techniques are constantly being developed in this field, allowing a better understanding of the types of compounds and concentrations entering areas of concern. Detection of these compounds in fish tissues will be needed to accurately assess exposure levels and environmental risks

associated with these pharmaceutical contaminants.

1.2.3 Implications for fish

With pharmaceuticals constantly being discharged through sewage effluent, many questions arise about the implications for wildlife exposed to low, sublethal concentrations of these drugs over long periods of time. Studies on fish exposed to various pharmaceuticals suggest fish are susceptible to changes in hormone levels (Gracia *et al.* 2007; Mennigen *et al.* 2008), offspring deformities (Foran *et al.* 2004; Nash *et al.* 2004; Parrott and Bennie 2009), reproductive dysfunction (Foran *et al.* 2004; Nash *et al.* 2004), decreased egg production (Lister *et al.* 2009), changes in social and feeding behaviours (Stanley *et al.* 2007), and cytotoxicity (Halling-Sorensen *et al.* 1998; Laville *et al.* 2004; Thibaut and Porte 2008). Many of these endpoints are seen at concentrations above what would be considered environmentally relevant. Two factors may affect the interpretation of environmentally relevant concentrations. First, additive and synergistic effects are important and should be considered. Many compounds within a particular drug class work along the same physiological pathway and mixtures of similar low-dose compounds may act on a biological system in a manner analogous to higher concentrations. Second, some of these compounds have been shown to bioaccumulate within the tissues of fish (Paterson and Metcalfe 2008), increasing the overall load to well above concentrations found in the surrounding waters. Whether fish can efficiently metabolize pharmaceuticals encountered in the environment is unclear at this time. The assumption that non-mammalian species are capable of drug metabolism is reasonable,

but the capacity and specific CYPs responsible for that metabolism will be more difficult to determine.

As discussed, the specific functions of individual CYPs outside of mammalian systems are not well understood and information is often inferred from mammalian data. Assumptions of similar function across taxa can be premature and problematic. Whether pharmaceuticals are able to induce or inhibit fish CYPs is yet to be determined.

1.3 Research questions and hypotheses

The goals of this project are to further our understanding of fish cytochrome P450s with respect to pharmaceutical contamination. This general objective has been packaged into three specific studies, presented as papers prepared for publication in chapters 2, 3 and 4.

Chapter 2 focuses on CYP induction and catalytic activity in fish liver microsomes. Juvenile rainbow trout (*Oncorhynchus mykiss*) and mummichog killifish (*Fundulus heteroclitus*) were exposed *in vivo* with compounds known to induce CYPs in mammals to test for induction over a suite of catalytic assays. In addition, β-naphthoflavone (BNF), known to induce CYP1A in fish, was used to help characterize the metabolism of these substrates in fish. Given that the nuclear receptor systems that facilitated CYP induction by the test compounds in mammals are present in fish, it was hypothesized that rifampicin, dexamethasone and pregnenolone-16α-carbonitrile would induce the metabolism of some of the substrates in killifish and rainbow trout. More

specifically, the substrates catalyzed by CYP2s and CYP3s in mammals (7-BR, 7-PR, AMMC, MAMC, BFC, MFC, BQ and DBF) would be induced by these compounds. BNF, being an AhR agonist was predicted to induce the metabolism of CYP1 substrates; specifically, it was hypothesized that EROD, MROD, BROD and CEC metabolism would be highly induced, with EROD and MROD showing particularly high induction by BNF.

Chapter 3 concentrates on CYP inhibition in fish. Using liver microsomes from BNF-treated rainbow trout, four pharmaceuticals were investigated for their potential to inhibit CYP-mediated reactions in fish. Erythromycin, ciprofloxacin, gemfibrozil and fluoxetine are pharmaceuticals that inhibit important drug-metabolizing proteins in mammals. These four compounds have been detected in the aquatic environment, making them environmentally relevant choices for investigation of CYP inhibition in fish. Control and BNF-treated rainbow trout liver microsomes were used to assess the inhibition of CYP-mediated catalytic reactions by erythromycin, ciprofloxacin, gemfibrozil and fluoxetine. Based on our knowledge of strong inhibition of mammalian CYPs, it was hypothesized that we would see inhibition of CYP-mediated reactions using fish liver microsomes. Specifically, ciprofloxacin would inhibit reactions catalyzed by CYP1A, gemfibrozil would inhibit typical mammalian CYP2 substrates; erythromycin would inhibit CYP3A-catalyzed metabolism and fluoxetine would inhibit more broadly, both CYP2 and CYP3 substrates.

Lastly, Chapter 4 documents a study investigating fluoxetine as a test compound

for *in vitro* hepatic drug metabolism in fish. A variety of fish species were investigated for their capacity to metabolize fluoxetine, a known environmentally relevant pharmaceutical. Based on previous *in vivo* fluoxetine metabolism studies in fish, it was hypothesized that we would see *in vitro* production of the demethylated metabolite, norfluoxetine, and that differences in the rate of demethylation would be found across the tested fish species.

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CHAPTER 2:

ASSESSMENT OF CYTOCHROME P450 FLUOROMETRIC SUBSTRATES WITH RAINBOW TROUT AND KILLIFISH EXPOSED TO DEXAMETHASONE, PREGNENOLONE-16α-CARBONITRILE, RIFAMPICIN, AND β-NAPHTHOFLAVONE

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Abstract

Cytochrome P450s (CYPs) are important xenobiotic-metabolizing proteins and their functions are well studied and understood in mammals. However, studies on CYP function in non-mammalian vertebrate systems are much less defined, and function is often inferred from mammalian data, assuming similar function across vertebrate taxa. In this study, we investigate whether *in vivo* treatment with known mammalian CYP inducers can alter the *in vitro* catalytic activity of fish microsomes using eleven fluorescent CYP-mediated substrates. We investigate the basal metabolism and induction potential for hepatic CYPs in two fish species, rainbow trout (*Oncorhynchus mykiss*) and killifish (*Fundulus heteroclitus*). Striking species differences were found in the baseline metabolism of these substrates. Killifish have significantly higher metabolic rates for all tested substrates except BQ and BFC (both mammalian CYP3A substrates); significant differences were also seen between male and female killifish. Treatment with dexamethasone (DEX), pregnenolone-16α-carbonitrile (PCN), and rifampicin (RIF) did not cause broad, measurable CYP induction in either fish species. DEX treatment (100 mg kg^{-1}) significantly induced CEC metabolism and RIF treatment (100 mg kg^{-1}) induced MROD activity in trout, although both were highly variable. PCN treatment (100 mg kg^{-1}) in female killifish showed significantly higher PROD activity. Trout treated with 10 and 50 mg kg^{-1} β-naphthoflavone (BNF), a CYP1A inducer, showed significantly induced activity across all substrates except DBF, BFC and BQ. MAMC, a typical CYP2D substrate in mammals, is not metabolized by untreated fish liver microsomes;

however, treatment with BNF significantly induced the metabolism of this substrate in trout. In addition, interesting correlations in fold-induction by BNF across these CYP substrates strengthens the argument that BNF induces multiple CYPs in fish liver, including, but not exclusively, the known BNF inducible CYP1s. At this time, the CYPs involved in the metabolism of these substrates in fish are still unknown. Many of these catalytic assays could be valuable tools for identification of the specific function of CYP subfamilies and individual isoforms in fish.

Keywords: cytochrome P450, fish, induction, metabolism, dexamethasone, rifampicin, pregnenolone-16α-carbonitrile, β-naphthoflavone

Contributions: This chapter has been formatted as a paper prepared for publication. Experimental work and data analysis was carried out by E.M.S under the guidance and supervision of J.Y.W.

2.1 Introduction

Cytochrome P450s (CYPs) are a superfamily of membrane-bound monooxygenase proteins involved in the biotransformation of many endogenous and exogenous compounds and have been characterized in both eukaryotic (Andersson and Forlin 1992; Bolwell *et al.* 1994; James and Boyle 1998; Walker 1998; Snyder 2000; Otyepka *et al.* 2007; Seliskar and Rozman 2007) and prokaryotic species (Urlacher and Schmid 2002; Lewis and Wiseman 2005). CYPs have been found in nearly all vertebrate tissues examined but are generally most prevalent in the digestive tract, specifically the liver (Debri *et al.* 1995). In mammals, proteins belonging to the first four families (CYP1-CYP4) are highly associated with drug activation and metabolism. However, drug metabolism studies are fundamentally lacking outside of mammalian species. Furthermore, the functions of CYPs outside of mammalian systems are not well known and information is often inferred from mammalian data, assuming similar function for putative orthologous proteins. Assumptions of similar function across taxa can be premature and problematic for several reasons. First, amino acid sequence similarity does not ensure functional conservation of a protein. Second, gene duplications can produce a different number of homologous genes across vertebrate lineages. Yet, CYP families and subfamilies, with the exception of the CYP2 subfamilies, are nearly identical in vertebrate taxa and the total numbers of CYP genes in vertebrate species is similar (Thomas 2007). For example, fish and mammalian lineages share all families except CYP39 (Nelson 2003). The assumption that non-mammalian species are capable of drug

metabolism is reasonable, but the specific CYP(s) responsible for that metabolism will be more difficult to determine.

A key aspect of CYPs is the induction of expression via nuclear receptors such that modulation of the CYP profile is often used as an indicator of exposure to environmental contaminants. CYP1A induction, specifically, is commonly used to identify the presence of polyaromatic hydrocarbons (PAHs) and other aryl hydrocarbon receptor (AhR) agonists, the specific nuclear receptor responsible for induction of CYP1 genes in all vertebrates (Hahn *et al.* 1998). In addition to PAHs, other contaminants present in aquatic systems have the potential to alter CYP activity. Pharmaceuticals are potent CYP-inducing agents, upregulating transcription of specific CYP genes via a suite of nuclear receptors. The mammalian CYP2 family is regulated through a number of receptors, including the retinoic acid receptor (RAR), the constitutive androstane receptor (CAR) and hepatocyte nuclear factor 4 (Honkakoski and Negishi 2000; Lewis *et al.* 2002). Pregnane X receptor (PXR) and glucocorticoid receptors are involved in mammalian CYP3 induction (Handschin and Meyer 2003). Similar induction pathways exist across vertebrate classes and with the exception of CAR, all of these nuclear receptors have been identified in fish (Moore *et al.* 2002; Maglich *et al.* 2003; Moore *et al.* 2003). PXRs respond to a structurally diverse group of xenobiotics and endogenous compounds in mammals and this characteristic appears to be conserved in fish (Moore *et al.* 2002). Collectively, these data suggest that fish CYPs are likely inducible by compounds that induce CYPs in mammals via the conserved nuclear receptor pathways;

however, the specific isoforms which would be responsive to such induction is still unclear.

In humans, CYP3A4 is the major CYP responsible for drug metabolism; other isoforms, namely, CYP2D6, 2C9 and 1A2 also play a large role in pharmaceutical metabolism (Hemeryck and Belpaire 2002). Orthologous genes to CYP 3A4, 2D6, 2C9 and 1A2 have been identified as major players in drug metabolism in other mammalian species. Mammalian CYP2 and 3 genes can be induced by pharmaceuticals such as carbamazepine (CBZ), dexamethasone (DEX), phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN) and rifampicin (RIF), although species differences are known to occur (Lu and Li 2001; Luo *et al.* 2002; Lee *et al.* 2006). Discrepancies exist with respect to typical mammalian CYP inducers and their effects in fish. PB is a strong inducer of CYP2s and 3s in mammals (Waxman and Azaroff 1992). However, in fish, which lack CAR (the nuclear receptor responsible for PB induction in mammals), CYP2 and 3 isoforms were not induced by PB in several studies (Addison *et al.* 1987; Ankley *et al.* 1987; Kleinow *et al.* 1990; Li *et al.* 2008) although PB induced CYP1A in fish hepatocytes (Sadar *et al.* 1996). β-naphthoflavone (BNF) is considered a prototypical ligand for the AhR and standard CYP1A inducer in both mammals and fish (Kloepper-Sams and Stegeman 1989; Haasch *et al.* 1994; Lemaire *et al.* 1996) and with few exceptions (Parente *et al.* 2009), is potent at inducing the CYP1A associated ethoxyresorufin-*O*-dealkylase (EROD) activity in nearly all species. Induction of fish CYPs has been documented by a variety of typical mammalian inducers; however, results

appear to be conflicting. For instance, both DEX and RIF induced zebrafish CYP3A65 in fry (Tseng *et al.* 2005), yet the sea bass CYP3A isoform, CYP3A79, was not affected at all by DEX (Vaccaro *et al.* 2007). *In vivo* CYP3A induction by RIF in fish appears promising (Li *et al.* 2008) but studies with this compound are limited.

It is crucial to understand and identify, specifically, which types of chemicals alter CYP expression and function in fish in order to understand basic fish physiology, the evolution of CYP function in vertebrates and for environmental concerns of pharmaceutical contamination. Over the last decade, hundreds of drugs, from human-use, agriculture and aquaculture, have been detected in the aquatic environment. Pharmaceuticals are designed to have a significant effect on biological systems and may present a risk to fish and other aquatic life, even at the low concentrations typical of aquatic systems. Whether the pharmaceuticals found in low concentrations in the aquatic environment are able to alter fish CYP levels is uncertain and cannot be predicted without an understanding of the induction pathways for fish CYPs.

Quantification of mRNA and protein content may be useful in identifying target pathways of specific compounds; however, these changes may not lead to meaningful alterations in protein activity. Furthermore, antibodies specific for fish CYPs are lacking and the development of relevant antibodies and application of qPCR approaches are expensive. The use of catalytic assays is effective at determining increased functional protein activity and these assays are relatively inexpensive. However, characterization of CYP-mediated substrate metabolism in fish is limited. Alkoxyresorufin-*O*-dealkylase

(AROD) substrates have been used in fish studies to determine changes in CYP activity. These substrates can be rather broad and determining more specific changes in CYP activity is needed. A number of new fluorometric substrates have been developed for use in mammalian systems but have yet to be broadly applied to non-mammalian species. For example, MAMC (7-methoxy-4-(aminomethyl)coumarin) and AMMC (3-[2-(N,Ndiethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin) are selective substrates for human CYP2D6 (Onderwater *et al.* 1999; Miller *et al.* 2000). 7-Benzyloxyquinoline (BQ) and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) have been developed as more selective replacement for 7-benzyloxyresorufin (7-BR), a common AROD substrate, as substrates for human CYP3A4 activity. Both BQ and BFC have been used to identify CYP3A activity in fish (Haasch *et al.* 1994; Burke *et al.* 1995; Thibaut *et al.* 2006). Dibenzylfluorescein (DBF) and 3-Cyano-7-ethoxycoumarin (CEC) are compounds that can be used as broader mammalian CYP substrates. For example, DBF is metabolized by mammalian CYP 3A4, 2C9 and is the first reported substrate for CYP2C8 in humans (Miller *et al.* 2000). Characterization of such substrates using fish tissues would be useful as a CYP-mediated functional comparison between mammalian and fish species.

This study was undertaken to compare the induction potential and catalytic activity of fish cytochrome P450s in liver microsomes from two fish species. We have determined the metabolism of eleven mammalian CYP substrates in rainbow trout (*Oncorhynchus mykiss*) and killifish (*Fundulus heteroclitus*) exposed to RIF, PCN, and

DEX; compounds known to induce important drug metabolizing CYPs in mammals. In addition, the metabolism of these substrates was tested using rainbow trout liver microsomes from fish treated with BNF to assess the catalytic activity across these substrates with a known fish CYP1 inducer. Using catalytic assays and a comparative approach, we will highlight trends and significant alterations seen in the *in vitro* metabolic activity across these CYP-mediated reactions and discuss the implications of assuming similar CYP function across taxa.

2.2 Methods

2.2.1 Animals

Mummichog killifish (*Fundulus heteroclitus*) were purchased from Aquatic Research Organisms (Hampton, NH, USA). Male and female killifish were held at 19-20°C in 10% recirculating seawater and fed staple flakes 5 days per week, supplemented with frozen brineshrimp. Juvenile rainbow trout (*Oncorhynchus mykiss*) were purchased from Humber Springs trout hatchery (Mono Mills, ON, Canada). Trout were kept at 12-15°C in flow-through water and fed floating trout pellets 3 times per week. All fish were held for a minimum of two weeks before use. Food was removed 24-48 hours before treatment.

2.2.2 In vivo CYP induction

β-naphthoflavone (BNF), pregnenolone-16α-carbonitrile (PCN), dexamethasone (DEX) and rifampicin (RIF) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Trout were exposed to 50 and 100 mg kg^{-1} body weight of PCN, DEX and RIF and 10, 25 and 50 mg $kg^{-1}$of BNF. Killifish were exposed to 100 mg $kg^{-1}$of PCN, DEX and RIF only. All compounds were dissolved in corn oil and administered via i.p. injection, under anesthetic (100-200 mg L^{-1} methanesulfonate salt, MS-222) with the injection volume not exceeding 10 μL g^{-1} total body weight. Control fish were injected with carrier only. All fish were sacrificed 48-hours after injection to allow for maximal CYP induction (Celander *et al.* 1997).

Livers were collected and placed directly on ice or flash-frozen in liquid nitrogen. Tissues were stored at -80°C until use. Rainbow trout livers were large enough to use single fish replicates; killifish replicates consisted of pooled individuals (3-4 fish per pool) due to their small liver size. Liver microsomes were prepared as in Stegeman *et al* (1995) and total protein content was determined using a bicinchoninic acid (BCA) kit, according to manufacturer's protocols (Pierce protein research products).

2.2.3 In vitro catalytic activity

CYP induction was determined using fluorescent-based catalytic assays for eleven substrates. The specificity of these substrates is known for mammalian CYPs but for many of these substrates has not been determined in non-mammalian species. Alkoxyresorufin-O-dealkylase (AROD) activities were determined using 7-ethoxyresorufin (7-ER), 7-benzyloxyresorufin (7-BR), 7-methoxyresorufin (7-MR) and 7-pentoxyresorufin (7-PR; Sigma-Aldrich, St. Louis, MO) according to previously

published methods (Hahn *et al.* 1993). The remaining seven substrates, 3-cyano-7-ethoxycoumarin (CEC), 7-methoxy-4-aminomethylcoumarin (MAMC), 3-[2-(N,Ndiethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-methoxy-4-trifluoromethylcoumarin (MFC), 7-benzyloxyquinoline (BQ) and dibenzylfluorescein (DBF; BD Biosciences, San Jose, CA), were used in assays optimized in our lab for use in fish, based on methods published for mammalian microsomes (Crespi *et al.* 1997; Miller *et al.* 2000; Stresser *et al.* 2000). A summary of the substrates with optimal concentrations, buffers and assay settings are listed in Table 1. All reactions were run at room temperature for 10 minutes in black 96-well format using kinetic assays. Fluorescence was measured using a Synergy II microplate reader and analysis done using Gen5 software (BioTek Instruments Inc.). Due to the small liver size of male killifish, there was insufficient material for all assays. Thus, PCN and DEX treated male killifish microsomes were used in those catalytic assays that showed significant induction for treated female killifish (EROD, MROD and PROD).

2.2.4 Statistical Analysis

Significant induction was determined using one-way or two-way analysis of variance (ANOVA) for trout and killifish, respectively. Data that did not pass assumptions for ANOVA were log-transformed. If log-transformed data did not qualify for parametric ANOVA, non-parametric Kruskal-Wallis one-way analysis of variance was used. All post-hoc tests were completed using the Student-Newman-Keuls method.

Linear regression analysis was run on fold-induction from controls for 10 and 25 mg kg^{-1} BNF treatment between substrates. All statistical tests were completed using Sigmaplot 11.0 (Systat Software Inc). Reactions that fell below limit of detection for respective standard curves were assigned a value equal to the limit of detection in pmol accumulated over the 10 min reaction time in order to perform statistical analysis.

2.3 Results

2.3.1 DEX, PCN and RIF treatments

Treatment with dexamethasone (DEX) yielded highly variable metabolism of many substrates (Table 2). Even with such high variability, rainbow trout treated with a high dose of DEX, had significantly higher metabolism of CEC, compared to controls (4.6-fold induction; log-transformed data, ANOVA; p=0.049). High DEX treatment in rainbow trout increased EROD and MROD activity, 3 and 2.3-fold over controls, although not statistically significant (ANOVA; p=0.09). Metabolism of these substrates by female killifish exposed to DEX did not reveal any significant differences from control fish, although high variability can been seen in metabolism of some substrates (Table 3).

Pregnenolone-16α-carbonitrile (PCN) did not significantly change the metabolism of any substrate by rainbow trout liver microsomes; some substrate metabolism by PCN-induced trout, namely BQ and 7-ER metabolism, are highly variable (Table 2). 7-PR metabolism by female killifish exposed to 100 mg kg^{-1} PCN had significantly higher rates of metabolism compared to controls (8.8-fold induction; Table 3). PROD was not

significantly different between control and treated male killifish, revealing a possible sex difference in the response to PCN treatment (Table 3).

Rifampicin (RIF) treatment in trout increased MROD activity at high dose (2.7-fold induction; one-way ANOVA; p=0.04). A high dose (100 mg kg^{-1}) of RIF in killifish did not significantly increase the metabolism of any tested substrates (Table 3).

2.3.2 BNF treatment

β-naphthoflavone (BNF) is a known CYP inducer in fish, and was used to help characterize CYP-function in these substrates using rainbow trout. Induction by moderate BNF treatment (25 mg kg^{-1}) was not significantly different from low (10 mg kg^{-1}) for any substrate and was not compared further in this analysis. Trout liver microsomes from BNF treated rainbow trout showed significantly increased metabolism of a number of CYP substrates (Figure 1). All four AROD substrate activities were increased by BNF treatment (Figure 1A). Of these four substrates, MROD activity showed the largest fold-induction, with 17 and 59-fold increases from control trout at low and high BNF treatment, respectively (log-transformed data; p<0.001; Figure 1A). CEC metabolism was significantly increased for both low (5.5-fold) and high (8-fold) BNF treatments (p<0.001; Figure 1B). AMMC metabolism assays produced no activity above background florescence for any sample. MAMC metabolism was significantly induced by both low and high BNF treatment (4 and 12-fold, respectively; log-transformed data; p<0.002; Figure 1B). Metabolism of MFC showed the most drastic increase, with over 19-fold and 60-fold induction at the low and high BNF dose, respectively (log-

transformed data; $p<0.001$; Figure 1B). The remaining three substrates (BFC, BQ and DBF) revealed no significant changes in activity (ANOVA, $p>0.05$).

Linear regressions, based on the fold induction over control activity, were run between substrates for those showing induced activity at 10 and 50 mg kg^{-1} BNF (Table 4). Fold-induction of EROD activity was correlated with only MFC at low BNF dose. All substrates, except 7-PR, were correlated with EROD induction at high BNF dose. The induction seen in 7-PR and CEC metabolism were correlated at both low and high BNF treatment. The increase in PROD activity was correlated with BROD at low BNF dose but not high dose. No other substrates showed correlation with the fold-induction of 7-PR metabolism. Induction of MFC and MAMC metabolism are correlated at both low and high BNF treatment. Induction of BROD activity is correlated with induction of CEC and 7-PR metabolism at low BNF dose only, and correlated with MFC and MAMC at high BNF dose only. Induction of MROD activity was correlated with MAMC at low dose but no substrates at high dose. The most striking change in correlation is with CEC and EROD: at low BNF dose, no relationship is seen at all, however at high BNF dose, a very strong correlation exists between the fold-induction of these two substrates (Table 4). Although all of these substrates show significant induction with BNF treatment, some substrate pairs show no correlations whatsoever in the induction of their metabolism (PR/ER, PR/MR, PR/MAMC, PR/MFC, MR/BR, MR/CEC, MR/MFC, CEC/MAMC, CEC/MFC; Table 4).

2.3.3 Species and sex comparison

Comparing baseline metabolism of these substrates by control rainbow trout and killifish liver microsomes reveals striking differences in enzymatic activity (Figure 2). Killifish liver microsomes metabolized most of these CYP-mediated substrates better than trout liver microsomes. Metabolism of 7-ER and 7-MR, in particular, was drastically higher in killifish (Figure 2A). Exceptions to this trend are metabolism of BQ and BFC, in which no significant differences were seen between the metabolism by trout, or killifish liver microsomes (Figure 2B). Note that the metabolism of BQ shows little variability between species. Metabolism of BQ is the highest activity in trout followed by EROD and MROD; EROD and MROD activity exceed BQ metabolism in killifish. The order in which each species metabolized these substrates differs; trout metabolized in the order: BQ > 7-ER >> 7-MR > MFC > 7-BR/7-PR/BFC > CEC > DBF. Killifish metabolized the substrates in the order: 7-ER > 7-MR > BQ >> 7PR/7BR > MFC/BFC/CEC > DBF. Neither MAMC nor AMMC were metabolized by control microsomes in either species.

Metabolism of 7-BR and 7-PR was similar for male and female killifish (Figure 2A). CEC and DBF were the only substrates that male killifish metabolized better than female killifish (Figure 2B). Metabolism of DBF was similar between female killifish and rainbow trout (Figure 2B). Yet, the overall catalytic activity of fish microsomes for CEC and DBF were very low when compared to the other substrates. Female killifish metabolized 7-ER, 7-MR, and MFC better than male killifish (Figure 2).

2.4 Discussion

This study has several important findings. First, we report a broad analysis of several fluorescent CYP substrates in two fish species; to our knowledge there are no studies with the metabolism of DBF, MFC, AMMC, or MAMC in any fish species. Second, we use eleven fluorescent catalytic assays to assess induction of hepatic CYPs by RIF, DEX, PCN and BNF, four commonly used CYP inducers in mammals. Thirdly, this study illustrates species and sex differences in baseline metabolism of these CYP-mediated substrates using juvenile rainbow trout and adult killifish. Collectively, we begin to uncover interesting relationships between induction and the tested substrates, suggesting functional differences in fish compared to mammals.

Alkoxyresorufin substrates (7-ER, 7-BR, 7-MR, and 7-PR) are commonly used CYP substrates in fish, mammals and other vertebrates. 7-ER, in particular is very widely used in vertebrates to assess CYP1A induction associated with PAH contamination. In mammals, 7-MR is used to distinguish between CYP1A1 and 1A2 activity with preferential metabolism of 7-MR by CYP1A2. In fish, both 7-ER and 7-MR are metabolized equally by the single CYP1A isoform (Gooneratne *et al.* 1997). Some salmonid species, however, possess two CYP1A isoforms, denoted CYP1A and CYP1A3 (Berndtson and Chen 1994). No preference is seen for 7-MR by this second CYP1A protein (Gooneratne *et al.* 1997), supporting the phylogenetic relationship of CYP1A3 (Morrison *et al.* 1998), which indicates that it is not homologous to mammalian CYP1A2. In fish, EROD and MROD are generally correlated and both are used as indicators of

CYP1 activity (White *et al.* 1997; Smeets *et al.* 2002; Parente *et al.* 2004). 7-BR is a broad substrate in mammals, metabolized by CYP1A, 2B and 3A proteins and is used as such in fish as well (Hartl *et al.* 2007). 7-PR-*O*-dealkylase (PROD) is used to identify CYP2B-like, or phenobarbital (PB)-like induction in mammals. Although a CYP2B ortholog is not present in fish, and PB has not been shown to induce PROD activity in fish, PROD activity is still commonly associated with CYP2B-like induction (Foster *et al.* 1998). PROD activity is generally very low or non-detectable in unexposed fish liver microsomes but is inducible (Haasch *et al.* 1994; Parente *et al.* 2004). The specific CYPs responsible for PROD activity in fish are not clear, but BROD and PROD activities are generally similar in undosed fish (Haasch *et al.* 1994; Iwata *et al.* 2002).

Both BQ and BFC are selective CYP3A substrates in mammals. Both have been used to identify CYP3A activity in fish (Haasch *et al.* 1994; Burke *et al.* 1995; Kullman *et al.* 2004; Thibaut *et al.* 2006) although recent data suggested that BFC, in particular is likely metabolized by a variety of CYPs in fish, including CYP1s (Scornaienchi 2009). Very few fish studies have used CEC, although it has been applied as a non-specific substrate in fish as it is in mammals (Padros *et al.* 2000). Benzo-*a*-pyrene induces both EROD and CEC-*O*-deethylase equally but the chemical tributyltin induces CEC metabolism without EROD induction, supporting the notion that multiple fish CYPs are responsible for its metabolism (Padros *et al.* 2000).

Nine of the eleven tested substrates were metabolized by untreated fish liver microsomes, with AMMC and MAMC being the exceptions. EROD activity was the

highest of the ARODs, followed by MROD. As reported in other fish studies (Iwata *et al.* 2002), BROD and PROD activity were similar in undosed fish. These substrates were consistently metabolized by all fish samples. The CYPs responsible for BROD and PROD activity in fish are still ambiguous and more specific characterization is necessary to establish accurate CYP screening when using these substrates for fish.

The mammalian CYP3A substrates, BFC and BQ, showed rates of metabolism different from one another in fish liver. BFC was not well metabolized or induced in the present study, although it appears inducible in some species (Haasch *et al.* 1994; Burke *et al.* 1995; Thibaut *et al.* 2006). BQ was also not inducible by the tested compounds, but it was well metabolized by fish liver microsomes with consistently high metabolism seen across both species evaluated. This suggests that fish are proficient at metabolizing BQ in the liver. Metabolism of BFC by killifish and trout was lower than human liver microsomes (Renwick *et al.* 2001). The rate of metabolism of BQ seen in this study is lower than the range found in microsomes from a number of different mammalian species (Baririan *et al.* 2006). Although BFC was not well metabolized by fish liver microsomes in this analysis, previous studies have reported higher levels of BFC metabolism in fish (Hegelund *et al.* 2004; Kullman *et al.* 2004; Kashiwada *et al.* 2005). Better characterization of the CYPs responsible for BFC and BQ metabolism in fish deserves further investigation. Care should be taken, however, in assuming that both of these substrates are metabolized by CYP3A in fish.

CEC metabolism was highly variable and showed evidence of species specificity.

Baseline CEC metabolism in trout was present but quite low compared to mammals (Ghosal *et al.* 2003). Killifish showed more reasonable metabolism of CEC. Because of the broad nature of this substrate in mammals, the species specificity seen here, and the fact that CEC has not been well investigated in fish, the CYPs responsible for its metabolism are unclear.

To our knowledge, this is the first study to test DBF, MFC, AMMC, or MAMC metabolism in fish. DBF is not well metabolized by fish liver microsomes and was not inducible by compounds tested in this study. This is not likely a practical candidate for CYP research in fish. MFC was not well metabolized by trout, although killifish seemed to metabolize it well. No detection of *in vitro* hepatic MAMC metabolism was found in untreated fish. Yet, both MFC and MAMC were inducible by BNF; MFC and MAMC may prove useful in future induction screening but not necessarily for baseline catalytic function in fish. Across numerous samples spanning two species, two sexes, and four CYP inducers, no metabolism of AMMC was detected. This substrate does not appear to be metabolized in the liver of fish.

In mammals, DEX, PCN and RIF typically induce CYPs via the pregnane X receptor (PXR) regulation (Honkakoski and Negishi 2000). Although a functional PXR has been identified in fish, few typical mammalian PXR agonists have been found to activate the ligand-binding domain (Moore *et al.* 2002). The ligands that have been shown to bind to the fish PXR do not generally produce large increases in functional proteins. In the present study, we see very little induction by DEX, RIF or PCN.

Variation exists in the response of these fish species to the tested CYP inducers; yet, in the midst of this variability significant differences are present. Significantly higher CEC metabolism is seen in trout treated with DEX. CEC is metabolized by broad range of CYPs in mammals (CYP1A2, CYP2Cs) yet metabolism of other typical mammalian CYP2C and 1A substrates (Table 1) are not induced by DEX in trout nor is CEC metabolism induced by DEX in killifish. DEX has shown little effect on expression levels of CYP3A in trout (Celander *et al.* 1989; Miranda *et al.* 1991; Lee *et al.* 1993), but increased CYP3A expression was successful in zebrafish (Padros *et al.* 2000). In trout liver microsomes, Haasch *et al* (1994) documented induced metabolism of a number of substrates, including PROD activity with only 2 mg kg^{-1} DEX. Perhaps the dose of DEX used in this study exceeded induction range in this species.

In this study, we see PROD activity in female killifish induced by PCN at levels which are orders of magnitude higher than controls, with no induction seen whatsoever in male killifish. PROD is indicative of 2B activity in mammals and is highly inducible by PB (Burke *et al.* 1985; Parmar *et al.* 1998). Fish lack the nuclear receptor, CAR, attributed to PB induction in mammals and PB does not induce 7-PR metabolism in fish. PROD activity is not well characterized in fish and the process by which PCN would induce female PROD activity is intriguing.

Grass carp CYP3As appear to respond to RIF and DEX induction (Li *et al.* 2008). Induced CYP3A protein expression has been documented by PCN in trout hepatic microsomes and in tilapia (Celander *et al.* 1989; Pathiratne and George 1996). Yet, in the

present study we see no induction in the typical mammalian CYP3A substrates tested. In mammals, PXRs are activated by a broad range of ligands. Fish PXRs have shown evidence of activation by many xenobiotics that activate the mammalian PXR (Moore *et al.* 2002). No clear pattern exists between the few substrates with induced activity and the mammalian CYP subfamilies generally induced by RIF, DEX and PCN. Across the literature, consistent induction by RIF, DEX and PCN is not found in fish. These compounds do not appear to strongly induce CYPs in either trout or killifish and are likely not good compounds for further assessment of induction pathways in fish. If compounds such as these are able to bind to the PXR in fish yet no functional protein induction is measured, perhaps the assays used to assess PXR-mediated CYP regulation in mammals are not appropriate for measuring the function of PXR-regulated CYPs in fish.

In contrast to RIF, DEX and PCN, BNF seems to induce the metabolism of nearly all substrates tested. Induction by BNF in trout liver microsomes is very broad, broader than what would be expected by CYP1 induction alone. 7-ER is the prototypical substrate used to assess CYP1A activity and therefore induction by BNF was expected. Recent data using heterologously expressed CYP1 proteins from zebrafish (*Danio rerio*) show that EROD activity is not catalyzed by CYP1A alone; other CYP1 enzymes, most significantly CYP1C2, are capable of 7-ER metabolism (Scornaienchi 2009). 7-MR is a CYP1A2 substrate and induction by BNF is well documented (Burke *et al.* 1994; Haasch *et al.* 1994). Metabolism of 7-MR displayed the second highest fold-induction by BNF.

Expressed zebrafish CYP1s metabolized 7-MR well (Scornaienchi 2009) and MROD activity has been well correlated to EROD activity in other fish studies (White *et al.* 1997; Smeets *et al.* 2002; Parente *et al.* 2004). Our data supports previous evidence that both 7-MR and 7-ER are metabolized by CYP1 enzymes.

7-BR and CEC are metabolized by a broad suite of CYPs in mammals, including CYP1s. Induction by BNF is therefore not unexpected. BROD induction by BNF was not seen in male of female English Sole (Miller *et al.* 2004). CEC is metabolized well by CYP1A2 and has been induced by BNF in human cells (Westerink *et al.* 2008). Induction of CEC metabolism by BNF in fish has not previously been investigated; however, increased metabolism of CEC has been correlated with the presence of other PAH contaminants (Stagg *et al.* 1995; Padros *et al.* 2000).

PROD activity is typically catalyzed by CYP2B proteins in mammals. 7-PR showed surprisingly high induction by BNF. 7-PR metabolism has not been fully characterized in fish and purified CYP1As from fish have shown only slight preference for 7-PR as a substrate (Da Silva *et al.* 2004; Scornaienchi 2009). BNF treatment does not induce PROD activity in mammals (Burke *et al.* 1985; Jenner and Timbrell 1994; Monshouwer *et al.* 1998); however, we see significant induction of PROD activity by BNF treatment in trout. Although researchers don't generally associate PROD activity with AhR-mediated induction, previous studies using BNF have found results consistent with ours and observe BNF induced PROD activity in trout (Haasch *et al.* 1994; Novi *et al.* 1998). PROD provides an excellent example of the functional differences between

mammals and fish and characterization of the CYPs involved in 7-PR metabolism in fish would be valuable.

MFC is typically a CYP2C/2E substrate in mammals with minimal metabolism attributed to CYP1A2 (Stresser *et al.* 2002; Ghosal *et al.* 2003; Mingoia *et al.* 2007). MFC displayed the highest fold-induction by BNF of any substrate tested in the current study. Fold-induction of MFC metabolism was even higher than seen in EROD. Expressed zebrafish CYP1A metabolizes MFC fairly well, although not to the extent of 7-ER or 7-MR (Scornaienchi 2009) suggesting that BNF associated CYP1 activity is unlikely to account for MFC induction in this study. BNF-induced increases in MFC metabolism have been noted in human liver cells although fold- induction was less than 10 and not beyond that of EROD (Khetani and Bhatia 2008).

AMMC and MAMC, typical CYP2D substrates in mammals, are not metabolized at all by undosed fish liver microsomes; however, treatment with BNF significantly induced the metabolism of MAMC in trout. Although CYP2s are present in fish, CYP2D isoforms are not and the function of CYP2s, in particular, in fish is still unknown. Zebrafish CYP1s do not metabolize MAMC (Scornaienchi 2009). Yet, BNF induced the metabolism of this substrate in trout suggesting that BNF-induced metabolism of MAMC is not CYP1-mediated.

BFC, BQ and DBF were the only substrates to experience no induction by BNF in this study. Scornaienchi (2009) found that expressed zebrafish CYP3A65 does not

metabolize BQ; BFC was metabolized better by expressed CYP1A than CYP3A65. Neither of these substrates are likely CYP3A-selective in fish as they are in mammals. DBF was metabolized by zebrafish CYP3A65 and CYP1B1, and slightly by CYP1A (Scornaienchi 2009); DBF is unlikely to be a selective CYP3A substrate in fish. Expressed CYP3A proteins in fish appear to metabolize a number of substrates but whether these are CYP3A-selective substrates in fish is not clear (Hegelund *et al.* 2004; Kullman *et al.* 2004; Kashiwada *et al.* 2005).

Amongst the substrates showing significant induction by BNF, some strong correlations were found. However, many substrates are significantly induced but show no correlation whatsoever with one another (i.e. 7-PR/7-ER; 7-PR/7-MR; 7-BR/7-MR; 7-MR/CEC; 7-MR/MFC; 7-PR/MAMC; 7-PR/MFC; MAMC/CEC; CEC/MFC). Correlations between certain substrate pairs at both BNF doses suggest that the same enzyme(s) were responsible for catalysis of the induced metabolism of these substrate pairs (7-ER/MFC; MFC/MAMC and CEC/7PR). This analysis also provides evidence that low and high BNF induction may be inducing a different suite of enzymes (i.e. CEC/ER is strongly correlated at high treatment but not at all in low BNF treatment). The correlation analysis on these substrates is supported by previous work in a number of substrates in fish. EROD and MROD activity are correlated in a number of fish species (Smeets *et al.* 2002; Parente *et al.* 2004). The lack of EROD/PROD and MROD/PROD correlations is supported by Parente *et al.* (2004), suggesting PROD is not catalyzed by CYP1A in fish and BNF is inducing CYPs outside of CYP1. Although few studies have

looked at CEC metabolism in fish, Stagg *et al.* (1995) investigated dab (*Limanda limanda*) living near oil platforms and found that increases in CEC metabolism correlated strongly with EROD; similar to what was found in the high BNF microsomes. Overall, the present data showing induction of a broad selection of CYP-mediated assays by BNF, coupled with the correlation analysis, and functional characterization of these substrates by expressed zebrafish CYPs (Scornaienchi 2009), strengthen the argument that the induction by BNF is suggestive of a suite of CYPs and cannot be attributed to the induction CYP1 alone.

Species and sex differences in baseline metabolism of these CYP-mediated substrates were observed here in juvenile rainbow trout and adult killifish. All substrates exhibited species differences in baseline metabolism except for BFC and BQ. Interestingly, these are the same substrates that are not induced by BNF in trout. Killifish have extremely high EROD and MROD activity, indicative of CYP1A activity. Metabolism of BQ is similar across species, and is the most highly metabolized substrate by trout.

Sexually dimorphic rates of metabolism were found in the baseline metabolism of a number of substrates in killifish. Hegelund and Celander (2003) found that CYP3A mRNA and protein expression was 2.5-fold higher in male killifish compared to females. The present data suggest no sexual dimorphism in metabolism of mammalian CYP3A substrates in killifish, providing further support that these may not be selective CYP3A substrates in fish. The metabolism of CYP1A substrates, however do show sexual

dimorphism (EROD and MROD). Although CYP regulation in fish is not fully understood, CYPs are often under hormonal control and differences between males and females are common (Gray *et al.* 1991; Andersson and Forlin 1992). These differences can be more apparent during breeding seasons (Devaux *et al.* 1998). Species and sexual differences in the baseline activity seen in this study highlight the importance of species comparisons. For environmental purposes, knowledge of a species' normal baseline CYP activity and seasonal changes would be useful in determining induction from environmental contaminants and affected populations.

In summary, we have found that at the doses used, DEX, PCN and RIF are not broad CYP inducers in the liver of rainbow trout or killifish. We have uncovered interesting relationships between CYP-mediated reactions commonly used in mammals, suggesting that fish likely metabolize them to a different capacity than mammals. More specifically, BFC and BQ, which are metabolized by the same protein in mammals, have very different baseline activities in these fish species. AMMC and MAMC are CYP2D6 selective in humans; in fish, however, AMMC is not metabolized at all and MAMC is only metabolized when induced. BNF, a prototypical CYP1 inducer in mammals is likely inducing a range of CYPs in rainbow trout. Typical CYP1 substrates like 7-ER and 7-MR were induced as expected. Typical mammalian CYP2 substrates, MAMC and MFC showed surprising induction by BNF with MFC having higher fold induction than either EROD or MROD. Lastly, large species differences exist between baseline metabolic capacity of killifish and rainbow trout, with BFC and BQ metabolism similar

across species. Many of the substrates tested in the present study are novel to fish and useful tools in assessing CYP activity in fish. Characterization of the specific CYPs responsible for their metabolism is necessary, particularly 7-PR, MFC, MAMC and CEC. Extrapolation of CYP isoforms from mammalian data is not reliable and functional differences are highly likely between fish species.

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2.6 Tables

Table 1. Fluorometric assay conditions for CYP activity in fish liver microsomes.

The known mammalian CYPs associated with the metabolism of each substrate are listed.

Substrate	Mammalian[a] CYPs	Optimal substrate concentration (μM)	Buffer	Excitation (nm)[b]	Emission (nm)[b]	LOD[c] (pmol)
7-ER	1A1>1A2,1B1	2	AROD buffer[d]	540/35	590/20	2.0
7-BR	1A, 2B, 3A	2	AROD buffer	540/35	590/20	2.0
7-MR	1A2	5	AROD buffer	540/35	590/20	2.0
7-PR	2B	5	AROD buffer	540/35	590/20	2.0
AMMC	2D6	50	0.5M KPO_4[e]	400/30	460/40	7.5
MAMC	2D6	50	0.5M KPO_4	400/30	460/40	2.5
CEC	1A2, 2C9, 2C19	10	0.5M KPO_4	400/30	460/40	0.3
BFC	3A4, 2C19	1000	0.5M KPO_4	400/30	528/20	0.1
MFC	2C9	100	0.5M KPO_4	400/30	528/20	0.1
BQ	3A4	1000	0.5M KPO_4	400/30	528/20	0.6
DBF	2C8, 2C9, 2C19, 3A4	10	0.5M KPO_4	485/20	528/20	0.1

[a] Primary CYPs responsible for the metabolism of this substrate in mammals
[b] Excitation and emission values displayed as wavelength/bandwidth
[c] Limit of Detection (LOD) on standard curve
[d] AROD buffer: 50mM Tris HCl, 1mM EDTA, 1mM DTT, 20% glycerol (pH 7.4)
[e] 0.5M KPO4 buffer (pH 7.4) as used by BD Biosciences, San Jose, CA (gentest.com)

Table 2. Metabolism of CYP substrates by rainbow trout (*Oncorhynchus mykiss*) liver microsomes. Trout were treated with low (50 mg kg^{-1}) and high (100 mg kg^{-1}) doses of dexamethasone (DEX), pregnenolone 16-carbonitrile (PCN) or rifampicin (RIF). Values represent the mean activity (pmol mg^{-1} min^{-1}) with standard deviation in parentheses.

SUBSTRATE	CONTROL $n = 4$	LOW DEX $n = 4$	HIGH DEX $n = 3$	LOW PCN $n = 4$	HIGH PCN $n = 4$	LOW RIF $n = 5$	HIGH RIF $n = 5$
7-ER	129 (47)	233 (137)	386 (200)	237 (115)	202 (78)	173 (116)	281 (106)
7-BR	3.1 (0.4)	3.2 (1.6)	3.2 (0.8)	4.2 (2.6)	4.6 (1.5)	6.6 (3.5)	6.2 (4.4)
7-MR	20 (7)	28 (12)	44 (19)	30 (14)	29 (5)	44 (20)	52 (9)*
7-PR	2.2 (0.5)	2.0 (0.2)	1.8 (1.1)	2.1 (0.3)	2.0 (0.6)	8.6 (5.9)	3.1 (2)
CEC	0.25 (0.08)	0.59 (0.32)	1.18 (1.06)*	0.28 (0.16)	0.32 (0.26)	0.47 (0.31)	0.51 (0.33)
AMMC	N/A [a]	N/A	N/A	N/A	N/A	N/A	N/A
MAMC	N/A [a]	N/A	N/A	N/A	N/A	N/A	N/A
BFC	2.4 (0.5)	3.2 (1.4)	2.7 (1.4)	3.2 (2.4)	3.1 (1.5)	3.4 (2.0)	3.1 (1.7)
MFC	3.9 (3.0)	3.8 (1.1)	5.8 (3.7)	3.2 (0.7)	4.5 (0.4)	3.1 (0.9)	5.5 (2.5)
BQ	215 (50)	192 (46)	156 (43)	239 (99)	266 (89)	200 (87)	231 (92)
DBF	0.20 (0.05)	0.17 (0.04)	0.13 (0.03)	0.18 (0.07)	0.27 (0.08)	0.25 (0.07)	0.26 (0.05)

[a] N/A – No detectable activity above background fluorescence.
*Significantly different from controls (one-way ANOVA, Student Newman-Keuls post-hoc test; $p<0.05$).

Table 3. Metabolism of CYP substrates by killifish (*Fundulus heteroclitus*) liver microsomes. Killifish were treated with 100 mg kg^{-1} of dexamethasone (DEX), pregnenolone 16α-carbonitrile (PCN) or rifampicin (RIF). Values represent the mean activity (pmol mg^{-1} min^{-1}) with standard deviation in parentheses. Two-way ANOVA on variance between sex and treatment within each substrate was run at α=0.05.

	CONTROL		DEX-treated		PCN-treated		RIF-treated	
Substrate	Female $n = 4$	Male $n = 3$ or 6[a]	Female $n = 3$	Male $n = 3$	Female $n = 3$	Male $n = 3$	Female $n = 2$	Male $n = 3$
7-ER	2933 (391)	1033 (758)[#]	1944 (932)	1030[b]	2909 (898)	1441 (835)[t]	3195 (55)	1515 (842)[#]
7-BR	37.7 (4.3)	48.3 (10)	42.4 (10)	--[c]	31.6 (17)	--	54.3 (33)	60.9 (24)
7-MR	712 (236)	267 (195)[#]	541 (228)	210[b]	692 (174)	283 (174)[#]	917 (85)	416 (261)[#]
7-PR	52 (10)	32 (22)	99 (68)	18 (6)	461 (249)*	23 (14)[#]	38 (17.5)	60.8 (12)
CEC	9.7 (8.0)	19.7 (6.4)	6.7 (2.7)	--	6.22 (4.5)	--	17.8 (14)	21.5 (18.7)
AMMC	N/A	N/A	N/A	--	N/A	--	N/A	N/A
MAMC	N/A	N/A	N/A	--	N/A	--	N/A	N/A
BFC	11.8 (8.8)	10.3 (1.6)	11.9 (4)	--	11.5 (5)	--	10.5 (1.2)	17.1 (6.4)
MFC	26.7 (9.3)	14.5 (5.9)	28.4 (19)	--	31.1 (10)	--	22.3 (13)	12.8 (8.4)
BQ	223 (49)	231 (51)	367 (14)	--	257 (62)	--	329 (19)	282 (128)
DBF	1.98 (2.5)	4.3 (3.2)	0.73 (0.2)	--	1.01 (0.6)	--	6.7 (7.7)	6.2 (2.4)

* Significant difference from female control
[#] Significant difference from female within the same treatment group
[a] 7-ER, 7-MR and 7-PR n=6; for all other substrates n=3
[b] limited sample, these assays were run with only a single replicate
[c] – assay was not run

Table 4. Linear regression analyses between CYP-mediated fluorometric substrates. Analyses were run on fold-induction of the metabolism of each substrate using liver microsomes from rainbow trout treated with 10 mg kg^{-1} (white background entries) or 50 mg kg^{-1} (grey background entries) of β-naphthoflavone compared to control activity. Bolded entries highlight statistical significance ($p < 0.05$).

	7-ER	7-BR	7-MR	7-PR	CEC	MAMC	MFC
7-ER		**R^2= 0.974** ***p=0.013***	**R^2= 0.926** ***p=0.038***	R^2= 0.773 *p=0.12*	**R^2= 1.000** ***p<0.0001***	**R^2= 0.940** ***p=0.03***	**R^2= 0.959** ***p=0.02***
7-BR	R^2= 0.480 *p=0.31*		R^2= 0.858 *p=0.074*	R^2= 0.881 *p=0.061*	R^2= 0.797 *p=0.107*	**R^2= 0.953** ***p=0.024***	**R^2= 0.993** ***p=0.004***
7-MR	R^2= 0.647 *p=0.19*	R^2= 0.450 *p=0.320*		R^2= 0.679 *p=0.176*	R^2= 0.468 *p=0.316*	R^2= 0.752 *p=0.133*	R^2= 0.804 *p=0.104*
7-PR	R^2= 0.5261 *p=0.275*	**R^2= 0.972** ***p=0.014***	R^2= 0.607 *p=0.221*		**R^2= 0.926** ***p=0.038***	R^2= 0.752 *p=0.133*	R^2= 0.860 *p=0.073*
CEC	R^2= 0.741 *p=0.139*	**R^2= 0.928** ***p=0.037***	R^2= 0.591 *p=0.231*	**R^2= 0.930** ***p=0.036***		R^2= 0.746 *p=0.136*	R^2= 0.817 *p=0.096*
MAMC	R^2= 0.751 *p=0.134*	R^2= 0.550 *p=0.251*	**R^2= 0.982** ***p=0.009***	R^2= 0.693 *p=0.168*	R^2= 0.710 *p=0.157*		**R^2= 0.979** ***p=0.01***
MFC	**R^2= 0.931** ***p=0.035***	R^2= 0.471 *p=0.314*	R^2= 0.864 *p=0.07*	R^2= 0.574 *p=0.243*	R^2= 0.708 *p=0.159*	**R^2= 0.923** ***p=0.039***	

2.7 Figures

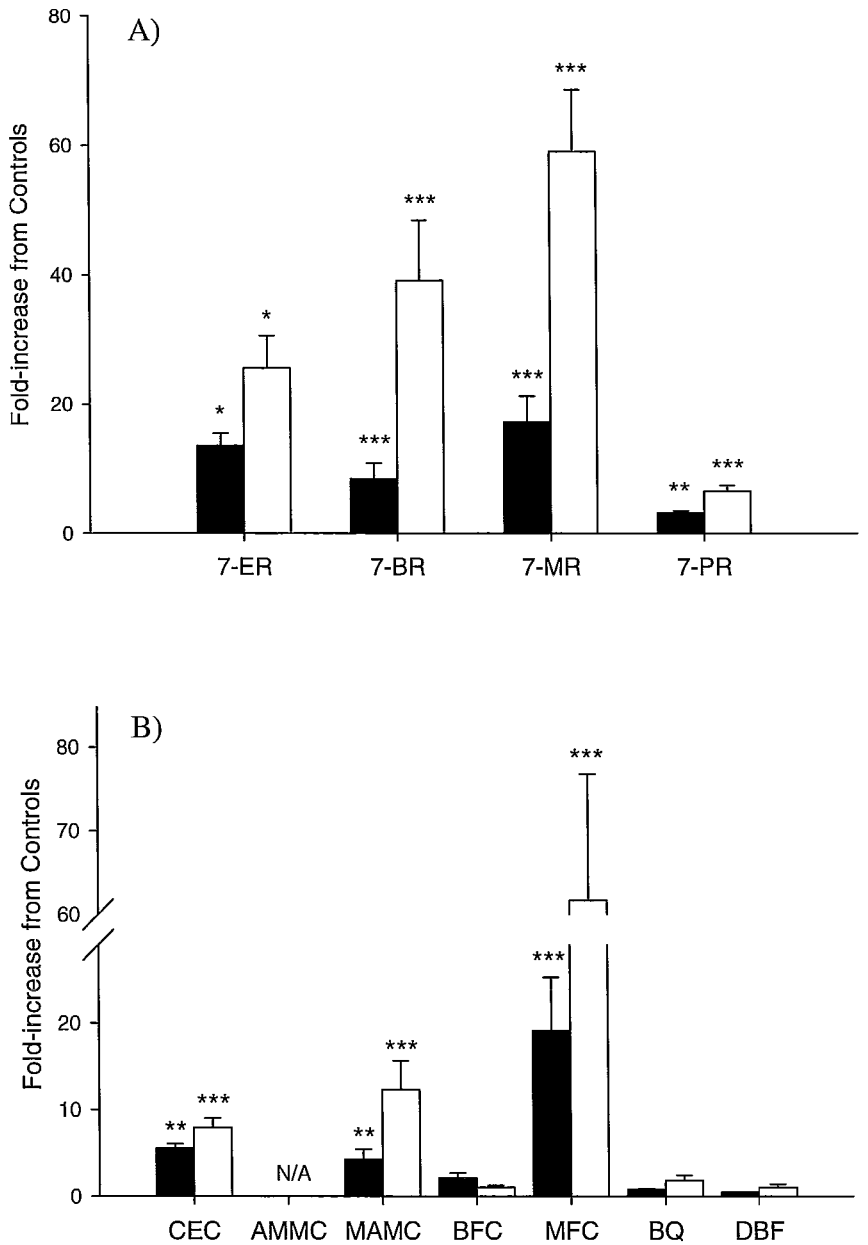

Figure 1. Induction of rainbow trout liver microsomes by low (10 mg kg^{-1}; black bars) and high (50 mg kg^{-1}; white bars) dose BNF. A) Metabolism of AROD substrates; B) metabolism of CEC, AMMC, MAMC, BFC, MFC, BQ and DBF. Data are displayed as fold-induction from controls with standard deviation. Asterisks indicate level of significance: *Kruskal-Wallis one-way analysis of variance on ranks, p=0.008; **One-way ANOVA $p<0.01$; ***One-way ANOVA $p<0.001$. N/A – No activity above background fluorescence.

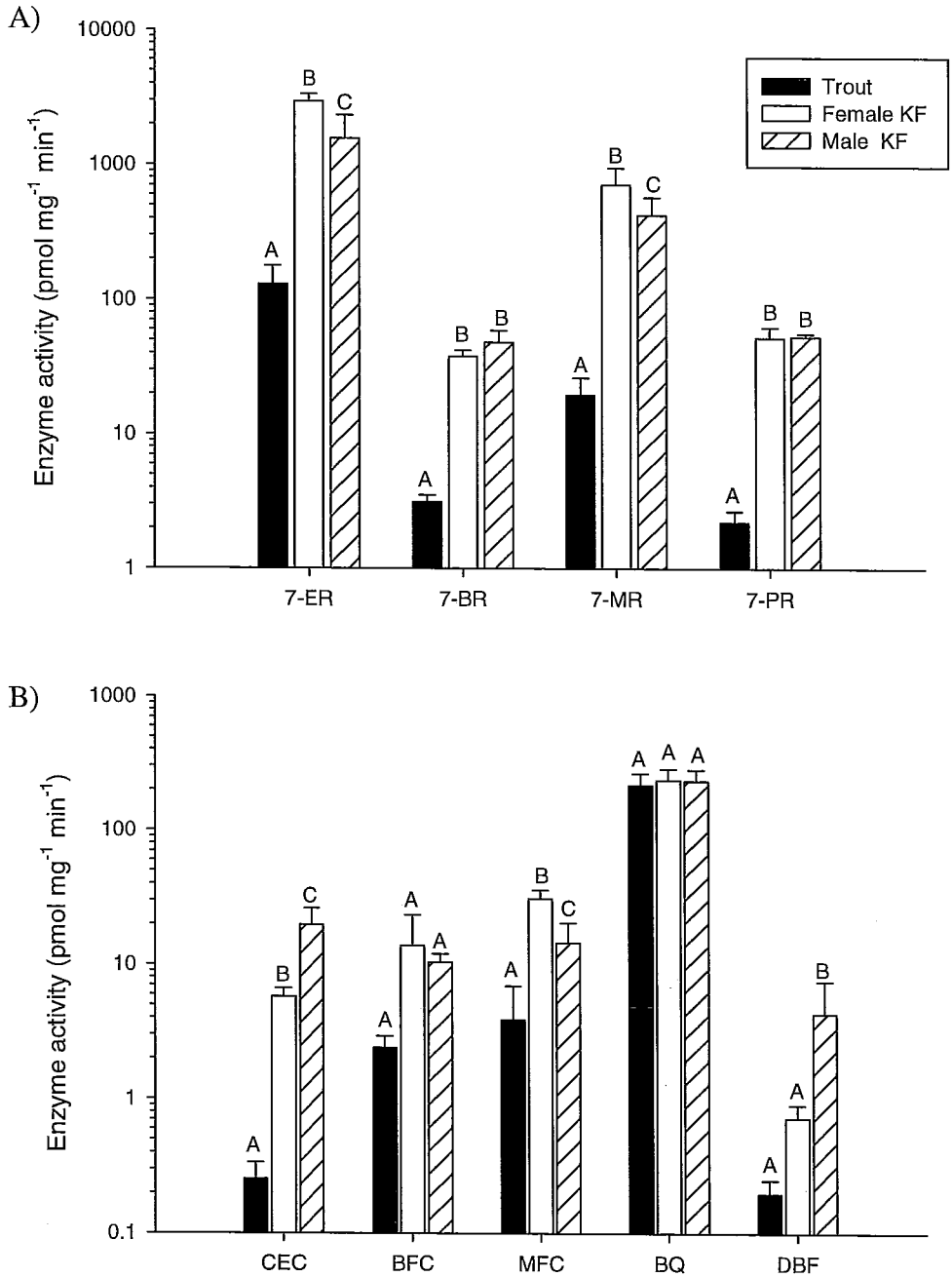

Figure 2. Comparison of *in vitro* substrate metabolism by rainbow trout and killifish liver microsomes A) Metabolism of AROD substrates; B) metabolism of CEC, BFC, MFC , BQ and DBF. Data are displayed on log-scale as pmol of product per mg protein per minute, with standard deviation. Letters indicate significant differences between groups, within each substrate (Two-way ANOVA between species and sex; α=0.05).

CHAPTER 3:

IN VITRO INHIBITION OF CYTOCHROME P450-MEDIATED REACTIONS BY GEMFIBROZIL, ERYTHROMYCIN, CIPROFLOXACIN AND FLUOXETINE IN FISH LIVER MICROSOMES

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Abstract

Pharmaceutical contamination in wastewater effluent is of increasing concern to aquatic organisms. In aquatic environments, pharmaceuticals are found in mixtures and therefore the potential for drug-drug interactions may be possible; however, studies on fish cytochrome P450 (CYP) inhibition by pharmaceuticals are lacking. Using liver microsomes from untreated and β-naphthoflavone (BNF)-treated rainbow trout, nine fluorescent CYP-mediated catalytic assays were used to assess *in vitro* CYP inhibition by four pharmaceuticals: fluoxetine, ciprofloxacin, gemfibrozil and erythromycin. A variety of patterns arose from these assays including two unexpected findings: first, inhibition in fish CYPs by the selected pharmaceuticals does not appear to be CYP-specific as would be predicted based on mammalian CYP inhibition. For instance, ciprofloxacin and erythromycin selectively inhibit CYP1A2 and CYP3A4 respectively in humans; however, data from the present study show inhibition of a number of CYP-mediated reactions by these pharmaceuticals in reactions with fish liver microsomes. Second, a number of reactions were found to be significantly activated with the addition of these compounds. For example, metabolism of 7-pentoxyresorufin (7-PR) was significantly activated by 10 and 100 μM gemfibrozil, ciprofloxacin and erythromycin in BNF-treated fish. In a number of reactions, control and BNF-induced trout liver microsomes responded differently to the addition of an inhibitor. For example, although BNF does not induce the metabolism of benzyloxy-4-trifluoromethylcoumarin (BFC), BFC metabolism was completely abolished by 100 μM of both gemfibrozil and ciprofloxacin in undosed fish only. With the exception of BFC, the lowest inhibitor concentration, 10 μM, did not

significantly inhibit the majority of reactions in undosed fish and estimated K_i values are quite large. These data demonstrate that *in vitro* hepatic CYP inhibition by these pharmaceuticals is possible in fish. Some compounds broadly inhibit fish CYP reactions and the patterns seen here are different than what would be expected based on CYP inhibition in mammals.

Keywords: fluoxetine, ciprofloxacin, gemfibrozil, erythromycin, cytochrome P450, CYP, enzyme inhibition, fish

Contributions: This chapter has been formatted as a paper prepared for publication written by E.M.S. Experimental design was carried out by J.Y.W and E.M.S and technical work and data analysis carried out by E.M.S and F.I.I. under the guidance and supervision of J.Y.W.

3.1 Introduction

The presence of pharmaceuticals within the aquatic environment was first reported in the mid-1970s (Hignite and Azarnoff 1977) and has since grown to be a major topic in environmental toxicology. Concentrations of such chemicals have been identified in aquatic systems ranging from nanograms per liter to micrograms per liter (Halling-Sorensen *et al.* 1998). 1998). Discharge from sewage treatment plants has been identified as a major source of pharmaceuticals and personal care products in the aquatic environment and it has been predicted that levels could approach kilograms per year in the near future (Lindberg *et al.* 2005). Pharmaceuticals are designed to have an inherent effect on biological systems, and even at low concentrations, may pose a real risk to aquatic organisms.

Cytochrome P450s (CYPs) are a superfamily of proteins, many of which are responsible for the metabolism of drugs and exogenous contaminants. CYPs from families 1 through 4 are highly associated with pharmaceutical metabolism; CYP 3A4 is responsible for 50% of human drug metabolism followed by 2D6, 2C9 and 1A2 at 25%, 15% and 5% respectively (Hemeryck and Belpaire 2002). Once outside of mammalian systems, however, the metabolic fate of many drugs is still unknown. In aquatic environments, pharmaceuticals are found in mixtures and therefore, drug-drug interactions are of concern. Studies of drug metabolism by fish P450s are limited, as is our knowledge of compounds involved in enzyme inhibition. Many pharmaceuticals are strong inhibitors of CYPs in mammals, yet few studies have looked at the effects of such

drugs on CYP activity in fish or other aquatic organisms.

In this study we assess the inhibition potential of four human pharmaceuticals, erythromycin, ciprofloxacin, gemfibrozil and fluoxetine, on CYP-mediated reactions in rainbow trout liver microsomes and expressed zebrafish CYP1s. These compounds have been selected because they are potent inhibitors of the dominant CYPs responsible for pharmaceutical-metabolism in mammals. In addition, these drugs have been detected in sewage treatment effluent and surface waters (Daughton and Ternes 1999; Hirsch *et al.* 1999; Kolpin *et al.* 2002; Metcalfe *et al.* 2003) and thus are environmentally relevant pharmaceuticals for study.

Laville *et al.* (2004) provided the first extensive look at the effects of pharmaceuticals on fish hepatocytes. Testing a broad range of drug classes *in vitro*, they found that following 24 hours exposure, fibrates and anti-depressants, in particular, were potent inhibitors of 7-ethoxyresorufin-*O*- dealkylase (EROD, an indicator of CYP1A activity). Studies are scarce, however, on fish CYP inhibition outside of CYP1A activity.

Ciprofloxacin inhibits CYP1A2 in mammals (Fuhr *et al.* 1992; Granfors *et al.* 2004). Although the capacity of this antibiotic to inhibit fish CYPs has not yet been assessed, enrofloxacin, the parent compound of ciprofloxacin, has been shown to inhibit a number of monooxygenase reactions, both *in vivo* and *in vitro* in sea bass (Vaccaro *et al.* 2003). Gemfibrozil is a potent inhibitor of CYP2C9 and modest inhibitor of CYP1A2 and 2C19 in humans (Wen *et al.* 2001). Fluoxetine effectively inhibits human CYP2D6,

2C19 and 3A4. In fish, gemfibrozil and fluoxetine have displayed both inhibition (Laville *et al.* 2004; Thibaut *et al.* 2006) and induction of EROD activity (Thibaut and Porte 2008). Inhibition of BFC-*O*-debenzyloxydase has also been reported by fluoxetine and gemfibrozil in carp liver microsomes (Thibaut *et al.* 2006). BFC is a CYP3A substrate in mammals and is often accredited to CYP3A in non-mammalian studies; however recent data suggests that it is likely more broadly metabolized by fish CYP enzymes (Scornaienchi 2009). Erythromycin is a strong inhibitor of human CYP3A4 but not CYPs 1A2 or 2C9 (Yamazaki and Shimada 1998; Okudaira *et al.* 2007). Erythromycin is often used as a CYP3 substrate in fish studies rather than an inhibitor (Novi *et al.* 1998; Bozcaarmutlu and Arinc 2008; Li *et al.* 2008). Expressed CYP3A from channel catfish intestine has displayed inhibition by erythromycin (James *et al.* 2005) although broad characterization of its inhibition potential in fish has yet to be determined. Collectively, these data suggest that these compounds are good candidates for further investigation of broad CYP inhibition in fish and are found in the aquatic environment making them environmentally relevant CYP targets in fish.

3.2 Methods

3.2.1 Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) were purchased from Humber Springs trout hatchery (Mono Mills, ON, Canada). Trout were held at 12-15°C in flow-through water and fed floating trout pellets 3 times per week. All fish were held for a minimum of two weeks before use. Food was removed 24-48 hours before treatment or sacrifice.

3.2.2 In vivo CYP induction

β-naphthoflavone (BNF) was purchased from Sigma-Aldrich, (St. Louis, MO, USA). Trout were exposed to 50 mg kg^{-1} body weight of BNF dissolved in corn oil and administered via i.p. injection, under anesthetic (100 mg L^{-1} methanesulfonate salt, MS-222). Injection volume did not exceeding 10 μL g^{-1} total body weight and control fish were injected with corn oil carrier only. All fish were sacrificed 48-hours after injection and livers were collected and flash-frozen in liquid nitrogen. Tissues were stored at -80°C until use. Liver microsomes were prepared as in Stegeman *et al* (1995) and total protein content was determined using a bicinchoninic acid (BCA) kit, according to manufacturer's protocols (Pierce protein research products).

3.2.3 In vitro inhibition of catalytic activity

Ciprofloxacin, gemfibrozil and fluoxetine was dissolved in DMSO and erythromycin was dissolved in methanol, to concentrated stock solutions (Sigma-Aldrich, St. Louis, MO). Stocks were diluted to working solutions using distilled water just prior to use in assays. CYP inhibition was determined using fluorescent-based catalytic activity assays for nine substrates with inhibitor concentrations of 0 (solvent control), 10 and 100 μM. The specificity of these substrates is known for mammalian CYPs but has not been determined in non-mammalian species for many of these substrates. Alkoxyresorufin-O-dealkylase (AROD) activities were determined using 7-ethoxyresorufin (7-ER), 7-benzyloxyresorufin (7-BR), 7-methoxyresorufin (7-MR) and 7-pentyloxyresorufin (7-PR; Sigma-Aldrich; St. Louis, MO) according to previously published methods (Hahn *et al.*

1993). The remaining five substrates, 7-methoxy-4-aminomethylcoumarin (MAMC), 3-cyano-7-ethoxycoumarin (CEC), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-methoxy-4-trifluoromethylcoumarin (MFC), and 7-benzyloxyquinoline (BQ) (BD Biosciences, San Jose, CA), were used in assays optimized in our lab for use in fish, based on methods published for mammalian microsomes (Crespi *et al.* 1997; Miller *et al.* 2000; Stresser *et al.* 2000). Limits of detection (LODs), assay settings, buffers, and substrate concentrations were as in Table 1, Chapter 2, with the exception of 7-BR, 7-MR, 7-PR and CEC substrate concentrations (presently 3 μM, 2 μM, 7 μM, and 20 μM respectively). All reactions were run at room temperature for 10 minutes in black 96-well format using kinetic assays.

Inhibition of AROD substrates was also investigated using heterologously expressed CYP1 proteins from zebrafish (*Danio rerio*). Expressed CYP1A, 1B1, 1C1 and 1C2 (Scornaienchi 2009) were used in place of microsomes in catalytic assays with 0, 10 and 100 μM ciprofloxacin, erythromycin, gemfibrozil and fluoxetine.

3.2.4 Data and Statistical Analysis

Percent inhibition was calculated based on individual trout run at zero inhibitor (100% activity) and averaged across fish within that treatment. Percent inhibition for BNF-treated fish was calculated separately from undosed trout, based on activity of BNF-treated microsomes with no inhibitor (100% activity). Data from each inhibitor were plotted on Dixon reciprocal plot to calculate K_i (inhibition constant; μM). Substrate inhibition by fluoxetine was run at multiple substrate concentrations and data plotted on

Lineweaver-Burk plot to characterize inhibition type: competitive (V_{max} stays constant, K_m increases), non-competitive (K_m stays constant , V_{max} decreases), uncompetitive (both K_m and V_{max} decreases) or mixed-type inhibition (K_m increases, V_{max} decreases).

Significant inhibitions were determined using one-way repeated measures (RM) Analysis of Variance (ANOVA) on log-transformed enzyme activity (pmol mg^{-1} min^{-1}) at 0, 10 and 100 μM inhibitor concentrations. If log-transformed data did not qualify for one-way RM-ANOVA, Friedman repeated measures Analysis of Variance on ranks was preformed. All post-hoc tests were completed using the Student-Newman Keuls method. Statistical tests were completed using Sigmaplot 11.0 (Systat Software Inc). Reactions that fell below limit of detection for respective standard curves were assigned a value equal to the limit of detection in pmol accumulated over the 10 min reaction time in order to perform statistical analysis.

3.3 Results

3.3.1 Inhibition of untreated and BNF-induced trout liver microsomes

Ciprofloxacin caused changes in the metabolism of all substrates except 7-ER (Figure 1A). Low concentration of ciprofloxacin (10 μM) caused significant inhibition BQ and BFC metabolism in undosed trout (12% and 59% respectively). High concentration of ciprofloxacin (100 μM) caused inhibition of CEC, 7-BR, and BQ metabolism (20%, 43%, and 46%, respectively) and completely abolished metabolism of BFC in undosed fish. Compared to baseline metabolism by BNF-treated fish, 10 μM ciprofloxacin caused both inhibition and activation of a number of substrates: 15-16%

inhibition of 7-BR, BFC and BQ metabolism, and 15-20% increased activity in 7-PR, CEC and MFC (Figure 1A). The highest concentration (100 μM) of ciprofloxacin caused significant and pronounced inhibition of MAMC, 7-BR and BQ metabolism in BNF-treated fish (37%, 45% and 47% respectively), and less pronounced inhibition of MFC, BFC and 7-MR metabolism (16-17% for all three substrates; Figure 1A). In BNF trout microsomes, significant increases in activity were seen in 7-PR, CEC and MFC metabolism with 10 μM ciprofloxacin (15-20% increases); PROD activity maintained elevated metabolism 100 μM ciprofloxacin in BNF-treated fish (22% increase; Figure 1A).

In undosed fish, erythromycin caused significant inhibition of the metabolism of a number of substrates. BFC and MFC were inhibited at both concentrations of erythromycin (53-70% and 19-25% respectively; Figure 1B). Metabolism of CEC, BQ and 7-MR were inhibited only at 100 μM (18, 29 and 35% respectively). In reactions with BNF-treated fish, 7-ER, 7-BR and BQ metabolism were significantly inhibited at both erythromycin concentrations (56-65%, 25-39% and 23- 34% decreases respectively). With BNF-treated trout, CEC metabolism was 24% higher with 10 μM erythromycin but showed no change with 100 μM (Figure 1B). 7-PR and MFC were metabolized significantly better with both 10 and 100 μM erythromycin (10-12% increase for MFC, 27-39% for 7-PR; Figure 1B).

Gemfibrozil caused significant changes of the metabolism of a number of substrates. In undosed fish, BFC, MFC and BQ metabolism were significantly inhibited

at both gemfibrozil concentrations (70-100%, 25-31% and 9-43% respectively; Figure 1C); BFC inhibition was very potent with this inhibitor. Metabolism of CEC was significantly inhibited 35% by 100 μM gemfibrozil. In reactions with BNF-treated fish, 7-ER, 7-BR, BQ and MAMC metabolism were significantly inhibited at both gemfibrozil concentrations (60-88%, 28-53%, 26-51% and 10-43% decreases in each substrate respectively; Figure 1C). Inhibition of EROD activity by gemfibrozil was high in the BNF treated trout microsomes but not undosed fish. With BNF-treated trout, 7-MR metabolism was significantly inhibited 19% by 100 μM gemfibrozil. Again, a number of substrates show activation with the addition of the inhibitor in BNF-treated trout assays: gemfibrozil caused 28-36% increased 7-PR metabolism and 5-15% increased MFC metabolism at both concentrations of gemfibrozil (Figure 1C). CEC metabolism was increased 32% with 10 μM gemfibrozil but showed no difference with 100 μM (Figure 1C).

In undosed trout assays, 10 μM fluoxetine caused no significant inhibition in the metabolism of any substrate tested; 100 μM fluoxetine caused inhibition of metabolism in all tested substrates: 7-ER (94%), 7-BR (75%), 7-MR (85%), 7-PR (25%), CEC (83%) and MFC (81% inhibition; Figure 1D). Due to limited sample, inhibition of BFC and BQ metabolism by fluoxetine were not run with undosed fish. In assays with BNF-treated microsomes, 10 μM fluoxetine caused inhibition of 7-MR, and 7-PR metabolism; 100 μM fluoxetine caused inhibition of nearly all substrates: 7-ER (78%), 7-BR (78%), 7-MR (75%), 7-PR (22%), CEC (70%) and MFC (78%) and MAMC (83% inhibition; Figure

1D). The exception being BFC, which showed 65% increase in activity with 100 μM fluoxetine.

Inhibition constants (K_i) were calculated on Dixon reciprocal plots for inhibition at 0, 10 and 100 μM inhibitor concentrations. All four inhibitors had Ki values in the μM range for most substrates tested (Table 1). K_i for inhibition by ciprofloxacin, erythromycin, and gemfibrozil were relatively large for most substrates (>100 μM). Inhibition of BFC metabolism by ciprofloxacin and gemfibrozil was very potent with a K_i <1 μM for undosed fish. For erythromycin, BFC inhibition had the lowest calculated K_i in undosed fish (68 μM) but was much higher in BNF-induced trout (>2mM; Table 1). BNF-treated fish had much higher inhibition constants in general, including several Ki values that were >1mM (Table 1). In BNF-treated fish, EROD inhibition by gemfibrozil had a K_i of only 20 μM, much lower than other substrates (Table 1). BNF-treated fish had very high K_i values for inhibition of BFC and MFC by gemfibrozil (1mM and 8.5mM, respectively (Table1). Overall, fluoxetine had the lowest Ki for nearly all substrates tested, although none were found to have K_i<1 μM (Table 1).

Inhibition by fluoxetine was run at additional substrate concentrations in order to assess the type of inhibition caused by fluoxetine (i.e. competitive, non-competitive, uncompetitive, mixed-type inhibition; see Appendix). Plotting these data on Lineweaver-Burk reciprocal plots revealed that 100 μM fluoxetine caused mostly mixed-type inhibition (Table 2). For some substrates, inhibition type was not clear from Lineweaver-Burk reciprocal plots. No substrates were found to experience a clear pattern representing

non-competitive inhibition. 7-MR was competitively inhibited in undosed fish but uncompetitive in BNF-treated fish. Uncompetitive inhibition was seen in 7-PR (undosed fish) and 7-BR (BNF-treated fish; Table 2).

3.3.2 Inhibition of AROD activity in expressed zebrafish CYPs

Expressed zebrafish CYP1A, 1B1, 1C1 and 1C2 were investigated for inhibition of AROD activity by chosen pharmaceuticals. No CYP was completely inhibited by any pharmaceutical at 10 or 100 μM (Figure 2). EROD activity catalyzed by CYP1A and 1C1 was activated by exposure to nearly all concentrations of inhibitors; only 100 μM of fluoxetine did not increase CYP1A associated EROD activity. CYP1B1 and 1C2-mediated EROD activity seem more susceptible to inhibition by the tested compounds. Fluoxetine inhibited 1B1 and 1C2 catalyzed EROD activity up to 70%; ciprofloxacin inhibited 1B1 and 1C2 up to 45% while inhibition by erythromycin and gemfibrozil was more modest (Figure 2A). With respect to BROD activity, CYP1C1 may be slightly more sensitive to inhibition by these compounds than the other CYP1s tested (Figure 2B). Concentration-dependent inhibition is clear across CYPs 1A, 1B1 and 1C1 associated BROD activity for all drugs, with up to 65% inhibition by 100 μM gemfibrozil (Figure 2B). MROD inhibition appears the most consistent across drugs and proteins with little activation (Figure 2C). CYP1A and 1B1-mediated MROD activity was inhibited by all compounds (up to 65% by fluoxetine; Figure 2C). CYP1C1-mediated PROD is inhibited by all tested drugs (up to 65% by gemfibrozil; Figure 2D). Gemfibrozil increases CYP1B1-mediated PROD at both 10 and 100 μM (>70% increase); at 100 μM

erythromycin inhibits CYP1B1-mediated PROD >60% (Figure 2D). CYP1C2-mediated PROD appears unaffected by the inhibitors at these concentrations (Figure 2D).

3.4 Discussion

Many of the previous studies on inhibition of cytochrome P450s (CYPs) in fish focus on the inhibition of 7-ethoxyresorufin-*O*-dealkylase (EROD), suggesting CYP1A inhibition. However, recent data with expressed CYP1s from zebrafish demonstrate that EROD is catalyzed not only by CYP1A but also by CYPs 1B1, 1C1 and 1C2 (Scornaienchi 2009). Previous reports on EROD inhibition is therefore not likely attributed to just CYP1A inhibition but more broad CYP1 inhibition. BFC and BQ are CYP3A substrates in mammals and activity in fish is solely attributed to CYP3A activity in most fish studies (Hegelund *et al.* 2004; Kashiwada *et al.* 2005). The assumption that these substrates are catalyzed by CYP3A in fish may be premature; BFC metabolism is higher for expressed zebrafish CYP1s compared to expressed CYP3A65 (Scornaienchi 2009). Thus, careful interpretation of CYP substrates and protein inhibition is needed in fish, where robust characterizations of CYP substrates and inhibitors are limited. The data presented in this study support the concept that the CYPs responsible for catalyzing the metabolism of these substrates may not be similar between mammals and fish.

The compounds selected for this study encompass inhibition of the important drug-metabolizing CYPs in mammals: CYPs 1A2, 2C9, 2C19, 2D6 and 3A4. Ciprofloxacin is a potent CYP1A2 inhibitor in mammals. To our knowledge, no studies have previously determined the inhibition potential of ciprofloxacin in fish. In this study,

we see modulation of a number of CYP-mediated reactions by ciprofloxacin. In microsomes, EROD activity was activated by ciprofloxacin, as were reactions with expressed CYP1A and 1C1. Expressed CYP1B1 and CYP1C2 catalyzed EROD were inhibited, suggesting that EROD activity in both undosed and BNF exposed trout microsomes were primarily catalyzed by CYP1A and CYP1C1. Inhibition of a number of CYP-mediated reactions, including EROD, have been reported for enrofloxacin, the parent compound of ciprofloxacin (Vaccaro *et al.* 2003). Ciprofloxacin does not appear to be a selective CYP1A inhibitor in fish, and provides evidence of more broad CYP inhibition in fish.

If structural and functional relationships were conserved between fish and mammals, we would expect that 7-MR, a CYP1A2 substrate in mammals, would be most inhibited by ciprofloxacin compared to other substrates. This is not reflected in microsomal inhibition of MROD. Expressed CYP1s show some level of inhibition, but the potency of CYP1A inhibition seen in mammals is not evident in fish. 7-MR and the expressed CYP1B1 and 1C2 are strongly inhibited by fluoxetine, a mammalian CYP 2D6, 2C19 and 3A4 inhibitor. Thus, mammalian CYP inhibitors need characterization in fish.

The strongest inhibition was found with BFC metabolism; nearly all activity was abolished in the presence of both ciprofloxacin and gemfibrozil in undosed fish. However, this was not found in reactions with BNF exposed trout where inhibition was more modest or lacking. BQ, metabolized by the same protein as BFC in humans

(CYP3A4), has a completely different inhibition profile from BFC; in fact, fluoxetine activated BFC metabolism but inhibits BQ metabolism supporting previous data that different CYPs are responsible for the metabolism of these two substrates (Chapter 2, Scornaienchi 2009). Interestingly, gemfibrozil and ciprofloxacin caused the same inhibition profile in BFC and BQ in both untreated and BFC microsomes. In mammals, these compounds do not inhibit the same CYPs and neither enormously reduces CYP3A activity. This provides further evidence that BQ and BFC are likely catalyzed by different CYPs in fish compared to mammals. BFC is likely metabolized by more than one CYP as inhibitors have a very different impact in reactions with BNF induced compared to control microsomes. Both BQ and BFC are metabolized by CYP3A in mammals and metabolism of neither substrate was induced with BNF treatment in fish (Chapter 2). However, this resemblance in catalytic function changes with introduction of inhibitors. In undosed fish, BFC is subject to potent inhibition by ciprofloxacin, erythromycin and gemfibrozil; in BNF-treated microsomes, this inhibition is far reduced and only significant with ciprofloxacin. Conversely, inhibition of BQ was not different between undosed and BNF-treated fish. In BNF-treated fish, fluoxetine increases the metabolism of BFC but inhibits BQ metabolism, again revealing a discrepancy in the assumed similar metabolism of these two substrates in fish. BFC inhibition by gemfibrozil has been previously in carp liver microsomes (Thibaut *et al.* 2006).

Gemfibrozil, a typical CYP2C9 inhibitor and modest inhibitor of CYP1A2, and 2C19 in mammals altered the metabolism of every tested substrate. Contrary to other

studies, which saw inhibition with 1mM gemfibrozil (Thibaut *et al.* 2006), the lower concentrations used in this study did not inhibit EROD activity in undosed fish. Only EROD activity in BNF-treated fish showed inhibition. In expressed CYP1s, only CYP1B1 was inhibited by gemfibrozil and CYP1A and 1C1 were activated. The reasons that gemfibrozil strongly inhibited BNF induced microsomal reactions when CYP1A and 1C1 were activated is not clear. While CYP1B1 is induced by BNF treatment, CYP1A and 1C1 are also strongly upregulated by BNF and the inhibition of CYP1B1 was not enough to account for that seen with microsomes. In BNF-treated fish, gemfibrozil activated PROD activity at both 10 and 100 μM. This PROD activation is seen also in expressed CYP1B1-mediated PROD activity. Although the pattern may suggest that gemfibrozil interacts with CYP1B1, inhibiting EROD activity but increasing PROD activity in fish, CYP1A is primarily responsible for EROD activity and inhibition of CYP1B1 alone does not account for this decrease in EROD activity.

Fluoxetine most strongly inhibits CYP2D6 and 3A4 in humans; it was the only compound to significantly inhibit EROD and PROD activity in untreated fish liver microsomes. Fluoxetine has been reported to inhibit EROD activity in fish hepatocytes and liver microsomes (Laville *et al.* 2004; Thibaut *et al.* 2006). Expressed zebrafish CYP1A-mediated EROD was not inhibited by fluoxetine, and is therefore not likely the sole CYP responsible for EROD activity in fish liver. MROD activity for CYP1A, 1B1 and 1C1 were inhibited by fluoxetine, although to a lesser extent in trout microsomes. Fluoxetine was far more potent at inhibiting the metabolism of MFC and CEC compared

to the other compounds.

In humans, over half of drugs are metabolized by CYP3A4 (Hemeryck and Belpaire 2002). Erythromycin is a broad-spectrum antibiotic and a persistent inhibitor of human CYP3A4. Few studies have looked at its inhibition potential in fish. These data provide evidence that erythromycin inhibits fish CYPs. BFC metabolism in undosed fish was inhibited but not to the same degree as by gemfibrozil or ciprofloxacin. MAMC was not inhibited by erythromycin, suggesting subtle differences between CYPs inhibited by erythromycin. The reactions inhibited by erythromycin are outside what would be expected of CYP3A inhibition. Erythromycin is likely a broader inhibitor of fish CYPs.

To our knowledge, this is amongst the first studies (Chapter 2) to assess MAMC metabolism in fish. MAMC (mammalian CYP2D6 substrate) is not metabolized by untreated fish liver microsomes (Chapter 2); MAMC was inhibited by gemfibrozil, ciprofloxacin and fluoxetine in BNF-treated fish. Of these, only fluoxetine is an inhibitor of CYP2D6 in mammals; however, fish do not possess CYP2D isoforms. The patterns in MAMC metabolism suggest a number of things. First, the protein(s) responsible for MAMC metabolism in fish is not normally active in untreated fish (Chapter 2). Second, BNF is capable of inducing the metabolism of a substrate normally metabolized by CYP2D in mammals. Third, CYP1A, CYP2C and CYP2D mammalian inhibitors are capable of inhibiting this induced activity in fish.

With the exception of BFC, the lowest inhibitor concentration, 10 μM, did not significantly inhibit the majority of reactions in undosed fish and estimated K_i values are

quite large. 100 μM is likely orders of magnitude higher than what would be found in the environment. However, many pharmaceuticals have been found to bioaccumulate in the tissues of wild fish (Brooks *et al.* 2005; Nakamura *et al.* 2008). Tissue-specific accumulation has also been documented, increasing the overall load on specific proteins, such as hepatic CYPs (Nakamura *et al.* 2008). Additive and synergistic effects are something that should be considered. These data suggest broad CYP inhibition by the tested compounds and therefore individual compound concentrations are not likely providing a full picture of environmental concern. The environmental relevance of inhibition by these compounds is therefore not straightforward. Pharmaceuticals are found in mixtures, and the dynamic of the mixture would dictate the outcome of induction, inhibition and activation of important xenobiotic-metabolizing proteins. In order to address if these compounds are of environmental concern, a better understanding of CYP function and characterization of catalytic assays in fish is vital.

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3.6 Tables

Table 1. Inhibition constants (K_i) for inhibition by ciprofloxacin, erythromycin, gemfibrozil and fluoxetine. Constants were calculated on Dixon reciprocal plots at 0, 10 and 100 μM inhibitor concentrations at optimal substrate concentration and are displayed in μM.

Substrate	Ciprofloxacin		Erythromycin		Gemfibrozil		Fluoxetine	
	Undosed	BNF	Undosed	BNF	Undosed	BNF	Undosed	BNF
7-ER	601	--[a]	--[a]	122	--[a]	20	30	20
7-BR	262	138	--[a]	227	940	115	22	22
7-MR	734	629	235	396	252	523	22	29
7-PR	362	--[a]	1787	--[a]	298	--[a]	313	473
CEC	292	1150	345	566	149	548	17	41
BFC	< 1	1128	68	2605	< 1	1006	--[b]	--[a]
MFC	1148	329	367	--[a]	377	8489	13	35
BQ	125	116	265	251	139	111	--[b]	157
MAMC	N/A[c]	181	N/A[c]	456	N/A[c]	136	N/A[c]	17

[a] – Estimate of K_i could not be calculated on Dixon plot
[b] – reaction was not run
[c] –N/A – undosed trout microsomes do not metabolize MAMC

Table 2. Characterization of inhibition by fluoxetine. Assessment of type of inhibition for undosed and BNF-treated rainbow trout liver microsomes exposed to 100 μM fluoxetine *in vitro*. Competitive, non-competitive, uncompetitive or mixed-type inhibition determined by Lineweaver-Burk reciprocal plots at concentrations given for each substrate.

Substrate	Substrate concentrations (μM)	Undosed	BNF-treated
7-ER	1, 2, 3	?[a]	Mixed-type
7-BR	2, 3, 5	Mixed-type	Uncompetitive
7-MR	1, 2, 3	Competitive	Uncompetitive
7-PR	7, 8,10	Uncompetitive	?
MAMC	10, 25, 50	N/A[b]	?
CEC	10, 20, 30	Mixed-type	Mixed-type
BFC	100, 500, 1000	--[c]	Mixed-type
MFC	10, 50, 100	Mixed-type	Mixed-type
BQ	100, 500, 1000	--[c]	Mixed-type

[a] ? – no clear type of inhibition
[b] N/A – undosed trout microsomes do not metabolize MAMC
[c] -- reaction was not run

3.7 Figures

Figure 1. Inhibition of CYP-mediated substrates in rainbow trout liver microsomes by pharmaceuticals. Data are expressed as mean percent (%) activity of control reactions run with no inhibitor (undosed or BNF) for 10 and 100 μM A) ciprofloxacin, B) erythromycin, C) gemfibrozil and D) fluoxetine. Reactions were run with undosed or BNF-treated (50 mg kg^{-1}) trout microsomes. * signifies significant different from respective control (one-way RM ANOVA; $p<0.05$). Error bars represent standard deviation; for each group N=3-5. [a] MAMC is not metabolized by untreated trout liver microsomes. [b] assay was not run.

Figure 1 A/B

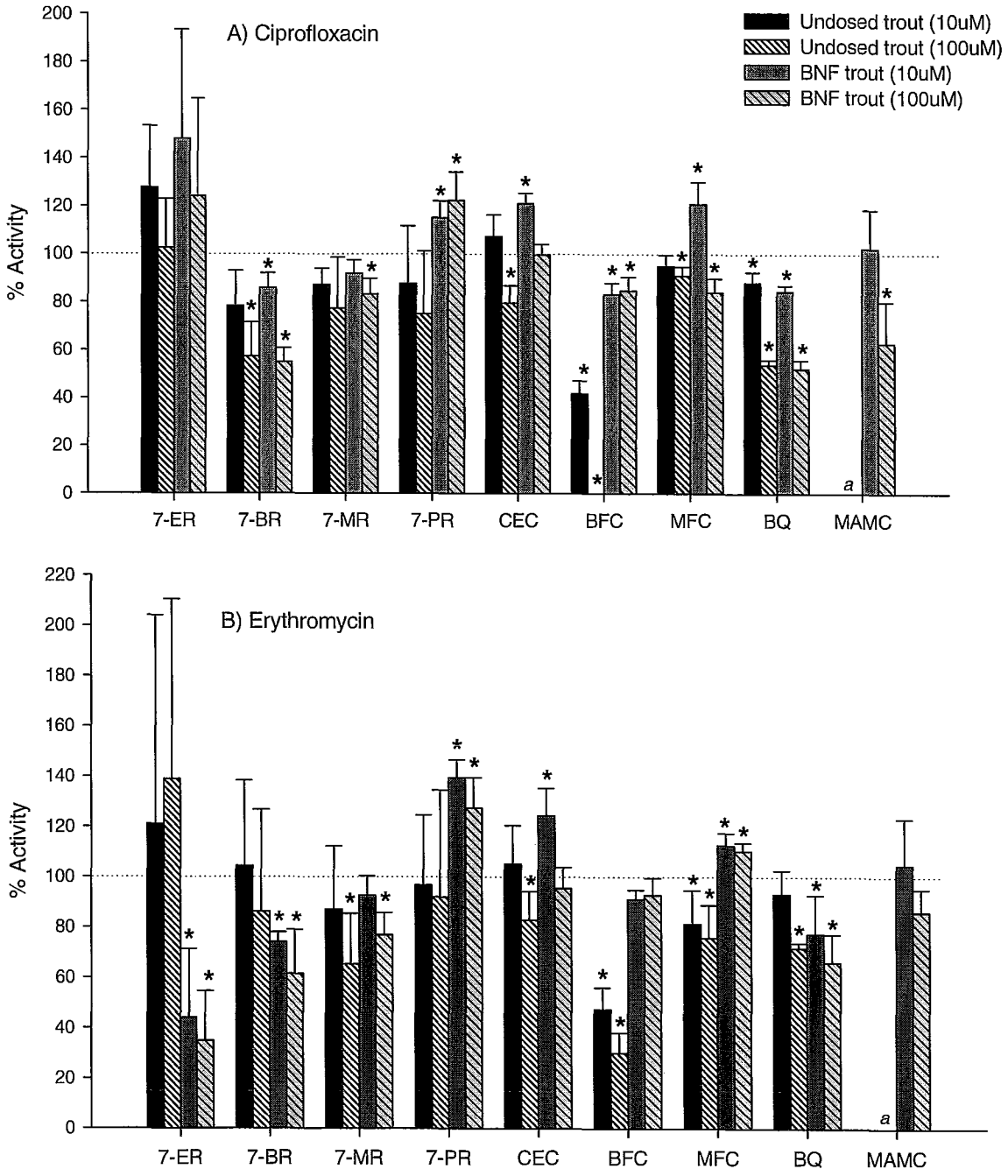

A) Ciprofloxacin
Undosed trout (10uM)
Undosed trout (100uM)
BNF trout (10uM)
BNF trout (100uM)
% Activity
200
180
160
140
120
100
80
60
40
20
0
7-ER
7-BR
7-MR
7-PR
CEC
BFC
MFC
BQ
MAMC
a
B) Erythromycin
% Activity
220
200
180
160
140
120
100
80
60
40
20
0
7-ER
7-BR
7-MR
7-PR
CEC
BFC
MFC
BQ
MAMC
a

Figure 1 C/D

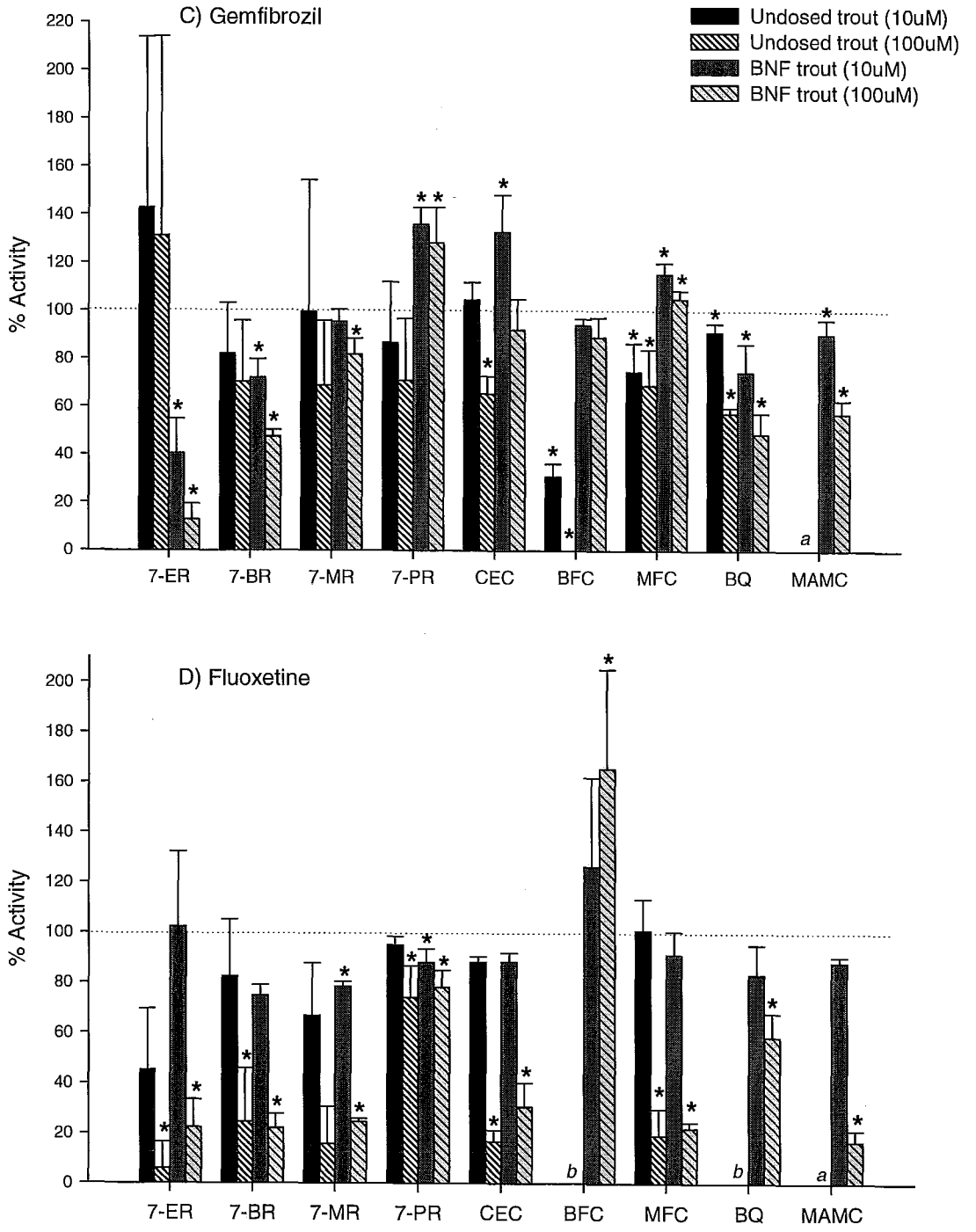

C) Gemfibrozil
Undosed trout (10uM)
Undosed trout (100uM)
BNF trout (10uM)
BNF trout (100uM)
% Activity
0
20
40
60
80
100
120
140
160
180
200
220
7-ER
7-BR
7-MR
7-PR
CEC
BFC
MFC
BQ
MAMC
a
D) Fluoxetine
% Activity
0
20
40
60
80
100
120
140
160
180
200
7-ER
7-BR
7-MR
7-PR
CEC
BFC
MFC
BQ
MAMC
b
b
a

Figure 2. Inhibition of zebrafish CYP1A, 1B1, 1C1 and 1C2 expressed proteins by 10 and 100 μM fluoxetine, ciprofloxacin, erythromycin and gemfibrozil. Data are expressed as mean percent (%) activity (± standard deviation) from controls for two replicate wells for A) 7-ethoxyresorufin-*O*-dealkylase (EROD), B) 7-benzyloxyresorufin-*O*-dealkylase (BROD), C) 7-methoxyresorufin-*O*-dealkylase (MROD) and D) 7-pentoxyresorufin-*O*-dealkylase (PROD).

Figure 2 A/B

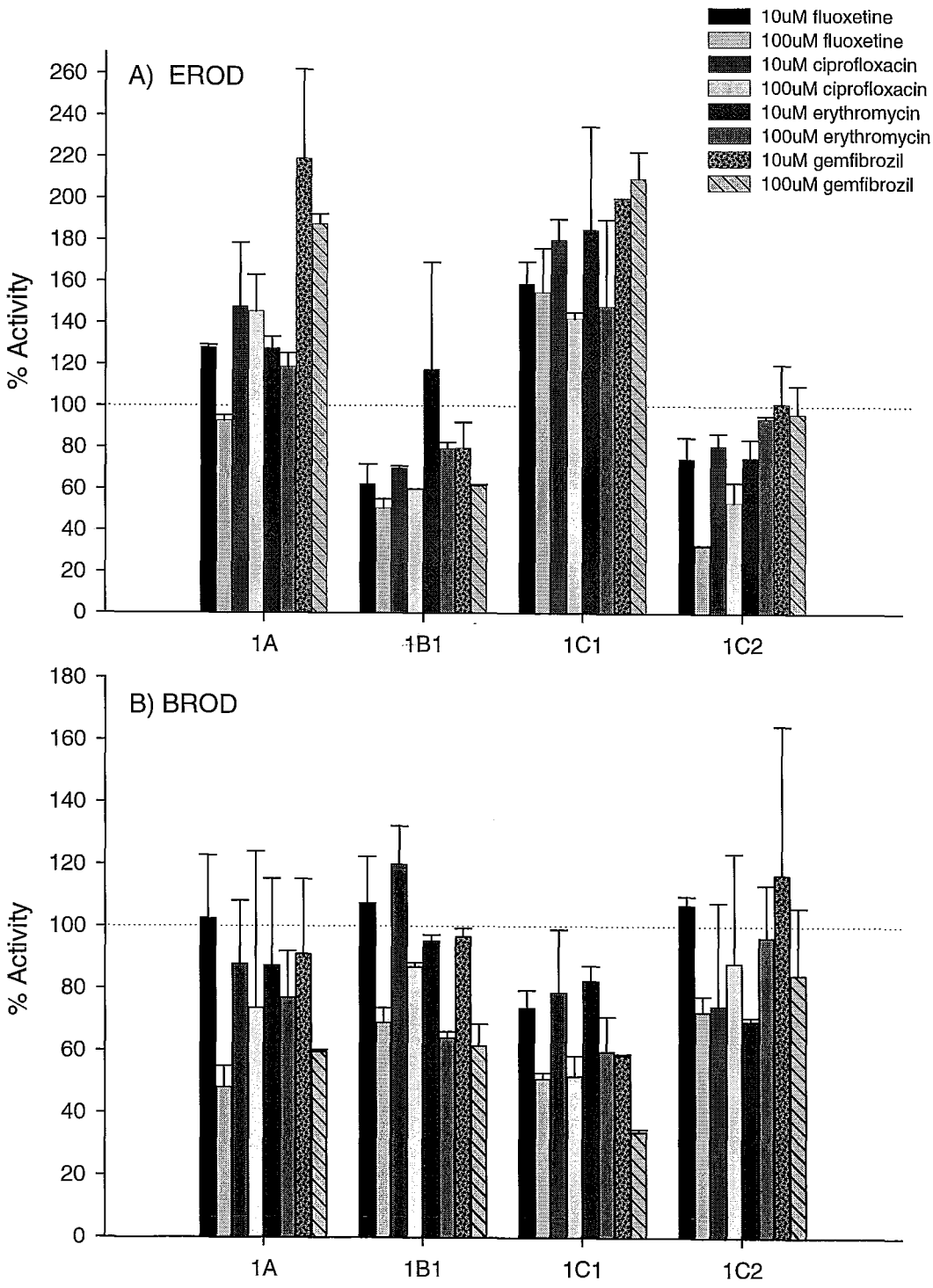
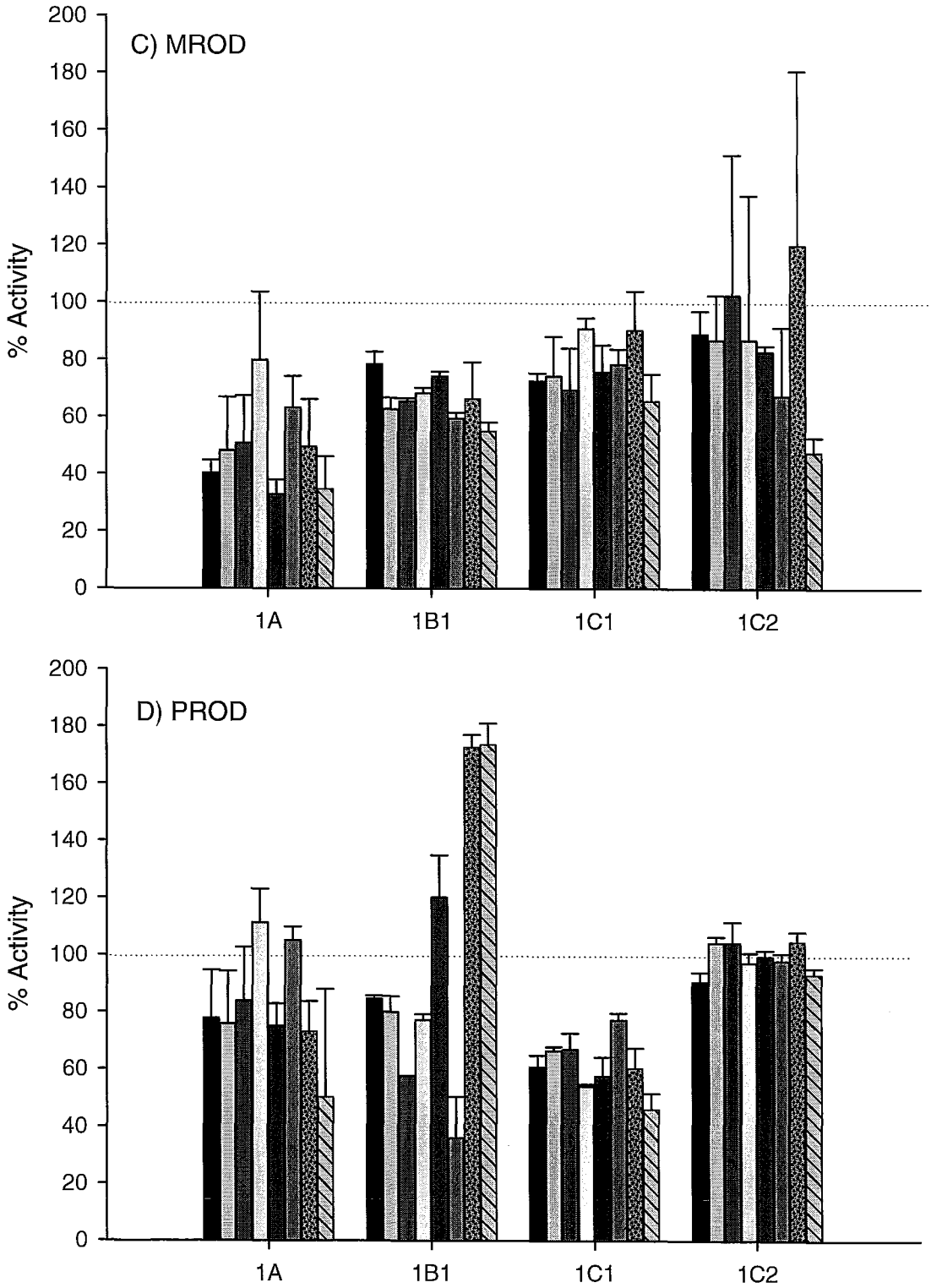

10uM fluoxetine
100uM fluoxetine
10uM ciprofloxacin
100uM ciprofloxacin
10uM erythromycin
100uM erythromycin
10uM gemfibrozil
100uM gemfibrozil
A) EROD
% Activity
260
240
220
200
180
160
140
120
100
80
60
40
20
0
1A
1B1
1C1
1C2
B) BROD
% Activity
180
160
140
120
100
80
60
40
20
0
1A
1B1
1C1
1C2

Figure 2 C/D


C) MROD
% Activity
200
180
160
140
120
100
80
60
40
20
0
1A
1B1
1C1
1C2
D) PROD
% Activity
200
180
160
140
120
100
80
60
40
20
0
1A
1B1
1C1
1C2

CHAPTER 4:

CROSS-SPECIES COMPARISON OF FLUOXETINE METABOLISM IN FISH LIVER MICROSOMES

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Abstract

The emergence of pharmaceuticals in wastewater effluent is of increasing concern to aquatic organisms. Selective serotonin reuptake inhibitors (SSRIs), used to treat chronic depression, appear in measurable concentrations in wastewater effluent. Fluoxetine (Prozac™) is a highly prescribed SSRI that has been used as a model compound to assess SSRI impacts on aquatic organisms. In this study, we have determined *in vitro* hepatic fluoxetine metabolism in several common fish model species: rainbow trout, goldfish, zebrafish and killifish. These species represent temperate and tropical, freshwater and marine species from diverse fish taxa. *In vitro* fluoxetine metabolism in rainbow trout liver microsomes shows a time-dependant loss of fluoxetine that is concomitant to an increase in norfluoxetine, the major mammalian metabolite. Although highly variable, fluoxetine does not appear to be well metabolized by hepatic microsomes. The loss of fluoxetine was higher than norfluoxetine production suggesting that norfluoxetine is not the only fluoxetine metabolite in fish. In fact, norfluoxetine was often not detected which may indicate that fluoxetine demethylation is a minor metabolic pathway in fish. The metabolism of fluoxetine in mammals is catalyzed by cytochrome P450 (CYP) enzymes. Rainbow trout were exposed to the known CYP inducers, carbamazepine (CBZ) and 3-methylcholanthrene (3-MC) to assess the potential for induction of hepatic fluoxetine metabolism in fish. Microsomal reactions from fish treated with 3-MC showed no detectable change in fluoxetine concentration; suggesting that CYP1 enzymes are not involved in fluoxetine metabolism in fish. Incubations with CBZ-treated trout microsomes revealed low, but detectable fluoxetine metabolism

suggesting that fluoxetine metabolism can be slightly induced. It is likely that multiple CYPs are responsible for the hepatic metabolism of fluoxetine in fish but the CYPs involved are unclear at this time. Investigation of metabolites other than norfluoxetine warrants future research.

Keywords: SSRI, Cytochrome P450, norfluoxetine, fluoxetine, carbamazepine, 3-methylcholanthrene, fish

Contributions: This chapter has been formatted as a paper prepared for publication written by E.M.S. Funding acquisition and experimental design was carried out in collaboration by J.Y.W and C.D.M. Species comparison data was collected and analyzed by E.M.S with analytical support provided by C.D.M. CBZ and 3-MC trout data was collected and analyzed by G.P and S.C under the supervision of C.D.M.

4.1 Introduction

Pharmaceuticals are discharged from wastewater treatment facilities into surface waters at rates estimated to reach kilograms per year(Daughton and Ternes 1999; Lindberg *et al.* 2005) (Daughton and Ternes 1999; Lindberg *et al.* 2005). Selective serotonin reuptake inhibitors (SSRIs), used to treat chronic depression, have been detected in wastewater and surface waters in Europe and North America (Kolpin *et al.* 2002; Metcalfe *et al.* 2003; Lamas *et al.* 2004; Vasskog *et al.* 2006) and in the tissues of fish collected near municipal wastewater discharge sites (Brooks *et al.* 2005; Chu and Metcalfe 2007). It has recently been established that this class of pharmaceuticals bioconcentrate in the tissues of fish (Paterson and Metcalfe 2008), more specifically, in the liver (Nakamura *et al.* 2008).

SSRIs are designed with a specific mode of action, targeting the reuptake of the brain neurotransmitter, serotonin, at the synaptic cleft. Fish produce serotonin, (Khetan and Collins 2007) and possess both serotonin receptors (Yamaguchi and Brenner 1997) and the serotonin transporter (SERT) which is the mode of action of SSRIs in mammals (Gould *et al.* 2007), making them a susceptible target for SSRIs, and the suite of biological and adverse effects that are associated with SSRI exposure in mammals. Fluoxetine, the active ingredient in Prozac™, is a highly prescribed SSRI found in the aquatic environment and is presently being used as a model compound in assessing SSRI impacts on aquatic organisms.

Fluoxetine has been shown to cause a broad range of effects in a variety of fish species. Recently, Lister *et al.*(2009) have shown that exposure of zebrafish to fluoxetine for seven days at environmentally relevant concentrations can significantly decrease egg production. Japanese medaka exposed to fluoxetine at concentrations as low as 0.1μg L^{-1} for four weeks showed significantly elevated plasma estradiol and developmental deformities among offspring (Foran *et al.* 2004). In goldfish, fluoxetine decreased isotocin transcript levels in the brain, the fish homolog of the mammalian neuropeptide oxytocin, suggesting a mechanistic link between fluoxetine exposure and reproductive dysfunction (Mennigen *et al.* 2008). Gulf toadfish had decreased plasma osmolality and increased urea excretion as a result of fluoxetine treatment (Morando *et al.* 2009). Changes in feeding behavior have been noted in fathead minnows (Stanley *et al.* 2007) and altered levels of neuropeptides that regulate feeding behavior in goldfish (Mennigen *et al.* 2009). These impacts seen in fish exposed to fluoxetine mirror many adverse effects of fluoxetine in humans, including appetite loss and sexual dysfunction (Brambilla *et al.* 2005).

While biological impacts of SSRI exposure have been investigated in aquatic species, studies on SSRI metabolism in fish are lacking. Recently, it has been documented that Japanese medaka are capable of *in vivo* fluoxetine metabolism and the production of norfluoxetine, the major fluoxetine metabolite in mammals (Nakamura *et al.* 2008; Paterson and Metcalfe 2008). Bioaccumulation rates were high for fluoxetine in medaka, and the half life was relatively long at 9.4 days (Nakamura *et al.* 2008;

Paterson and Metcalfe 2008). In humans, the half-life of fluoxetine is 1-4 days (Hiemke and Hartter 2000), suggesting that metabolic conversion of fluoxetine is significantly less in fish than in mammals.

Xenobiotic biotransformation is a multi-phase process, the first of which usually involves the cytochrome P450 (CYP) superfamily of enzymes (Andersson and Forlin 1992; Xu *et al.* 2005). In humans, CYP 3A4 is responsible for 50% of all human drug metabolism followed by 2D6, 2C9 and 1A2 at 25%, 15% and 5% respectively (Hemeryck and Belpaire 2002). Enzymes from the CYP2 and CYP3 families are responsible for SSRI metabolism in mammalian species. In humans, fluoxetine is primarily metabolized to norfluoxetine through demethylation by CYP 2D6 and to a lesser extent, by CYPs 2C9, 3A4 and 2C19 (Figure 1), yet fish lack CYP2C and 2D homologs. It has been reported that fish are capable of metabolizing some typical mammalian CYP2 and CYP3 substrates (reviewed in Buhler and Wang-Buhler 1998). However, studies of drug metabolism in fish are extremely limited and the metabolic fate and CYPs responsible for the metabolism of fluoxetine, and other drugs, in fish are largely unknown.

The present study was designed to investigate the *in vitro* hepatic metabolism of fluoxetine by fish using a comparative approach. Liver microsomes from a range of model fish species were used to assess basal metabolism of fluoxetine in fish. Fish species used were rainbow trout, mummichog killifish, zebrafish and goldfish. To investigate the potential to alter *in vitro* hepatic fluoxetine metabolism, trout were

exposed to the CYP inducers carbamazepine and 3-methylcholanthrene. The rates of metabolism in untreated and exposed fish microsomes were compared to that found in rat or rabbit liver microsomes. Resolving the capacity of aquatic vertebrate species to metabolize fluoxetine, and other environmentally relevant pharmaceuticals, will contribute to our understanding of the ecological risks associated with the release of pharmaceuticals, specifically SSRIs, into the aquatic environment.

4.2 Methods

4.2.1 Animals

Goldfish (*Carassius auratus*; 2-7g body weight) were purchased from a local pet store, held at room temperature in recirculating dechlorinated tap water and fed staple flakes daily. Mummichog killifish (*Fundulus heteroclitus*; 1.5-5g body weight) were purchased from Aquatic Research Organisms (New Hampshire, USA). Killifish were held at 19-20°C in 10% recirculating seawater and fed staple flakes 5 days per week supplemented with frozen brineshrimp. Rainbow trout (*Oncorhynchus mykiss*) were purchased from Humber Springs trout hatchery (100-130 g body weight; Mono Mills, Ontario) or Lynnwood Acres Trout Farm (180-230 g body weight; Campbellcroft, Ontario). Trout were kept at 10-15°C in flow-through water and fed floating trout pellets 3 days per week. Zebrafish (*Danio rerio*; 200-500 mg body weight) were purchased from DAP International (Etobicoke, Ontario). Zebrafish were kept in a semi-recirculating system at 28-29°C and fed three times per day, alternating between tropical flake food and fresh brineshrimp (*Artemia*). All fish were held for a minimum of two

weeks before use. Rainbow trout livers were large enough to use single fish replicates; other species were pooled due to their small liver size. Goldfish were juveniles and consisted of 21-25 fish per pool. Sexually mature zebrafish and killifish were separated into male and female pools. Zebrafish and killifish pools consisted of 35-42 and 8-13 fish, respectively.

Livers from male Sprague-Dawley rats were kindly provided by Dr. S. Raha (McMaster University). Liver from four rats were pooled and used as a mammalian control in metabolism assays. Commercially available rabbit liver microsomes (Sigma-Aldrich, St. Louis, MO) that express high CYP2B activity were purchased and used for optimization and as a mammalian comparison.

4.2.2 In vivo P450 induction with 3-MC or CBZ treatment

Female rainbow trout, *Oncorhynchus mykiss* (180-230 g) were held in filtered Otonabee River water. Food was withdrawn from the fish at 2 days prior to injection. Five fish per treatment were injected i.p. with carbamazepine (CBZ) or 3-methylcholanthrene (3-MC) at a dose of 20 mg kg^{-1} in corn oil. At five days post-injection, the fish were sacrificed by an overdose of MS-222.

4.2.3 Fluoxetine metabolism assay

Livers were collected and either placed directly on ice or flash-frozen in liquid nitrogen and stored at -80°C until microsomal preparation. Liver microsomes were prepared as in Stegeman *et al.* (1995) and stored at -80°C until use in fluoxetine

metabolism assay. Total protein content was determined using Bicinchoninic Acid kit (BCA; Pierce protein research products – Thermo Fisher Scientific), according to manufacturer's protocols (Smith *et al.* 1985).

Rabbit liver microsomes were used to optimize the conditions for *in vitro* metabolism of fluoxetine. These parameters included the microsome concentration, pH, NADPH concentration, buffer, and incubation time. Microsomes were pre-incubated for 5 minutes at 25°C (fish) or 37°C (mammal) with $MgCl_2$, NADPH (final concentrations in reaction of 3 mM and 1 mg mL^{-1} respectively) in 20 mM $KP0_4$ (pH 7.4). Fluoxetine was added at nominal final concentrations of 45, 80 or 160 $\mu g\ L^{-1}$ to initiate reaction. Microsomes made up one half of the final reaction volume (i.e. 500 µL in 1 mL final reaction volume or scaled down proportionally). Sub-samples of 100 µL were taken from experimental tubes at desired time points and immediately added to 200µL of internal standard to stop the reaction (200μgL^{-1} fluoxetine-*d5* in acetonitrile; C/D/N Isotopes Inc. Pointe-Claire, Quebec, Canada). Samples were immediately centrifuged at 400*xg* for 10min to precipitate proteins and the aqueous layer was aspirated into auto-sampler vials and analyzed for fluoxetine and norfluoxetine using LC-MS/MS.

4.2.4 LC-MS/MS analysis

Fluoxetine and norfluoxetine were analyzed as described previously by Chu and Metcalfe (2007). Briefly, all analyses were performed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) with an atmospheric pressure chemical ionization (APCI) source. LC-APCI-MS/MS analysis was conducted with an Agilent

1100 HPLC coupled to an Applied Biosystems Q-Trap tandem mass spectrometer (MDS Sciex, Concord, Ontario, Canada) equipped with an APCI ionization source. LC-MS/MS instruments were operated in positive ion mode for analysis using multiple-reaction monitoring (MRM) for transition ions. Fluoxetine, norfluoxetine and the internal standard, fluoxetine-d_5 were monitored with ion transitions (m/z) of 310.2 > 43.9, 296.2 >134 and 315.3 >43.9, respectively. Chromatographic separation of analytes was carried out on a Genesis C18 column (150 × 2.1 mm i.d., 4 μm particle size, Chromatographic Specialties, Brockville, Ontario, Canada) coupled with a guard column with the same packing material (4 mm × 2.0 mm, Phenomenex Torrance, Ontario, Canada). HPLC conditions include 25 μL injection volume and gradient elution with mobile phases A (10 mM ammonium acetate in water) and B (10 mM ammonium acetate in 95 % acetonitrile and 5 % water) at a flow rate of 200 μL min^{-1}. Quantification was performed using an internal standard method with a minimum five point calibration curve spanning the range of analyte concentrations in the samples. The responses to the internal standards in the samples were used to correct for recoveries and/or matrix effects. Limits of quantitation (LOQs), defined as 10:1 signal-to-noise were 0.05 and 0.15 μg L^{-1} for fluoxetine and norfluoxetine, respectively. Limits of detection (LODs), defined as a 3:1 signal-to-noise ratio were 0.02 and 0.06 μg L^{-1} for fluoxetine and norfluoxetine, respectively.

4.3 Results

4.3.1 Time and Concentration Dependant Metabolism of Fluoxetine

The concentration of fluoxetine decreases over time in reactions with both CBZ-

treated rainbow trout and rabbit microsomes (Figure 2). The reaction is linear to approximately 30 minutes and then plateaus; maximal fluoxetine metabolism is seen by 60 minutes in reactions with both rabbit and trout liver microsomes (Figure 2). Norfluoxetine was detected in all treatments with rabbit microsomes and CBZ induced trout microsomes within 10 minutes of incubation (Figure 3). Norfluoxetine showed an increase in the reaction mixture that paralleled the loss of fluoxetine. Concentrations of norfluoxetine continue to increase until 40-50 minutes of incubation (Figure 3). At an initial substrate concentration of 160 $\mu g\ L^{-1}$, fluoxetine metabolism, as measured by the percent loss of fluoxetine in the reaction, reached 13% by 30 min and 17% by 60 min with CBZ- treated trout microsomes. The percent of initial fluoxetine converted into norfluoxetine in the same incubation tubes reached less than 2% by 30 minutes and 2.9% by 60 minutes (Table 1). Incubation at the same substrate concentration with rabbit microsomes showed faster fluoxetine metabolism, with 69% decrease in substrate concentration by 30 minutes and 87% by 60 minutes. The norfluoxetine production was also much higher with rabbit microsomes, with 14% of initial fluoxetine converted into norfluoxetine by 30 minutes incubation and 16% by 60 minutes (Table 1).

Fluoxetine metabolism and norfluoxetine production is concentration dependant. Both rabbit and trout microsomal preparations had increased metabolic capacities at the higher substrate concentrations. For CBZ-induced rainbow trout microsomes, doubling the substrate concentrations resulted in an 8% increase in total substrate metabolized (Table 1; Figure 2); the rate of fluoxetine demethylation into norfluoxetine increased

approximately 1.4-fold (Figure 3A). Increasing the fluoxetine concentration from 45 to 160 $\mu g\ L^{-1}$with rabbit microsomes resulted in a 6% increase in total substrate metabolized and 2.4-fold increase in the rate of fluoxetine metabolism to norfluoxetine (Table 1; Fig 3B).

4.3.2 Comparative fluoxetine metabolism

To assess intrinsic metabolic capabilities, four untreated fish species were selected. Rainbow trout represent a large, freshwater salmonid species. Zebrafish and goldfish are both freshwater cyprinids, but live in tropical and temperate waters, respectively. Killifish belong to the family Fundulidae and are a common marine species used for our comparison. Collectively, these species represent commonly used laboratory fish. These species span a variety of body sizes, and consequently, liver sizes, resulting in the need to pool individual livers in all but rainbow trout for our analyses.

In vitro metabolism of fluoxetine by unexposed fish liver microsomes was extremely variable. Data are displayed for single samples to show intra and inter-specific variability (Table 2). Only five samples out of the 23 incubated with untreated fish liver microsomes showed detectable amounts of norfluoxetine after 30 or 60 minutes of incubation. Contrary to our assessment of substrate concentration effects in CBZ-treated rainbow trout and commercial rabbit liver microsomes, increasing the substrate concentrations did not yield significantly higher rates of fluoxetine metabolism or norfluoxetine production in any consistent pattern (Table 2). Fluoxetine metabolism was rarely over 1% for undosed rainbow trout but appeared to be much higher for both male

and female killifish (Table 2), where samples lost between <1 to 48% of the initial fluoxetine over the incubation time. Norfluoxetine production was nearly uniformly below the limit of detection and did not show a strong relationship with fluoxetine metabolism. Rat liver microsomes metabolized fluoxetine to detectable levels of norfluoxetine in both replicates; however, the percent of fluoxetine loss per mg protein is still not in proportion to detection of its demethylated metabolite, norfluoxetine (Table 2).

4.3.3 Induced fluoxetine metabolism

In the incubations with CBZ-induced trout microsomes and rabbit microsomes, fluoxetine concentrations declined over the incubation period (Table 1) and norfluoxetine was detectable and quantifiable. Incubation with trout treated with 3-MC did not significantly change fluoxetine concentrations and no metabolite was detected, similar to reactions with undosed trout liver microsomes (Table 2). At fluoxetine concentrations of 160 and 45 $\mu g\ L^{-1}$, rabbit liver microsomes metabolized >50% of the initial fluoxetine substrate, metabolism that is much higher than that seen in rat liver microsomes (Table 2). In comparison, CBZ-induced trout liver microsomes metabolized only 17% and 11% of initial substrate in the treatments with 160 and 80 $\mu g\ L^{-1}$ fluoxetine, respectively (Table 1). At the nominal concentration of 160 $\mu g\ L^{-1}$, the rate of fluoxetine metabolism by trout microsomes was < 1% of that determined for rabbit microsomes. Reactions containing rabbit liver microsomes metabolized approximately 15% of the initial fluoxetine substrate to the demethylated metabolite norfluoxetine. By contrast, CBZ-induced trout liver microsomes metabolized only 2 to 3% of the initial fluoxetine

substrate to norfluoxetine. CBZ-induced trout showed higher and more consistent metabolism of fluoxetine compared to unexposed trout (Table 2).

4.4 Discussion

The current study is among the first to look at fluoxetine metabolism in fish and, to our knowledge, is the first to determine *in vitro* hepatic microsomal fluoxetine metabolism for any fish species. Liver microsomes from trout induced with carbamazepine (CBZ) produce a time and concentration dependant decrease in fluoxetine and increase in norfluoxetine, suggesting that hepatic metabolism of fluoxetine is possible in fish. Several untreated fish species were selected, encompassing a variety of native habitats and taxa, to test intrinsic differences in basal fluoxetine metabolism in fish. Reasonable substrate concentrations and reaction times were selected based on the established time-course and concentrations tested using CBZ-induced trout and rabbit microsomes.

As with both the CBZ-induced trout and rabbit microsomes, fluoxetine metabolism was apparent by loss of fluoxetine over time in most fish species tested. Yet, the samples showed a high amount of variability, both between species and across intra-specific samples. Individual variability has been noted in populations of *Fundulus heteroclitus*, with evolutionarily adaptive changes in gene expression thought to account for much of this variability (Oleksiak *et al.* 2005; Crawford and Oleksiak 2007). Although sample variability does not allow us to determine statistical differences across species, some species appear to have higher rates of metabolism of fluoxetine compared

to others. Killifish, in particular, have higher percent loss of fluoxetine, with the exception of one sample (killifish 2; Table 2). Rainbow trout appear to have much lower capacity for fluoxetine metabolism than the other species tested. Such differences have been noted across mammalian species (Walker 1978), but similar characterization and comparison is lacking for fish. If adaptive changes can be seen amongst individuals within fish populations (Oleksiak *et al.* 2005; Crawford and Oleksiak 2007), inter-specific variation merits similar attention.

While nearly all samples showed a loss of fluoxetine with time, demethylation of fluoxetine to norfluoxetine comprised only a small proportion (i.e. < 30%) of the total biotransformation and loss of fluoxetine over time, if there was norfluoxetine production at all. Although fluoxetine metabolism with untreated trout liver microsomes is clearly variable, norfluoxetine production is not detectable in most samples. These data indicate that the loss of fluoxetine is much larger than production of the demethylated metabolite, norfluoxetine. This is apparent in CBZ-treated rainbow trout (Table 1) and all undosed fish species (Table 2), suggesting that there may be production of multiple fluoxetine metabolites which have not been investigated. It has been suggested that other fluoxetine metabolites are present in human fluoxetine metabolism, however norfluoxetine is the dominant metabolite and any others are often considered negligible (Li *et al.* 2004). *O*-Dealkylation of fluoxetine into trifluoromethylphenol has been documented in mammals (Liu *et al.* 2002) and may be a candidate for future research in fish. Clearly, norfluoxetine is unlikely to be the primary fluoxetine metabolite in fish as nearly all

undosed fish samples had no detectable norfluoxetine in spite of demonstrable loss of fluoxetine. This is particularly evident in reactions with microsomes from killifish 7, which had nearly 50% loss of fluoxetine without detection of norfluoxetine.

Paterson and Metcalfe (2008) established *in vivo* uptake and depuration of fluoxetine in Japanese medaka and found the half-life of fluoxetine to be 9.5 days. The current study supports their predictions that the long half-life in fish is likely a result of lower biotransformation capacity relative to mammals. Nakamura *et al.* (2008) identified bioaccumulation of fluoxetine in fish tissues, with the liver having higher concentrations of both fluoxetine and norfluoxetine compared to whole body, after 30 day exposure to fluoxetine. In contrast to these studies, a great majority of samples analyzed presently showed no detection of norfluoxetine. The *in vitro* incubation time in the present study did not exceed 60 min, whereas these *in vivo* exposures have spanned over many days. Based on our *in vitro* data, the production of norfluoxetine is predicted to be extremely slow *in vivo* and coupled with the clear *in vivo* accumulation over time, suggests that the excretion of norfluoxetine is low in fish.

Rainbow trout treated with CBZ, but not 3-MC, metabolized fluoxetine in a time and concentration dependent manner and at rates that were higher than the untreated trout, suggesting that CYP induction can increase the metabolism of fluoxetine and production of the metabolite norfluoxetine. In mammals, CBZ induces CYP1A2 and CYP3A4 (Hemeryck and Belpaire 2002). Induction of benzylether-resorufin (BzRes) dealkylation has been seen in rainbow trout hepatocytes treated with CBZ, without the

induction of ethoxyresorufin-*O*-dealkylation (EROD), suggesting CBZ may induce CYP2 or CYP3 genes in fish (Gagne *et al.* 2006) but not CYP1s. 3-MC treatment in both mammals and fish is associated with arylhydrocarbon receptor (AhR)-mediated CYP1 induction (Forlin 1980; Thomas *et al.* 1983; Stuchal *et al.* 2006). Incubation of fluoxetine with 3-MC-treated trout microsomes revealed no detectable fluoxetine metabolism, suggesting that CYP1 induction, including CYP1A (the homolog to the mammalian CYP1A1 and 1A2 genes), does not increase fluoxetine metabolism in trout liver. Incubation with CBZ-treated rainbow trout liver microsomes, however, revealed detectable norfluoxetine after only 10 minutes suggesting that CBZ treatment in fish induces the demethylation of fluoxetine to norfluoxetine in the liver.

It is likely that multiple CYPs are responsible for the metabolism of fluoxetine in fish liver. Identifying which CYPs are dominant in this process will be more difficult to determine, particularly since the primary metabolite of fluoxetine in fish is unknown and unlikely to be norfluoxetine. Determination of the specific CYPs induced by CBZ in fish would help to identify which CYPs are the catalyst of demethylation of fluoxetine to norfluoxetine. Drug metabolism studies are fundamentally lacking outside of mammalian species. Several CYP proteins are responsible for the metabolism of fluoxetine in mammals (Figure 1) and it would not be unreasonable to predict the same to be true in fish. Building on the framework surrounding drug metabolism in fish, it is apparent that the capacity of fish liver microsomes to metabolize fluoxetine *in vitro* is minimal and variable. These data provide a broad comparison across species, including

two mammalian species and highlight the importance of using an inter-specific comparative approach in novel research. More studies are needed on the intrinsic ability of fish to metabolize pharmaceutical contaminants. Hepatic metabolism of fluoxetine in fish may involve the production of a different fluoxetine metabolite altogether that could account for the loss of fluoxetine seen without detection of norfluoxetine in most untreated fish samples.

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4.6 Tables

Table 1. Fluoxetine metabolism in treated rainbow trout liver microsomes. Reactions consisted of various nominal fluoxetine concentrations incubated with liver microsomes from rainbow trout treated with 20 mg kg^{-1} of carbamazepine (CBZ) or 3-methylcholanthrene (3-MC). Rabbit microsomes are shown for *in vitro* mammalian comparison. Nominal, initial and final fluoxetine concentrations are expressed as mean ($\mu g\ L^{-1}$) ± standard deviation. Percent norfluoxetine indicates the percentage of the initial fluoxetine substrate detected as norfluoxetine after 60 min of incubation.

Species	Treatment	Nominal concentration	n	Initial concentration	Final concentration	% norfluoxetine
Trout	CBZ	80	3	82.5 ± 0.6	73.0 ± 0.3	2.9 ± <0.1
	CBZ	160	3	160.1 ± 0.3	132.7 ± 1.1	2.4 ± <0.1
	3-MC	160	3	153.0 ± 13.4	150.8 ± 5.8	<0.1[a]
Rabbit	N/A[b]	45	2	44.3 ± 0.1	19.6 ± 0.1	15.4 ± <0.1
	N/A	160	2	162.0 ± 0.8	61.3 ± 0.2	16.2 ± <0.1
Blank[c]		80	3	79.3 ± 0.4	80.0 ± 0.7	ND[d]

[a] Less than limit of quantitation (LOQ)
[b] Commercially available microsomes.
[c] No microsomes added to incubation tubes.
[d] ND- Norfluoxetine not detected in incubation tubes.

Table 2. Cross-species comparison of fluoxetine metabolism. Species, sex, incubation time, and nominal fluoxetine concentrations are presented following incubation with untreated liver microsomes from several species. Data are arranged as separate samples to show high variability amongst analyzed samples. Changes are displayed as percent decrease in fluoxetine over total incubation time (i.e. 30 or 60 minutes) and percent of initial substrate detected as norfluoxetine after the same incubation period. Percent change in fluoxetine and the percent substrate detected as norfluoxetine are normalized for mg of total protein in the sample.

Sample	Sex	Incubation time (min)	Nominal Fluoxetine ($\mu g\ L^{-1}$)	% decrease in fluoxetine over total incubation time	% initial fluoxetine concentration detected as norfluoxetine
Goldfish 1	juvenile	60	45	24	2.35
Goldfish 2	juvenile	30	80	<1.0	ND[a]
Goldfish 3	juvenile	30	80	1.4	ND
Goldfish 4	juvenile	30	80	8.3	1.69
Trout 1	juvenile	60	45	6.6	1.89
Trout 2	juvenile	30	80	14.8	ND
Trout 3	juvenile	30	80	<1.0	ND
Trout 4	juvenile	30	80	<1.0	ND
Trout 5	juvenile	30	80	<1.0	ND
Zebrafish 1	juvenile	60	45	--[b]	3.99[c]
Zebrafish 2	male	30	80	17	ND
Zebrafish 3	male	30	80	<1.0	ND
Zebrafish 4	male	30	80	4.1	ND
Zebrafish 5	female	30	80	13	ND
Zebrafish 6	female	30	80	20	ND
Zebrafish 7	female	30	80	<1.0	ND
Killifish 1	male	60	45	--[b]	6.63[c]
Killifish 2	male	30	80	<1.0	ND
Killifish 3	male	30	80	23	ND
Killifish 4	male	30	80	20	ND
Killifish 5	female	30	80	9.6	ND
Killifish 6	female	30	80	19	ND
Killifish 7	female	30	80	48	ND
Rat 1	male	60	45	10.5	3.01
Rat 2	male	30	80	1.6	4.82

[a] ND- No detection.
[b] -- Initial time point was not measured therefore unable to calculate change in fluoxetine.
[c] Initial time point was not measured; this value is approximated based on final fluoxetine concentration at 60min.

4.7 Figures

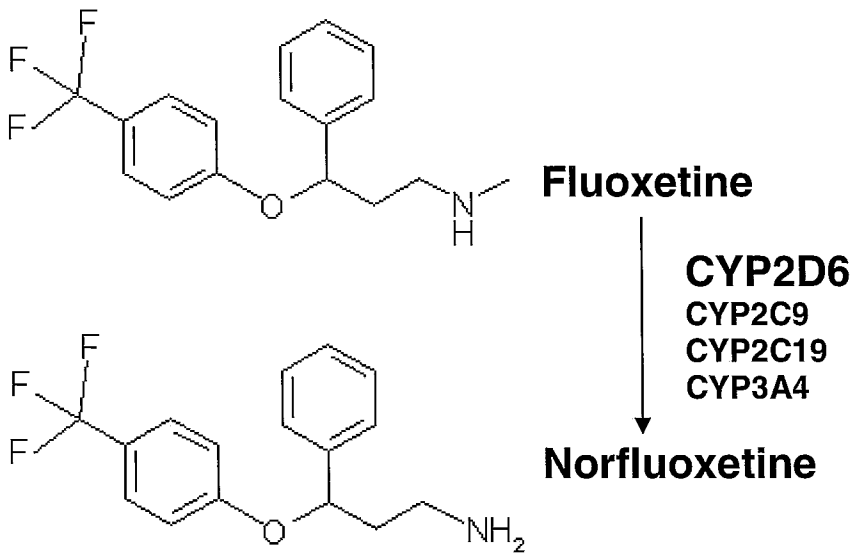


Figure 1. The chemical structure of fluoxetine and its active demethylated metabolite, norfluoxetine. The cytochrome P450 (CYP) enzyme isoforms associated with the demethylation of fluoxetine to norfluoxetine in humans are listed. The larger font size for CYP2D6 signifies this isoform as the dominant human CYP responsible for this metabolism.

Figure 2. Change in concentrations of fluoxetine (μg L^{-1}) over time in treatments with rabbit microsomes and carbamazepine-induced rainbow trout liver microsomes. Microsomes were incubated with: A) 160 μg L^{-1} fluoxetine, or B) 45 (rabbit) or 80 (trout) μg L^{-1} fluoxetine. Solid and dashed lines represent the best fit non-linear regressions for reactions with trout and rabbit liver microsomes, respectively. Individual values indicate the means of 3 and 2 replicates (± 1 standard deviation) for rainbow trout and rabbit treatments, respectively.

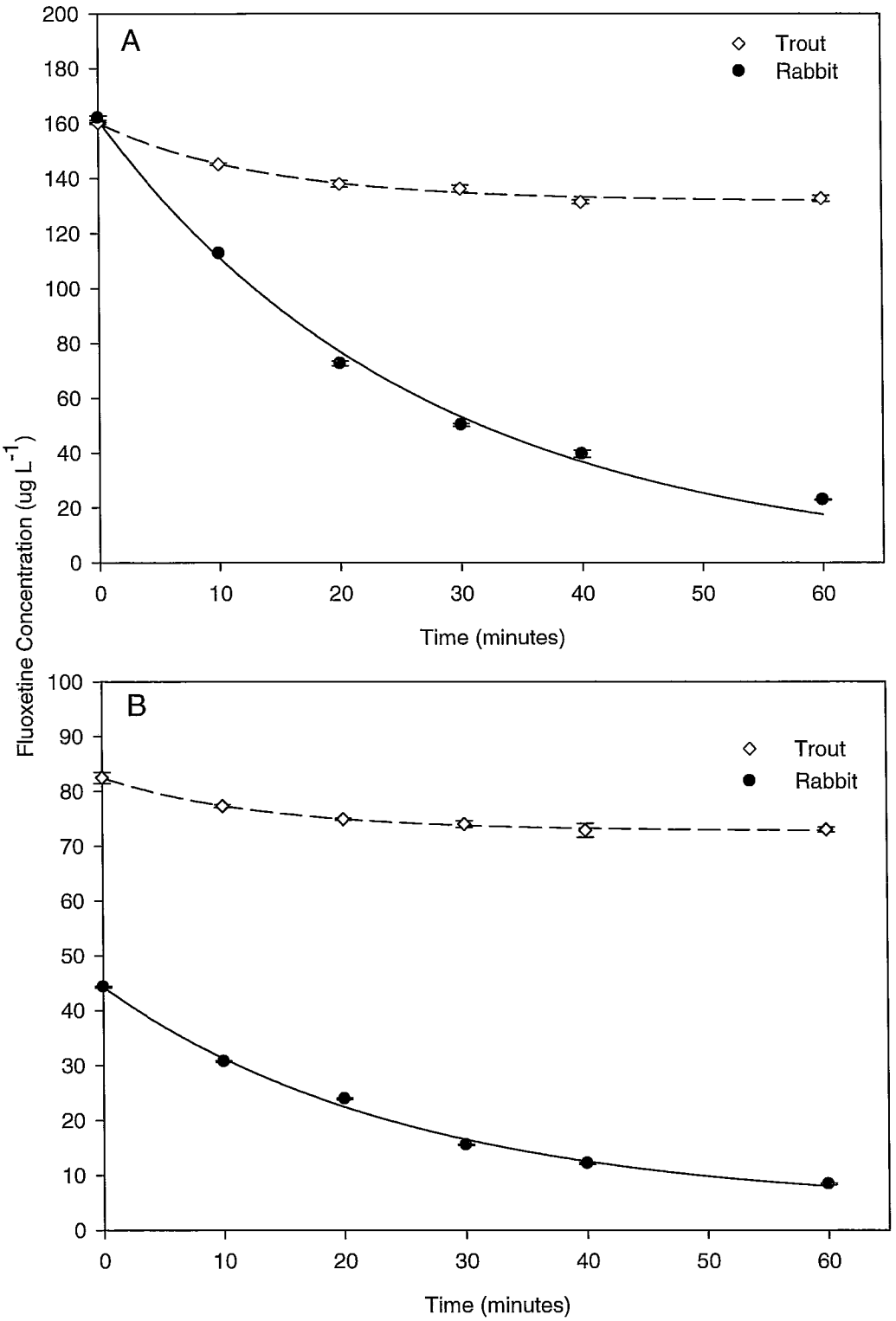
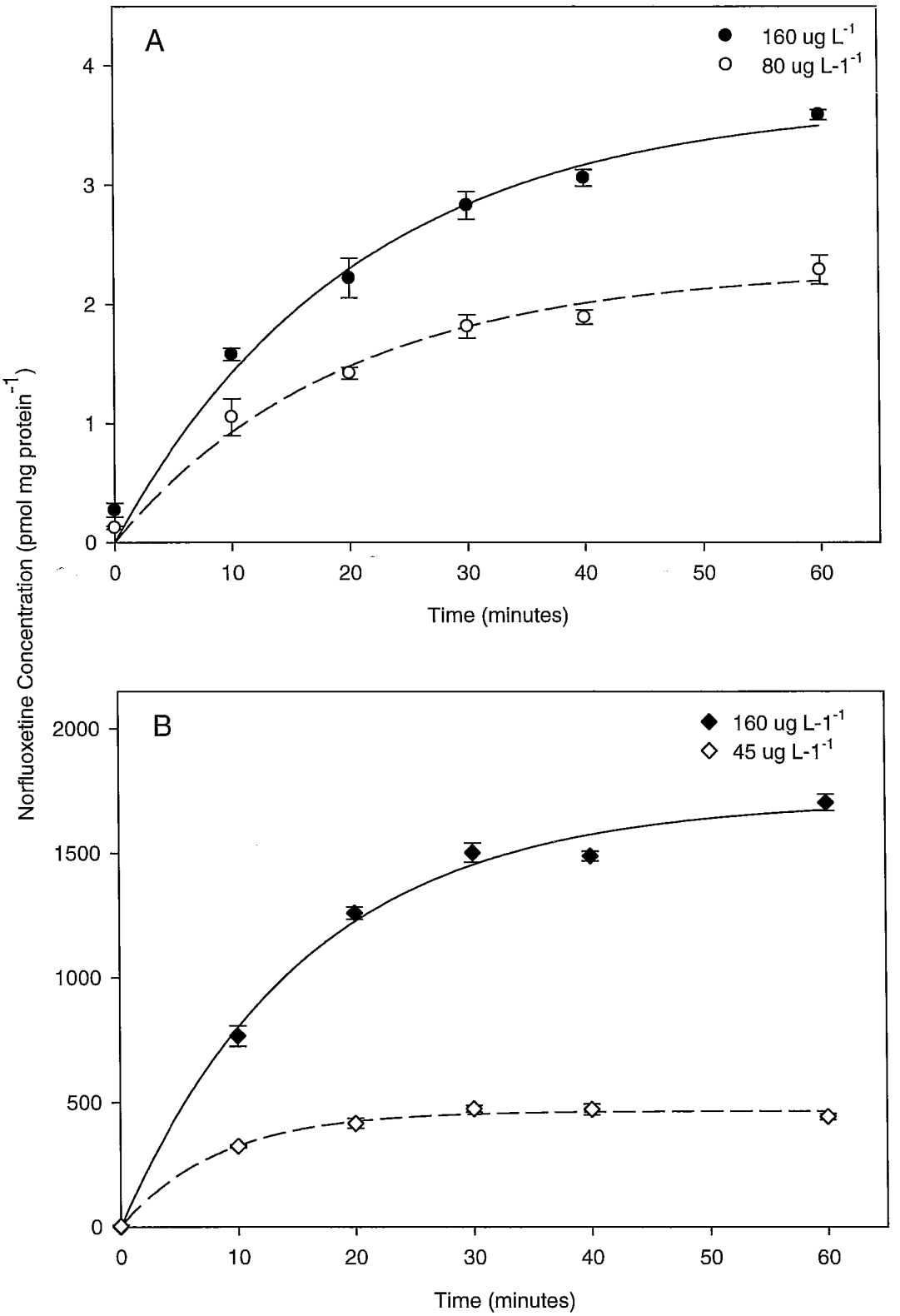

A
Trout
Rabbit
Fluoxetine Concentration (ug L-1)
Time (minutes)
B
Trout
Rabbit
Time (minutes)

Figure 3. Rate of norfluoxetine formation (pmol mg^{-1} protein) over time in treatments with carbamazepine-induced rainbow trout liver microsomes or rabbit liver microsomes. A) Rainbow trout microsomes were incubated with 80 or 160 μg L^{-1} fluoxetine. B) Rabbit microsomes were incubated with 45 or 160 μg L^{-1} fluoxetine. Solid and dashed lines represent the best fit non-linear regressions for the high and low fluoxetine concentrations, respectively. Individual values indicate the means of 3 and 2 replicates (± 1 standard deviation) for rainbow trout and rabbit treatments, respectively.


A
160 ug L-1
80 ug L-1-1
Norfluoxetine Concentration (pmol mg protein-1)
Time (minutes)
B
160 ug L-1-1
45 ug L-1-1
Time (minutes)

CHAPTER 5:

GENERAL DISCUSSION

5.1 Metabolism by cytochrome P450s

Beyond catalyzing the metabolism and biosynthesis of hormones, vitamins and other endogenous compounds, the cytochrome P450s (CYPs) play a central role in detoxification and the metabolism of exogenous compounds, including environmental contaminants and pharmaceuticals. In humans, CYP 3A4 is responsible for 50% of all human drug metabolism followed by 2D6, 2C9 and 1A2 at 25%, 15% and 5% respectively (Hemeryck and Belpaire 2002). Of these four important drug-metabolizing CYPs, fish possess CYP1A and 3A homologs but CYP2s are more diverse and no CYP2C or 2D isoforms are found in fish. Functional characterization of fish CYPs is not straightforward. Although fish and mammals possess similar proteins and fish are capable of metabolizing some typical mammalian substrates, it should not be assumed that the same proteins are consistently able to metabolize identical substrates between vertebrate taxa.

In this thesis, we have examined a number of fluorometric CYP-catalyzed assays, and fluoxetine, a model selective serotonin reuptake inhibitor (SSRI), to assess CYP-mediated metabolism in fish. Overall, we raise questions about extrapolating known mammalian data to fish CYP function. 7-ethoxyresorufin-*O*-deethylase (EROD) is the most common assay used in vertebrates to assess CYP1A activity. In mammals,

metabolism of 7-methoxyresorufin (7-MR) is used to distinguish between CYP1A1 and 1A2 activity with preferential metabolism of 7-MR by CYP1A2. In fish, both 7-ER and 7-MR are metabolized equally by the single CYP1A isoform (Gooneratne *et al.* 1997; Scornaienchi 2009). Some salmonid species possess two CYP1A isoforms, denoted CYP1A and CYP1A3 (Berndtson and Chen 1994); no preference is seen for either of these substrates by this second CYP1A protein, providing functional evidence that CYP1A3 is not homologous to mammalian CYP1A2 (Gooneratne *et al.* 1997). Recent data suggests that CYP1s outside of CYP1A are able to metabolize these substrates in fish (Scornaienchi 2009).

BQ and BFC are selective CYP3A substrates in mammals. Both have been used to identify CYP3A activity in fish (Haasch *et al.* 1994; Burke *et al.* 1995; Thibaut *et al.* 2006) although recent data is suggesting that BFC is likely metabolized by a variety of CYPs in fish, including CYP1s (Scornaienchi 2009). Although these substrates are similarly metabolized in mammals, we observed that rates of metabolism of BFC and BQ were different from one another in fish liver (Chapter 2). BFC was not well metabolized in undosed fish of either species; BQ was well metabolized by both trout and killifish liver microsomes with consistent rates of metabolism seen between species. In chapter 3, we find further evidence that BQ and BFC are metabolized by different CYPs. The response to inhibition is drastically different between undosed and BNF-treated microsomes in the BFC assays, whereas the profile of BQ inhibition by all inhibitors was the same in undosed and BNF-treated microsomes. BFC and BQ are not likely

metabolized by the same CYPs. Although overall BFC metabolism was not induced, it is clear that the dynamic of CYPs responsible for its metabolism change with BNF treatment

We have branched beyond CYP-substrates previously used in fish research and have assessed the metabolism of MFC, AMMC, or MAMC in trout and killifish (Chapter 2). These substrates are protein-specific in mammals and used in drug-discovery to test CYP-inhibition (BD Bioscience, Gentest.com). MFC, a CYP2C9 substrate in humans, displayed species differences in metabolism. Trout did not metabolize this substrate well, although killifish seemed more capable. Being that this substrate has not been assessed in fish, the CYPs responsible for its metabolism seen here are unknown.

CYP2D6 is the second most-important drug-metabolizing protein and is the major CYP responsible for fluoxetine metabolism in humans. In mammals, AMMC and MAMC are selective substrates for CYP2D activity. No detection of *in vitro* hepatic AMMC or MAMC metabolism was found in untreated fish; metabolism of MAMC was inducible by BNF but AMMC was not (Chapter 2). Fluoxetine metabolism was low in all fish species tested but was measurable and inducible (Chapter 4). This implies that fish possess the capacity to metabolize human CYP2D6-selective substrates but the CYPs responsible are not highly expressed in the liver of fish and these reactions are catalyzed by multiple CYPs in fish. Furthermore, across numerous samples spanning two species, two sexes, and four CYP inducers, no metabolism of AMMC was detected. This substrate does not appear to be metabolized in the liver of fish. The mechanism underlying the

capacity for one CYP2D6 substrate to be metabolized while the other is not is intriguing.

In chapter 4, we extend beyond catalytic assays traditionally used to assess CYP function and investigate the *in vitro* hepatic metabolism of fluoxetine in a number of fish species. Here, we see minimal metabolism compared to mammalian controls and high species and sample variability. Rather than a kinetic, fluorometric assay, as was used in identifying metabolism in chapters 2 and 3, here we measure metabolism using LC-MC, measuring both substrate (fluoxetine) and product (norfluoxetine). The benefit to this assay is we are able to see a decrease in our substrate without formation of the product. This is more time-consuming and expensive but we are able to see a decrease in fluoxetine over time. Hepatic metabolism of fluoxetine in fish may involve the production of a different fluoxetine metabolite than mammals and that could account for the loss of fluoxetine without detection of norfluoxetine in most untreated fish samples. It is likely that multiple CYPs are responsible for the metabolism of fluoxetine in fish liver. Several CYP proteins are responsible for the metabolism of fluoxetine in mammals and it would not be unreasonable to predict the same to be true in fish. Identifying which CYPs are dominant in this process will be more difficult to determine, particularly since the primary metabolite of fluoxetine in fish is unknown and unlikely to be norfluoxetine.

5.2 Induction of CYP-mediated reactions

Induction of CYP1A is regulated via the arylhydrocarbon (AhR) receptor, a cytosolic transcription factor that binds compounds like PAHs, PCBs and other dioxin-

like molecules. Once bound, the AhR-complex translocates into the nucleus, dimerizes with ARNT (AhR nuclear translocator) and binds to the XRE (xenobiotic response element), initiating transcription of the target gene (Beischlag *et al.* 2008). 3-MC and BNF are AhR agonists known to induce CYP1A in vertebrates. In chapter 2, we assess BNF induction using a suite of CYP-catalyzed assays and find increases suggestive of induction beyond just CYP1A. As discussed, EROD activity is most commonly used in assessing PAH-type contamination.

Structurally diverse xenobiotics and pharmaceuticals are known to alter CYP3A gene transcription levels in mammals by binding to nuclear receptors like the pregnane X receptor (PXR) or the constitutive androstane receptor (CAR) (Moore *et al.* 2002). A functional CAR has yet to be found in fish, although a single gene, similar to both PXR and CAR, was found in the pufferfish genome, possibly an indication that CAR evolved after the divergence of higher vertebrates, or has been lots in some or all fish (Moore *et al.* 2002; Maglich *et al.* 2003; Moore *et al.* 2003). Although a functional PXR has been identified in fish, few typical mammalian PXR agonists have been found to activate the ligand-binding domain (Moore *et al.* 2002). The ligands that have been shown to bind to the fish PXR do not generally produce large increases in functional proteins. In chapter 2, we investigated three pharmaceuticals which are PXR ligands in mammals. Consistent with some previous studies, no large changes in CYP activity were seen in rainbow trout or killifish liver microsomes. Overall, fish appear to be considerably less sensitive to typical mammalian PXR agonists. Perhaps expression of CYP3A in fish is regulated by a

different mechanism altogether.

Phenobarbital (PB) is a strong inducer of CYP2s and 3s in mammals, in particular CYP2B, with regulation via CAR (Waxman and Azaroff 1992). PROD activity is generally associated with CYP2B-like, or PB-like induction in mammals (Foster *et al.* 1998). Fish do no have CYP2B orthologs and PB has not been shown to induce PROD activity in fish. PROD activity is generally very low or non-detectable in unexposed fish liver microsomes but is inducible by other compounds (Haasch *et al.* 1994; Parente *et al.* 2004). In chapter 2 we see PROD induced by BNF in trout. In addition to PROD induction, other mammalian CYP2 substrates were also induced by BNF in our study, namely MAMC, MFC, and CEC (although CEC is metabolized by CYP1A2 in mammals as well). Although our knowledge of CYP2 function and regulation in fish is scarce, it could be postulated that in fish, the XREs associated with AhR-mediated regulation are upstream of more CYPs than previously thought, leading to induction of CYPs outside of typical CYP1A when exposed to agonists like BNF.

5.3 CYP inhibition

In chapter 3 we investigated the potential for fluoxetine, gemfibrozil, ciprofloxacin and erythromycin to inhibit CYP-catalyzed reactions in fish liver microsomes. The inhibitors selected for this study encompass inhibition of the important drug-metabolizing CYPs in mammals: CYPs 3A4, 2D6, 2C9 and 1A2. With the exception of BFC, the lowest inhibitor concentration, 10 μM, did not significantly inhibit the majority of reactions in undosed fish; 100 μM is likely orders of magnitude higher

than what would be found in the environment. In collaboration with the metabolism and induction patterns seen in chapter 2, this inhibition study helps give insight into the modulation of fish CYPs and differences compared to typical mammalian CYPs responsible for the metabolism of the fluorescent CYP-catalyzed assays.

In chapter 3, we investigated the type of inhibition caused by fluoxetine using Lineweaver-Burk plots. This characterization was assessed for undosed and BNF-treated (50 mg kg^{-1}) fish. In doing such investigation, low (10 mg kg^{-1}) BNF-treated fish was also plotted on Lineweaver-Burk graphs for characterization of inhibition by fluoxetine (data not shown). An interesting pattern was encountered in the low BNF dosed-fish for 7-ER and BQ. The point on the graph at which a normal enzyme (no inhibitor) will intersect with the inhibited enzyme can help determine the type of interaction (see Appendix). For these assays, the control reaction (no inhibitor) did not intersect with 100 μM fluoxetine in a way that clearly showed such characterization. However, when reactions run with 10 μM fluoxetine were plotted against 100 μM fluoxetine, the resulting intersection was characteristic of competitive-type inhibition. This suggests that 10 μM fluoxetine inhibits some, but not all CYPs responsible for 7-ER and BQ metabolism and that these remaining enzymes experience competitive-type inhibition with 100 μM fluoxetine in the well. This pattern also occurred in control fish metabolizing 7-BR. Interpretation of these patterns is difficult without an understanding of which CYPs are specifically induced by BNF, those inhibited by fluoxetine or the CYPs responsible for the metabolism of these substrates but provides evidence of

complex interactions amongst these variables.

5.4 Species comparison

A central theme over the last three chapters is looking at similarities and differences in metabolism, induction and inhibition across a number of fish species. Rainbow trout represent a large, freshwater salmonid species. Zebrafish and goldfish are both freshwater cyprinids, but live in tropical and temperate waters, respectively. Killifish belong to the family Fundulidae and are a common marine species used for our comparison. Collectively, these species represent commonly used laboratory fish. By testing catalytic activity in a number of fish species, we can more realistically study CYPs within the fish lineage as a whole, taking into account species variability in the interpretation of our data. In chapter 4, intrinsic fluoxetine metabolism was investigated over the four aforementioned fish species. In this study we see not only inter-specific variability but individual variability as well. Individual variability has been noted in populations of *Fundulus heteroclitus*, with evolutionarily adaptive changes in gene expression thought to account for much of this variability (Oleksiak *et al.* 2005; Crawford and Oleksiak 2007). In chapter 4, some species appeared to have higher rates of metabolism of fluoxetine compared to others. Killifish displayed perhaps greater loss of fluoxetine and rainbow trout appeared to have much lower capacity for fluoxetine metabolism than the other species tested. We see this difference in chapter 2 as well, with killifish metabolizing the majority of substrates better than trout. Such differences have been noted across mammalian species (Walker 1978), but similar characterization

and comparison is lacking for fish. If adaptive changes can be seen amongst individuals within fish populations (Oleksiak *et al.* 2005; Crawford and Oleksiak 2007), inter-specific variation merits similar attention.

5.5 Revisiting research questions and hypotheses

The goals of this project were to further our understanding of fish cytochrome P450s with respect to pharmaceutical contamination. In Chapter 2, my first hypothesis was that DEX, PCN and RIF, would induce the metabolism of typical mammalian CYP2 and CYP3 substrates in fish. Second, I hypothesized that BNF would induce the metabolism of CYP1 substrates in fish. Neither of these hypotheses was fully supported by the data. Although BNF did induce the metabolism of typical CYP1 substrates (namely EROD and MROD), it also induced the metabolism of nearly every other substrate tested. DEX, PCN and RIF did not prove successful in the broad induction of CYP3 metabolism in fish.

In chapter 3, it was hypothesized that we would see inhibition of CYP-mediated reactions using fish liver microsomes. Specifically, ciprofloxacin would inhibit reactions catalyzed by CYP1A, gemfibrozil would inhibit typical mammalian CYP2 substrates; erythromycin would inhibit CYP3A-catalyzed metabolism and fluoxetine would inhibit more broadly, both CYP2 and CYP3 substrates. Results did not support the CYP-selective inhibition predicted. Activation of the metabolism of many substrates was seen by all inhibitors. The inhibition seen across known mammalian CYP-mediated reactions was not what was predicted based on mammalian data.

Lastly, in chapter 4, it was hypothesized that we would see *in vitro* metabolism of fluoxetine and production of the demethylated metabolite, norfluoxetine. With the decrease in fluoxetine over time, we did not see the predicated appearance of norfluoxetine across fish species, suggesting the possibility of another fluoxetine metabolite in fish. We also predicted that species-differences would occur in baseline fluoxetine metabolism across fish. This may be true, and is evident in the metabolism of other substrates (chapter 2), but unpredicted individual variability was found in fluoxetine metabolism across fish samples.

5.6 Future directions

The present research provides evidence that there are still many unknown aspects of CYP function in fish. Development of appropriate CYP antibodies would be useful in determining specific changes in protein expression in fish for comparison to the catalytic rates of substrate metabolism, particularly after induction by chemical exposure. In addition, expressed CYP proteins would be useful in assessing the metabolism of substrates, such as those used here, and the potential for compounds to inhibit fish CYPs. We need a better understand of basic CYP function and modulation in fish in order to determine environmental implications of pharmaceutical contamination with respect to such changes.

In continuation of this research, the first short term goals would be to use the zebrafish expressed CYP1 proteins to assess inhibition by our selected inhibitors on the remaining substrates. So far only the AROD substrates have been investigated with

respect to this inhibition. Second, BNF appears to induce CYPs outside of the predicted CYP1s. This may be a candidate for investigating the possible induction of fluoxetine metabolism in fish. Third, fish CYP1A and 1C1 antibodies are available and could prove useful in assessing whether these CYPs are being induced by the compounds tested in this study. A long-term future direction that may prove more difficult is characterization of CYP-specific assays for fish. Expressed fish CYP1, CYP2 and CYP3 proteins would likely be useful in such investigation. This would prove extremely useful for high-throughput assessment of induction and inhibition of fish CYPs.

5.7 References

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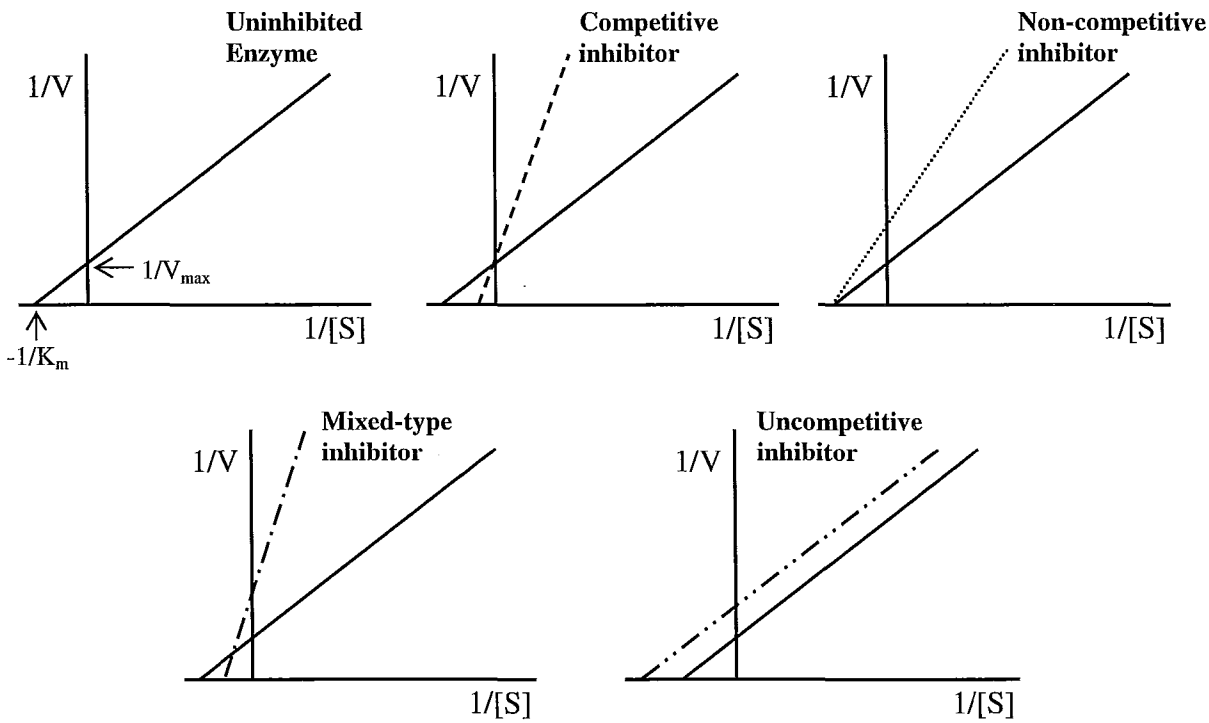
APPENDIX



Lineweaver-Burke plots showing characterization of different times of inhibition. Competitive inhibition will show intersection at $1/V_{max}$; non-competitive inhibition will show intersection at $-1/K_m$; mixed-type inhibition will intersect between these. Uncompetitive inhibition will show parallel lines which do not