EXPRESSION OF CYTOCHROME P450 3C AND 3B GENES IN TELEOSTS

EXPRESSION OF CYTOCHROME P450 3C and 3B FAMILY GENES IN MODEL FISH SPECIES

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ABSTRACT

Cytochrome P450s (CYPs) are enzymes that are found throughout the three domains of life. They function in the metabolism of endogenous and exogenous compounds. CYPs are extensively studied in mammalian systems due to their importance in drug metabolism. Mammals have only one CYP3 subfamily, subfamily A, and are known by their ability to metabolize over 50% of human pharmaceuticals. As such, they are highly expressed in detoxification organs like the liver and intestine. Fish CYP3s are not well understood. CYP3s have diversified in fish and subfamilies A, B, C and D constitute the CYP3 clade in fish. In this study, CYP3C1, CYP3C2, CYP3C3 and CYP3C4 in zebrafish (*Danio rerio*) and CYP3B4, CYP3B5 and CYP3B6 in medaka (*Orzyias latipes*) were quantified in hepatic and extrahepatic organs. CYP3C genes were quantified throughout development. All CYP3B and 3C isoforms were detected in all organs except CYP3B4 in male organs and in female brain. CYP3C1-C3 were maternally acquired and expressed in all embryonic stages. Higher expression of some of the isoforms occurred in the liver and intestine of zebrafish and medaka. This is indicative of a possible role in xenobiotic metabolism. Most interestingly, expression of CYP3C1 and CYP3B5 was high in the olfactory rosette, an organ that is required to sense odours to mediate proper behaviors involved in breeding, feeding, and migration. Differences in expression between males and females gonad was observed, suggesting a possible role for estrogen in gene regulation. Further research will contribute to characterizing the upstream response elements in order to understand whether estrogens or other compounds are responsible for CYP3 regulation in fish. To the best of our knowledge,

this study is the first to quantify the expression of CYP3C and CYP3B genes in different organs and throughout development. This knowledge will contribute to understanding the potential function these unique families of CYPs serve for fish.

ABBREVIATIONS

AHR – Aryl hydrocarbon receptor

BaP – Benzo[a]pyrene

BFC – Benzyloxy – 4 – (trifluoromethyl) - coumarin

BQ – 7- Benzyloxyquinoline

CAR – Constitutive androstane receptor

CT – Cycle threshold

CYPs – Cytochrome P450s

EF1-α – Elongation factor alpha

ER – Estrogen receptor

ERE – Estrogen response element

EROD – Ethoxyresorufin-O-deethylase

EST – Expressed sequence tag

EF1-α – Elongation factor alpha

hpf – Hours post fertilization

Olf. Ros. – Olfactory rosette

PAHs – Polycyclic aromatic hydrocarbon

PCB – Polychlorinated biphenyls

PXR – Pregane X receptor

qPCR – Quantitative polymerase chain reaction

RT-PCR – Reverse transcriptase polymerase chain reaction

RPL-7 – Ribosomal protein L7

RXR – retinoid X receptor

TCDD – 2,3,7,8 - tetrachlorodibenxodioxin

VDR – Vitamin D receptor

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 2, Tables

LIST OF FIGURES

Chapter 2, Figures

Chapter 2, Supplementary Figures

CHAPTER 1:

GENERAL INTRODUCTION

Cytochrome P450 Enzymes

Cytochrome P450 enzymes (CYPs) are a superfamily of heme proteins found in all domains of life. They are critical for the synthesis and metabolism of a number of important biological signaling molecules including steroidal hormones, prostaglandins and fatty acids and are critical for xenobiotic metabolism. CYPs catalyze a monooxygenase reaction that promotes substrate hydroxylation. CYPs contain an active site composed of a heme group. Each individual substrate (RH) binds in close proximity to the heme group. Once docked, the substrate induces a structural change in the active site that allows for the transfer of an electron from NAD(P)H (Denisov et al., 2005). The charge on the iron is reduced from a ferric (Fe^{2+}) to ferrous (Fe^{3+}) state, favouring the recruitment of an oxygen molecule to form a complex with the substrate ((Fe2+-02 (RH); Guengerich, 2001). The Fe^{2+}-0_2 complex is reduced by the addition of second electron from NADPH. This event favours the recruitment of two protons. As a result, the oxygen bond is broken to form a P450 compound 1 complex ($(Fe\text{-}O)^{3+}$) with the RH and water is released (Denisov et al., 2005). The oxygen on the P450 compound 1 complex is transferred to the substrate, resulting in the release of a hydroxylated product (ROH).

When improper docking of a substrate occurs, the transfer of electrons from NADPH to the CYP active site occurs but without the production of a hydroxylated product. Instead the reaction results in an uncoupling event and the formation of reactive oxygen species

such as superoxides and peroxides (Guengerich, 2001). An abundance of reactive oxygen species indicates a strong uncoupled reaction and no product yield.

Methods of Measuring CYP Enzymatic Activity

The method of measuring CYP activity is highly dependent on the CYP and substrate being tested. For example, a fluorescent catalytic assay is commonly used to measure CYP metabolic activity. The fluorescent catalytic assay involves incubating the enzyme of interest, a tissue fraction, or cells, with a substrate whose product is fluorescent. The rate of conversion of a substrate into a fluorescent product can be easily measured (Scornaienchi et al., 2010b). However, this method depends highly on whether or not a fluorogenic substrate exists for the CYP being studied and the specificity of the substrate for a single CYP. In the case of certain CYP isoforms, specific flourogenic substrates are not known or do not exist.

Analytical approaches (e.g. mass spectrometry and/or gas chromatography) allow the direct detection of substrate consumption or product production by the isoform of interest (Malins et al., 2006). This method can be tedious, long and very expensive but can provide detailed information on multiple product formation. For example, CYP1 enzymes can produce multiple products (BaP-1,6-dione, BaP-3,6-dione, 3-hydroxy BaP) during the metabolism of the substrate benzo[a]pyrene (BaP) (Scornaienchi et al., 2010b). Analytical approaches demand a unique assay that must be adequately optimized to fit the individual substrate or product(s) tested. Alternatively, the co-factor NADPH (and NADP+ product) may be detected with UV-VIS spectrophotometry as a generic assay for

any CYP/substrate combination (Harskamp et al., 2012). The rate of metabolism can be determined by measuring the amount of NADPH consumed by the enzyme when exposed to a certain substrate (Gorsky et al., 1984). However, because there is NADPH consumption without product formation during uncoupled reactions, this method could give false positives. In order to differentiate between coupled and uncoupled reactions, fluorescent probes (e.g. dichlorofluorescein) that are sensitive to peroxides can be utilized to measure the amount of reactive oxygen species produced (Harskamp et al., 2012). Reactions with substrate metabolism should produce high NADPH consumption and low reactive oxygen species production (Harskamp et al., 2012).

Cytochrome P450 Nomenclature

Cytochrome P450s have been named based on their location in the cell and their ability to absorb light at a wavelength of 450nm when bound to carbon monoxide. The CYP Nomenclature Committee devised a naming system to name each CYP by amino acid sequence similarity. When naming individual genes, the word "cytochrome P450" is abbreviated to "CYP". Using CYP3C1 as an example, a number is given to signify the gene family (3), followed by a capital letter to signify the subfamily (C) and another number to differentiate between different isoforms (1). CYPs with more than 40% similarity in amino acid sequence are grouped into the same family. CYPs with more than 55% similarity in amino acid sequence are grouped into the same subfamily. Though this nomenclature system is typically adequate to reflect the relationship within families, it fails to portray the evolutionary relationship among families (McArthur et al., 2003).

Most phylogenetic studies have agreed and supported this nomenclature approach (Goldstone et al., 2010; Nelson et al., 2004), with a few important exceptions (Wassmur et al., 2010; Yan and Cai, 2010).

Expression Patterns of CYPs

Cytochrome P450s are found through out the organism, with some isoforms more abundant in certain tissues than others. The common sites of high CYP expression are the liver and the small intestine but expression is seen in many other organs as well (Ding and Kaminsky, 2003).

The sites of location in which a certain isoform are present can be indicative of its function. The enzymes can play a role in the 'defensome' (Goldstone et al., 2006) by metabolizing exogenous compounds when expressed in organs like the liver, gastrointestinal tract or kidney (Carriere et al., 2001; Ioannides and Parke, 1990; Pelkonen et al., 1986). Although CYP subfamilies can be composed of several closely related isoforms, yet, expression patterns can differ. For example, the human CYP3As consist of four isoforms; CYP3A43 (Domanski et al., 2001; Gellner et al., 2001; Westlind et al., 2001), CYP3A4, CYP3A5, CYP3A7 (Thummel and Wilkinson, 1998). The human CYP3A genes are highly similar in sequence but can vary in function and location. Of the three CYP3As, CYP3A4 is most abundant in the adult human liver, accounting for 30% of all total P450s in the liver (Shimada et al., 1994) and is also expressed in the gut (Kolars et al., 1994; Watkins et al., 1987). This CYP plays a critical role in drug metabolism (Guengerich, 1999). CYP3A7 appears only in the fetal liver, the adult

endometrium and placenta (Schuetz et al., 1993). CYP3A5 has polymorphic expression, with the protein being expressed in the liver and kidney of only some humans (Guengerich, 1999). CYP3A5 does contributes to drug metabolism in humans but not to the same extent as CYP3A4 (Williams et al., 2003). CYP3A5 is the predominant CYP3A isoform in the human lung (Anttila et al., 1997; Kivistö et al., 1996). Lastly, CYP3A43 is expressed in very low levels in the liver and assumed to have no function (Westlind et al., 2001).

The CYP1A subfamily consists of two mammalian isoforms, CYP1A1 and CYP1A2. These two members have an approximately 70% amino acid identity and differing expression patterns (Guengerich et al., 1995). The appearance of these isoforms in their specific organs is indicative of their function. Both CYP1A1 and CYP1A2 appear in the liver, but CYP1A2 is more abundant (Zanger and Schwab, 2013) and it is responsible for metabolizing and activating 20% of pharmaceuticals (Wang and Zhou, 2009). CYP1A1 is detected in small amounts in the liver but appears in extrahepatic tissues, such as the intestine, and acts as a detoxification system by clearing out harmful substances (Ding and Kaminsky, 2003; Uno et al., 2004; Zanger and Schwab, 2013). CYP1A1 is highly inducible in liver and plays an important role in metabolism of polycyclic aromatic hydrocarbons (PAHs) (Drahushuk et al., 1998; Eltom and Schwark, 1999; Schulz et al., 1996). Fish only have one CYP1A gene, named CYP1A1. CYP1A1 is used as a biomarker for aquatic pollution as its abundant expression in the liver, gut and gill is due to its defense mechanism against PAHs and dioxin-like compounds (Hahn and Stegeman, 1994; Husøy et al., 1996; Jönsson et al., 2007; Van Veld et al., 1988). Matsuo

et al. (2008) demonstrated the expression of CYP1A as well as CYP2K1, CYP2M1, and CYP3A2 in the olfactory rosettes and gills of rainbow trout (*Salmo gairdneri*), important uptake sites for water contaminants (Scott et al., 2003), and suggested a role for all four CYPs in xenobiotic metabolism.

Some CYPs are more critical for catabolism and anabolism of endogenous compounds than exogenous compounds. The expression pattern of CYPs involved in regulation of important biological signaling molecules are often more restricted or in organs that do not play a role in xenobiotic metabolism such as brain and gonads. For example, CYP3A5 is expressed in the prostate and has been shown to play a critical role in steroidal metabolism (Finnström et al., 2001; Koch et al., 2002; Yamakoshi et al., 1999). CYP3A43 shares a >71% amino acid identity with the other human CYP3As, but it is expressed in the testis and liver was shown to metabolize testosterone and contribute very little to drug metabolism (Domanski et al., 2001; Westlind et al., 2001; Zeigler-Johnson et al., 2004). CYP19 catalyzes estrogen synthesis and the enzyme is expressed in the brain, gonads and placenta (Simpson, 2004). Zebrafish CYP19 is expressed in the vitellogenic follicles in the ovary, hypothalamus and telencephalon of the brain and plays an important role in producing estrogens from androgens for reproductive and developmental purposes (Chiang et al., 2001; Simpson, 2004). Understanding where and in what quantity CYPs are expressed can provide some insight on the function of the individual CYP.

Estrogen Metabolism and Estrogen Response Elements

Estrogen is an important hormone for many species. It is involved in sexual maturation, female sexual differentiation and other processes (Tsuchiya et al., 2005). The actions it is able to play are dependent on estrogen interaction with two estrogen receptors (Gustafsson, 2003; Matthews and Gustafsson, 2003; Tsuchiya et al., 2005). Its metabolism and regulation must be well controlled in order to ensure proper function. In mammals, CYP1A/B and CYP3A and CYP19 play critical roles in estrogen regulation (Tsuchiya et al., 2005). CYP19 produces estradiol; an important biological signaling molecule which transcriptionally activates a large suite of genes (Tsuchiya et al., 2005). Estradiol is converted to inactive metabolites by CYP1A/B and CYP3A that can be easily excreted in urine or feces (Tsuchiya et al., 2005). CYP1A and CYP3A biotransform most of the estradiol in the liver to 2-hydroxyestradiol and 4-hydroxyestradiol, in an approximate ratio of 4:1 (Tsuchiya et al., 2005). Induction studies by 2,3,7,8-tetrachlorodibenzodioxin (TCDD) have suggested that CYP1A/B mediated estradiol metabolism was predominately regulated by the aryl hydrocarbon receptor (AHR) pathway (Spink et al., 1998; Tsuchiya et al., 2005; Whitlock Jr, 1999). The metabolism of estradiol by CYP3A is most likely regulated by nuclear receptors such as the pregnane X receptor (PXR) (Goodwin et al., 2002; Tsuchiya et al., 2005), at least in mammals. However, some research suggests that CYP1B1, expressed in estrogen target tissues, is induced by estrogen through an estrogen receptor (ER) (Tsuchiya et al., 2004). An estrogen response element (ERE) was identified upstream to the CYP1B1 gene and the binding of ER to the ERE caused increased CYP1B1 mRNA expression (Tsuchiya et al.,

2004). Thus, estrogen regulation in mammals appears to be mediated by multiple CYP isoforms, whose expression can be altered by a suite of nuclear receptors.

Estrogen regulation in fish is not as well characterized as in mammals. Though the liver is the predominant site of estrogen metabolism, many other organs have been shown to contribute to estrogen regulation and metabolism, such as the brain, gonads and gill (Butala et al., 2004; Callard et al., 2001). Like in mammalian CYP1A, teleost CYP1A1 is one of the important estrogen metabolizing CYPs. CYP-mediated 17β-estradiol metabolism in teleosts, such as white flounder (*Pseudopleuronectes americanus*) and scup (*Stenotomus chrysops*) has been observed, favouring the formation of 2-hydroxyestradiol (Snowberger and Stegeman, 1987). Like the mammalian CYP1A, zebrafish CYP1A also produce greater levels of 2-hydroxyestradiol than 4-hydroxyestradiol (Scornaienchi et al., 2010a). However, unlike mammalian CYP3A4, zebrafish CYP3A65 showed lower estradiol metabolism compared to its mammalian counterpart (Scornaienchi et al., 2010a). The current research suggests that CYP1A may be a dominant estrogen metabolizer in the zebrafish liver (Scornaienchi et al., 2010a). Current research suggests that regulation of fish CYP1A seems to be controlled by the aryl hydrocarbon receptor (Billiard et al., 2006; Hahn et al., 1994).

Estrogen production in teleosts is catalyzed by two aromatase genes, CYP19a and CYP19b. Fish CYP19a is mostly expressed in the ovary while CYP19b is expressed in the brain (Callard et al., 2001). Estrogens are produced from androgens and interact with estrogen receptors, which cause transcriptional upregulation of genes after the ligand activated ER has bound to an ERE (Matthews and Gustafsson, 2003), as expected based

on mammalian studies. The CYP19b promoter site consists of a two ERE's, an ERE half site (ERE1/2), a TATA box and a nerve growth factor inducible-B protein responsive element and gene expression was induced with exposure to estrogen (Callard et al., 2001). No EREs were found upstream to CYP19a (Callard et al., 2001) and gene induction of zebrafish CYP19a by estrogen is very small (Cheshenko et al., 2007).

Cytochrome P450s, Family 3

The CYP 3 gene family (CYP3s) contains multiple subfamilies but only one subfamily, CYP3A, is found in mammals (Qiu et al., 2008). CYP3A enzymes are characterized by a flexible active site that is able to bind several compounds with no structural similarity (Ekroos and Sjögren, 2006). CYP3As are important for the synthesis, metabolism and catabolism of hormones (Burk and Wojnowski, 2004). The flexible active site of CYP3A4 allows it to metabolize over 50% of pharmaceutical drugs in humans, making it one of the most important human CYPs (Guengerich, 1999). CY3A4 is a major enzyme involved in human Phase I metabolism and highly expressed in the liver and the intestine (Burk and Wojnowski, 2004). In Phase 1 metabolism, CYPs are involved in changing a substrate, usually a drug, into an active or inactive form by adding an oxygen atom to it. Due to the importance of this enzyme subfamily in drug metabolism, CYP3As dominates as the most studied of the CYPs in humans.

Unlike mammalian CYP3s, the CYP family 3 genes in fish are from four subfamilies: CYP3A, CYP3B, CYP3C and CYP3D (Yan and Cai, 2010). CYP3A isoforms exist in several different species across the vertebrate lineage, however, there is

usually only one CYP3A isoform identified for every fish species. CYP3B, CYP3C and CYP3D genes are lineage specific and have only been identified in teleost fish. CYP3Cs, which have four isoforms, have been found in zebrafish (Yan and Cai, 2010). To date, there are seven CYP3B isoforms identified in teleost fish, four of which (CYP3B3-CYP3B6) occur in medaka (Yan and Cai, 2010). Lastly, only one CYP3D isoform is known (CYP3D1) and occurs in a few fish species (e.g., stickleback (*Gasterosteus aculeautus)*, tiger pufferfish (*Fugu rubripes)*, spotted green puffer (*Tetraodon nigroviridis*) but was not found in the genomes of medaka or zebrafish (Yan and Cai, 2010).

One of the first CYP3 genes in fish was found in Atlantic cod (*Gadus morhua*) and expression patterns of the gene bore some similarities to the mammalian CYP3As genes (Husoy et al., 1994). However, differences between mammalian CYP3s and fish CYP3s became apparent with the characterization of the zebrafish CYP3A65 (Tseng et al., 2005). Like the mammalian CYP3A, high expression was found in the liver and gut (Tseng et al. 2005). Expression of CYP3A65 was induced by mammalian CYP3A inducers, such as dexamethasone and rifampicin, known inducers the PXR pathway (Goodwin et al., 2002; Tseng et al., 2005). Unlike the mammalian CYP3A4, the zebrafish CYP3A65 was also induced by TCDD, implying that its regulation was dependent on the AHR pathway (Chang et al., 2013; Tseng et al., 2005). Results from knocking down PXR and AHR expression individually suggested that CYP3A65 regulation is dependent on both pathways (Chang et al., 2013).

Heterologously expressed CYP3A65 was tested for the capacity to metabolize 7-

benzyloxy-4-(trifluoromethyl)-coumarin (BFC) and 7-benzyloxyquinoline (BQ), which are considered specific substrates of mammalian CYP3A activity (Scornaienchi et al., 2010b). However, CYP3A65 did not metabolize BFC as effectively as the mammalian CYP3A4 and CYP3A65 failed to metabolize BQ altogether (Scornaienchi et al., 2010b). Smith et al. (2010) investigated BQ metabolism in fish liver microsomes and found that the liver was capable of BQ metabolism but concluded that the metabolism was not mediated by the same CYP that metabolizes BFC. These studies suggest that BFC is not the specific CYP3A substrate they are in mammals and that fish CYP3A may not metabolize BQ at all.

There is evidence for differences in regulation and expression within the four CYP3 subfamilies in fish and with the CYP3A in mammals. For example, unlike CYP3A65 and CYP3A4, CYP3C1 expression was not induced by rifampicin, dexamethasone or by TCDD, which suggests the PXR and AHR pathway are unlikely involved in regulation of CYP3C1 (Corley-Smith et al., 2006). The expression of CYP3C1 was high in a wide range of organs with comparably high expression in the liver, intestine and ovary (Corley-Smith et al., 2006).. The expression patterns during embryonic development were different than those observed for CYP3A65. Expression of CYP3C1 was not seen in the gut until 120 hours post fertilization (hpf) but CYP3A65 was detected much earlier (Corley-Smith et al., 2006; Tseng et al., 2005). The contrasting characteristics between the two genes suggest varying functions. There is little insight into the expression or function of CYP3B and CYP3D genes (which are products of genomic assemblies) as no experimental testing has been done on these genes.

Evolution of CYP3s

According to the available data, it is believed the common ancestor of the CYP3s diverged from other CYPs approximately 800 million years ago (Gonzalez, 1990). As mentioned previously, the CYP3 family consists of CYP3A-D, where CYP3A occurs in most vertebrate species and CYP3B, CYP3C, and CYP3D likely occur only in fish (Yan and Cai, 2010).

The evolution and function of the CYP3As is extensively studied and well understood in mammals. However, very little information is available on CYP3s in fish, which are the most diversified clade of CYP3s. The teleost CYP3s cluster together and separate from the mammalian and reptilian CYP3s, in a single clade consisting of the fish CYP3A-D genes. The fish CYP3As are more related to the other CYP3 subfamilies within teleost fish than they are to mammalian CYP3As (Yan and Cai, 2010). The phylogenetic data does not support the nomenclature of fish CYP3As; a rare case where phylogeny and nomenclature conventions do not agree (Wassmur et al., 2010; Yan and Cai, 2010). In general, nomenclature in the CYP3 family appears to be problematic. Some genes in the family do not meet the 40% amino acid similarity cut off but in those cases, phylogenetic analyses have identified that they are still more closely related to the CYP3s than any other CYP family (Yan et al. 2010).

Zebrafish and Medaka as Model Organisms for CYP Functional Studies

The zebrafish, a small tropical fish from the Indian rivers, and the medaka, a native fish of Southeast Asia, are popular model organisms in biological studies. Both are

very small animals that can be easily cultured in the lab. They are a favourite for biological research because they have:

- a short generation period (Iwamatsu, 2004; Wixon, 2000)
- external fertilization (Wixon, 2000; Wittbrodt et al., 2002)
- continuous spawning (Tokarz et al., 2013),
- a short developmental time coupled with a clear chorion that allows direct observation of embryogenesis (Wixon, 2000; Wittbrodt et al., 2002),
- a fully sequenced genome, and
- tolerance of a variety of environmental conditions (Abbot, 2013; Tokarz et al., 2013)

Zebrafish and medaka are increasingly valuable for both whole organismal and developmental toxicology (Abbott, 2013; Ankley and Johnson, 2004). Since CYPS are important for both toxicology and steroidogenesis, knowledge of the presence, expression patterns, regulation, and function of CYP genes will be needed in these species. Considering the availability of genomic data for these species, measures of gene expression can be more easily developed and hypotheses regarding regulation of expression raised in these species than other fish species that lack these resources. Both fish are asynchronous breeders and have a very similar and well-understood hypothalamus-pituitary-gonadal axis, allowing for facilitated steroidogenesis studies (Ankley and Johnson, 2004). Both of these species can be manipulated to suit many research purposes making them valuable tools in studying CYP mediated toxicology and steroidogenesis. Since zebrafish and medaka have a different complement of CYP3C

subfamilies (CYP3Cs are in zebrafish and CYP3Bs are in medaka), comparative studies are needed with both species to determine the similarities and differences across these important model species and whether the function of the CYP3C and CYP3B subfamilies are overlapping.

Objectives of This Study

The apparent importance of mammalian CYP3s in drug metabolism and steroid catabolism and anabolism drives my research interest towards understanding the function of the unique CYP3s in fish. It has been assumed that fish CYP3A genes have a similar function to mammalian CYP3A isoforms, yet the biological roles of the unique CYP3 subfamilies in fish is unclear. Understanding the patterns of expression of these enzymes is important in providing insight on the function that CYP3s may play in fish.

The expression pattern of CYP3s in adult organs of zebrafish (CYP3Cs) and medaka (CYP3Bs) was investigated; developmental expression of CYP3Cs was determined in zebrafish. Investigating which organs show high expression of the CYP enzyme might provide some insight on the function of the CYP. For example, high expression in the gill could suggest a role in metabolizing waterborne compounds and high expression in the ovary could suggest a role in metabolizing estrogen. Knowing at which developmental time point expression of a CYP is seen can further provide insight on function. For example, the gene's appearance between 24 hpf to 40 hpf, the time in which gills develop, could indicate the gene's importance in the gill (Kimmel et al., 1995). Overall, the goal of this study is to quantify CYP3 mRNA expression in several

organs and developmental time points, study expression patterns and determine if expression of CYP3cs in zebrafish overlap with expression of CYP3Bs in medaka.

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CHAPTER 2:

Title:

Expression of Cytochrome P450 3C and 3B Family Genes in Model Fish Species

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L.S.: Designed experiment, collected and analyzed data, wrote manuscript

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ABSTRACT

Cytochrome P450 (CYP) enzymes in the CYP3 family are highly expressed in detoxification organs like the liver, intestine and kidney and play a very important role in the metabolism of pharmaceuticals and other xenobiotics. In fish, the CYP3 family is diversified with additional subfamilies, CYP3B, CYP3C, and CYP3D, not found in mammalian species. In this study, the expression patters of novel teleost CYP3 genes were examined in two common model fish species, medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). Primers were designed for zebrafish CYP3C1, CYP3C2, CYP3C3, CYP3C4 and medaka CYP3B4, CYP3B5 and CYP3B6 genes and the expression of each isoform was quantified with quantitative real time PCR in multiple internal organs from male and female adult fish. CYP3C gene expression was determined in zebrafish embryos at several developmental time points. Expression in all organs was detected for all tested isoforms, except for CYP3B4 expression in male organs. CYP3C1, CYP3C3, CYP3B4, CYP3B5, and CYP3B6 were more highly expressed in liver and/or intestine from at least one gender, suggesting a putative role for these genes in xenobiotic metabolism. Interestingly, expression of CYP3C1 and CYP3B5 in olfactory rosette was comparable to the level of liver expression of those genes. Differences in expression between sexes existed for all studied isoforms. Expression of CYP3C1 and CYP3C4 were higher in the female while CYP3C2 and CYP3B5 were higher in the male gonads. CYP3B5 and CYP3B6 expression was generally higher in the female organs than in the male organs. CYP3C1-3 expression was in all the developmental stages examined; CYP3C1, CYP3C2, and CYP3C3 genes appear to be maternally deposited genes as they

are present in the embryo at the 4-8 cell stage. Expression of CYP3C1 and CYP3C3 decreased from the maternal load later in development while CYP3C2 expression remained constant. Overall, the expression of CYP3Cs and CYP3Bs was variable between the two families. The expression pattern suggests that some, but not necessarily all of these CYP isoforms may be involved in xenobiotic metabolism.

INTRODUCTION

Cytochrome P450s are important proteins found throughout all domains of life. CYPs catalyze a monooxygenase reaction and are predominately expressed in liver and intestine, where they play an important role in metabolizing xenobiotic compounds (Hahn and Stegeman, 1994; Van Veld et al., 1988; Wang and Zhou, 2009). However, extrahepatic expression, such as in the brain and gonads, predominates for CYPs with a critical role in steroid production and metabolism (Finnström et al., 2001; Koch et al., 2002; Yamakoshi et al., 1999).

CYP enzymes have a standard nomenclature where all CYPs with 40% and 55% amino acid similarity are grouped into the same gene family or subfamily, respectively (Nelson et al., 2004). While nomenclature is not explicitly based on evolution within the CYP superfamily, phylogenetic analyses have typically (Goldstone et al., 2010; Nelson et al., 2004) but not exclusively (Wassmur et al., 2010; Yan and Cai, 2010) supported the nomenclature system. A notable exception is the CYP3 family, where fish CYP3A genes do not phylogenetically cluster with mammalian CYP3A genes but instead cluster with the CYP3D, CYP3B and CYP3C subfamilies (Wassmur et al., 2010; Yan and Cai, 2010).

In mammals, CYPs in families 1-4 are well studied and are predominately responsible for exogenous compound metabolism. CYP1s are well known to metabolize planar aromatic compounds such as polycyclic aromatic hydrocarbons (PAHs) and polycholorinated biphenyls (PCBs) (Drahushuk et al., 1998; Eltom and Schwark, 1999; Schuetz et al., 1992). CYP1s and CYP2s are also critical in mammalian drug metabolism. CYP1A2 is responsible for metabolizing ~20% of pharmaceuticals in mammals (Wang and Zhou, 2009) and CYP2D6, CYP2C9 and CYP2C19 are capable of metabolizing 15%, 17% and 10% of drugs, respectively (Zanger and Schwab, 2013). One very important family involved in drug metabolism is the CYP3s. For example, the human CYP3A4 enzyme is capable of metabolizing 50% of pharmaceuticals and is highly expressed in the liver and the gut (Guengerich, 2008). CYP3A4 is induced by rimfampicin and dexamethasone, compounds that are capable of activating the pregnane-X-receptor pathway (PXR) (Goodwin et al., 2002). In mammals, CYP3s are only from one subfamiliy, CYP3A. Mammalian CYP3As have a very flexible active site allowing for the docking of many structurally unrelated compounds (Ekroos and Sjögren, 2006).

The evolution of the CYP3 family in vertebrates is quite interesting. Non-mammalian vertebrate species, including fish, have CYP3A genes. Unlike mammals, fish have multiple CYP3 subfamilies, namely the CYP3A, CYP3B, CYP3C and CYP3D subfamilies. Most fish species have one or more CYP3As, while CYP3B, CYP3C and CYP3D genes to date, only appear in certain teleost fish (Yan and Cai, 2010). Both CYP3B1 and CYP3B2 have been reported in fugu (*Fugu rubripes*) (Nelson, 2003; Yan and Cai, 2010) and the spotted green puffer (*Tetraodon nigroviridis*) (Yan and Cai, 2010).

CYP3B3, CYP3B4, CYP3B5, and CYP3B6 were reported in medaka (Yan and Cai, 2010). Zebrafish do not contain CYP3B or CYP3D genes but have four (CYP3C1-4) CYP3C genes (Corley-Smith et al., 2006; Goldstone et al., 2010; Yan and Cai, 2010). CYP3D1 has been reported in fugu and stickleback (*Gasterosteus aculeatus)* (Yan and Cai, 2010). The evolutionary relationships between the teleost CYP3 subfamilies do not agree with the assigned nomenclature (Yan and Cai, 2010). Indeed, teleost CYP3A genes phylogenetically cluster with the CYP3B, CYP3C, and CYP3D subfamilies (Yan and Cai, 2010). Within the fish CYP3 clade, fish CYP3A and CYP3D are more closely related and CYP3B and CYP3C genes cluster together (Yan and Cai, 2010).

CYP3A65 expression and regulation has been characterized in zebrafish (Tseng et al., 2005) and was different from the mammalian CYP3As. Like mammalian CYP3As, CYP3A65 was highly expressed in the liver and gut and the gene was induced by rimfampacin and dexamethasone (Tseng et al., 2005). Unlike mammalian CYP3A4, expression of CYP3A65 was also induced by 2,3,7,8-tetrachlorodibenzodioxin (TCDD), a ligand of the aryl hydrocarbon receptor (AHR) (Tseng et al., 2005). Functional studies of heterologously expressed CYP3A65 have investigated the capacity of this enzyme for metabolism of specific substrates of the mammalian CYP3A4, 7-benxyloxy-4(trifluromethyl)-coumarin (BFC) and 7-benxyloxyquinoline (BQ) (Scornaienchi et al., 2010). CYP3A65 was capable of BFC metabolism but with less specificity than expected; zebrafish CYP1s were also capable of significant BFC metabolism (Scornaienchi et al., 2010). CYP3A65 was unable to metabolize BQ (Scornaienchi et al., 2010) and BFC and BQ metabolism do not appear to correlate in liver microsomes (Smith and Wilson, 2010).

Hepatic studies suggested that perhaps BQ metabolism was mediated by a CYP other than CYP3A (Smith and Wilson, 2010).

Little data are available on the other teleost CYP3 subfamily enzymes. Reverse transcriptase PCR suggested that there was high expression of CYP3C1 in liver and gut, but expression was also detected in other organs such as the eye, gill, heart, ovary and spleen (Corley-Smith et al., 2006). CYP3C1 was not induced by rifampicin, dexamethasone or TCDD, which suggested the regulation of CYP3C1 was distinct from CYP3A65 or CYP3A4 (Corley-Smith et al., 2006). Expression data on CYP3Bs and CYP3Ds are unavailable as these genes are only a product of genomic assemblies.

CYP3A is important for mammalian xenobiotic metabolism is apparent and it is assumed that the CYP3As play a very similar biological role in fish. However, the CYP3 family is diversified in fish and data on expression, regulation or function of these proteins are very limited. This study aims to understand the expression pattern of the novel CYP3C and CYP3B genes in multiple fish organs and the CYP3C genes during fish development. Knowledge of the site of expression may help with understanding the functional role of the CYP. For example, high expression in the liver and intestine usually suggests a role for xenobiotic metabolism. Considering the wide use of both zebrafish and medaka in aquatic toxicology studies, we have characterized the CYP3B (medaka) and CYP3C (zebrafish) complement in each species to compare expression patterns across novel teleost CYP3 subfamilies.

MATERIALS AND METHODS

Fish Care

Zebrafish were maintained in a semi-recirculating housing system with a minimum of 15% daily water replacement and automated dosing with sodium bicarbonate or salts (Instant Ocean, Spectrum Brands, USA) to maintain tank water at 28.5°C, pH 7-8, and 450-470 μS conductivity. The fish were acclimated to a 14:10 light, dark cycle. Fish were fed three times a day; twice with commercial fish food (Tetramin Tropical Flakes, Tetra, USA) and once with live artemia (GSL Brine Shrimp, US). All fish care was provided in accordance to McMaster University's animal care regulations using approved protocols.

Medaka were maintained in a semi-recirculating housing system with a minimum of 15% daily water replacement. Temperature was maintained at 28°C and the salt concentration at 20 ppt. Fish were fed three times a day, with commercial fish food and live artemia. All fish care was in accordance to Trent University's animal care regulations under approved protocols.

Zebrafish embryos were collected through whole tank matings using an embryo trap placed into the tank prior to onset of first light. Embryos were removed from the trap and placed in petri dishes (50 embryos per 100mm dish) with E3 media (5mM NaCl, 0.17mM KCl, 0.33 mM $CaCl_2$ and $MgSO_4$) and developmentally staged under a Zeiss Axiolab microscope (Carl Zeiss, Hallbergmoos, Germany, 100X magnification) according to Kimmel et al., (1995). Embryos were maintained in an incubator at 28.5°C

until they grew to specific developmental stages. Media was replaced as needed after removal of debris and the discarded chorions post-hatching.

Tissue Collection and Analysis

Tissues were dissected from 3 female and 3 male fish per pool (4 pools per gender) to provide tissues for RNA extraction and subsequent PCR for all CYP3 genes. The tissues selected were brain, eye, gill, gonad (testis or ovary), heart, intestine, kidney, liver, olfactory rosettes, and spleen. Organs of interest were chosen based, in part, on an analysis of the expression of CYP3C and CYP3B genes using existing zebrafish and medaka EST (expressed sequence tag) libraries (see below).

Zebrafish embryos were pooled, transferred to a cryovial, all excess E3 media was removed, and the pool was snap frozen in liquid nitrogen and stored at -80°C. Four pools of zebrafish were collected at the 4-8 cell stage (1hpf; 80 embryos/pool), 30-50% epiboly stage (4.7-5 hpf; 100 embryos/pool), 60% somite stage (12 hpf; 100 embryos/pool), prim-6 stage (25hpf; 50 embryos/pool), and hatching stage (48 hpf; 50 embryos/pool).

RNA was extracted and purified using Trizol (Invitrogen, Carlsbad, CA) with two ethanol rinses to ensure clean samples. RNA samples were treated with DNAse I (invitrogen, Carlsbad, CA). 25% of all zebrafish and medaka total RNA samples were assessed for RNA integrity using Agilent's 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) in the Farncombe Metagenomics Facility (McMaster University, Hamilton, ON). RNA quantity and purity were assessed for all RNA samples using a NanoDrop 2000c (Thermo Scientific, Wilmington, DE). Only those samples with a

260/280 ratio greater than 1.8 were used in downstream applications. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, Calrsbad, CA) and treated with RNAse OUT (Invitrogen, Calrsbad, CA) as described by the manufacturer.

Primer Design and Sequencing

Zebrafish (CYP3C) and medaka (CYP3B) sequences were obtained from Genbank and Ensembl, respectively. Table 1 lists the accession numbers and chromosome locations for each of the sequences used for primer design. CYP3C and CYP3B sequences were aligned using Clustal X (Thompson et al., 1997) and unique primers (Table 2) were designed with Primer 3 (Rozen and Skaletsky, 1999), targeting the regions of the genes with the highest sequence dissimilarity across the genes in a given subfamily. Where possible, primers for CYP gene expression were specifically designed for their use in quantitative (or real time) PCR using the following conditions: amplicon size 70-200 base pairs (bp), ~60°C primer annealing temperature and 18-25 bp primer length. CYP3C2, CYP3C3 had primer lengths ranging around 30bp and CYP3B4 yielded a product of 211bp. Housekeeping gene primers for RPL-7 and 18S RNA in medaka were obtained from Zhang and Hu (2007). Zebrafish housekeeping gene primers were from Craig et al., (2007) for EF1-α and from Alsop and Vijayan (2008) for β-actin.

CYP3 and housekeeping genes were amplified from adult organs and embryos using the unique primers (Table 2) and Platinum Taq polymerase (Invitrogen, Calrsbad, CA). The amplified material was run on 1% agarose gel (Sigma-Aldrich, St Louis, MO) and visualized by ethidium bromide (Sigma-Aldrich, St Louis, MO) under UV light. PCR

gel products from the liver and intestine of each CYP3 gene were excised and purified using QAIEX II Gel Extraction Kit (Qiagen, Toronto, ON). Purified DNA amplified by CYP3C4 was ligated into a pGEM-T Easy Vector (Promega, Madison, MI), transformed into *E.coli* JM109 cells and cultured on LB/ampicillin/IPTG/X-Gal plates. White colonies were selected and incubated in LB/Amp media for one day. Plasmids were collected and purified using PureLink Mini Prep Kit (Invitrogen, Carlsbad, CA) and sent for sequencing at Mobix Lab (McMaster University, Hamilton, ON) to confirm that primers were amplifying the gene of interest.

Quantitative PCR

All samples for each primer set used in quantitative PCR (qPCR) included no template (NTC) and no reverse transcriptase (NRT) controls. qPCR reactions were run in duplicate on a Stratagene Mx3000 thermocycler (Agilent Technologies, Santa Clara, CA) using LuminoCT SYBR Green qPCR Ready mix (Sigma-Aldrich, St Louis, MO). Reference genes, β-actin and elongation factor alpha (EF1-α) were used to normalize gene expression of CYP3Cs in zebrafish adult organ and developmental tissue, respectively. The reference gene RPL-7 and 18S RNA was used for normalization of expression of CYP3B genes in medaka adult tissue. Zebrafish embryo qPCR data were also normalized to the total RNA. A 5-8 point standard curve was developed for each qPCR assay based on a dilution of template; the dilution factor was calculated in accordance to the primer's critical threshold value. Template used for the standard curve was from either pooled cDNA from the highest expressing organs (all housekeeping

genes, CYP3C1) or from purified product for each primer set (CYP3C2, CYP3C3, CYP3C4; CYP3B4, CYP3C5, CYP3C6). The standard curves for all genes had an $R^2 > 0.9$ and 90%-110% efficiency (Table 3). All primer sets were optimized for annealing temperature using gradient qPCR, primer concentration and template volume. The resultant amplified product was run on 1% agarose gel and a melting curve analysis was included to analyze primer specificity.

EST Library Searching

Expressed sequence tag (EST) data were retrieved from NCBI's UniGgene (Pontius et al., 2003; Sayers et al., 2010) to determine tissue specificity and potential organs with expression of CYP3B and CYP3C genes in medaka and zebrafish, respectively. The EST libraries were accessed for each CYP3 gene of interest via BLAST on NCBI (Boguski et al., 1993) by accession number or chromosome location and common ESTs were clustered and viewed in Unigene (Pontius et al. 2003). EST data was normalized for the size of the EST library and expressed in transcripts per million and visualized as digital northern blots (EST profiles).

Statistical Analysis

Statistical analyses of gene expression were preformed using SigmaStat Version 12.2 (Systat Software, San Jose, CA) and GraphPad Prism version 5 for Mac OS X (GraphPad Software, San Diego, CA). All data was normalized using a log transformation. A one-way ANOVA and a Fischer LSD test were used to test for

differences in expression between liver and other organs within each gender and across developmental times; statistical tests with $p<0.050$ were considered significant. A two-tailed t-test was used to determine differences in expression between the same organ in male and female and tests with $p<0.050$ were considered significantly different between genders.

RESULTS

Sequence Similarity

CYP3C and CYP3B sequences were aligned and an identity matrix was constructed using the coding sequence of all CYP3C and CYP3B genes. CYP3C sequences shared an amino acid identity of ≥70% between the four isoforms (Table 4A). CYP3C2 and CYP3C3 shared the highest (90.6%) amino acid identity (Table 4A) of any CYP3 genes examined. The amino acid sequence identities between the various CYP3B genes were ~70-75% (Table 4B). Amino acid identity between CYP3C and CYP3B sequences was 50% or more (data not shown).

EST Library Data

The total number of ESTs available for zebrafish and medaka were 1 488 275 and 666 891, respectively. The EST data was normalized by library size and expression was compared across all libraries available for CYP3C1, CYP3C2, CYP3C3 and CYP3C4. CYP3C1 was found in all major organs except the gills (Table 5A), heart, and muscle (data not shown). CYP3C2 and CYP3C3 were only found in the gill and olfactory

rosettes, respectively (Table 5A). CYP3C4 was found in the whole adult library (data not shown) but not in any specific organ library (Table 5A). The expression of the CYP3C genes varied across developmental time points; there was no developmental expression identified for CYP3C2 and CYP3C4 (Table 5B). Interestingly, CYP3C1 expression was found in multiple developmental stages (gastrula, hatching, larval) but not in the egg (Table 5B). It is worth noting that the EST library size was quite variable in zebrafish and was very small (<10 000) for some organs (e.g. bone, intestine and skin had 7629, 4157, and 9394 ESTs, respectively). No ESTs were found for medaka CYP3Bs.

Reverse Transcriptase PCR

Prior to quantitative PCR, RT-PCR was attempted to ensure that the correct product was amplified and to identify which organs expressed CYP3 genes. Considering the lack of CYP3C4 expression in any specific tissue EST library, RT-PCR was meant to confirm if quantitative PCR should be pursued for all genes and all organs. RT-PCR resulted in the amplification of CYP3C1, CYP3C2, CYP3C3 and CYP3C4 from several organs for both the male and the female (Table 6). For females, CYP3C1, CYP3C2, and CYP3C4 were present in all organs except the olfactory rosette; CYP3C3 was seen in all organs except in the gill, heart, and olfactory rosette (Table 6A). For the male organs, very small expression of CYP3C1 was seen in the intestine, testis and kidney; CYP3C4 was observed in all organs except the brain, intestine, and liver (Table 6B). CYP3C2 and CYP3C3 were amplified by RT-PCR in all organs from the male (Table 6B).

RT-PCR resulted in the amplification of very faint bands of CYP3C1-4 in most of the developmental stages (data not shown) but no detectable amplification was seen in the hatching phase for CYP3C1, CYP3C3 or CYP3C4. Amplification of CYP3C2 was not detected at the 4-8 cell stage even after 35 cycles of RT-PCR (data not shown).

Three primer sets were developed for CYP3B3 but the correct product was never amplified. CYP3B3 primers resulted in either nonspecific amplification or amplification of CYP3B5 (data not shown). CYP3B4, CYP3B5 and CYP3B6 were amplified from several male and female organs by RT-PCR (Table 6). Like CYP3Cs, the expression of CYP3Bs varied amongst males and females and between different isoforms, yet each isoform was expressed in multiple organs.

Quantitative PCR

Quantitative PCR assays were developed and optimized for 7 CYP genes (CYP3C1, CYP3C2, CYP3C3, CYP3C4, CYP3B4, CYP3B5, and CYP3B6); the assay performance for each CYP3 gene and the housekeeping genes is shown in Table 3. Additional species-specific housekeeping gene assays were based on published primers (Zhang and Hu, 2007; Craig et al., 2007; Alsop and Vijayan, 2008). RPL-7, β-actin and EF1-α were used to normalize expression in medaka organs, zebrafish organs and zebraffish development, respectively; these housekeeping genes were chosen because they were the most stable genes within these experimental groups. For each quantitative PCR assay, the efficiency was 90-110% with R^2 values for the regression line >0.9 (Table 6). The no template and no reverse transcriptase controls did not show significant

amplification although primer dimers were visible in CYP3B4 no template and no reverse transcriptase control wells. Quantitative PCR data was analyzed in two ways: 1) the level of expression was expressed relative to the liver, and the expression in extra-hepatic organs were compared to liver within a single gender, using a one-way anova (i.e. liver versus extrahepatic organ expression in males or females); 2) the level of expression was expressed relative to the male liver, and the expression between genders was determined for each organ using two-tailed t-test (i.e. male versus female expression). Supplemental figures are provided to show the data and statistics for the first analyses; Figures 1-4 and Figures 6-8 show the expression data relative to male liver only. In several cases (noted below), olfactory rosettes were not included in statistical analyses because of low sample size.

CYP3C Gene Expression in Adult Organs

Quantitative PCR resulted in the amplification of CYP3C1 (Figure 1), CYP3C2 (Figure 2), CYP3C3 (Figure 3) and CYP3C4 (Figure 4) in all of the organs that were investigated. CYP3C1 expression was significantly higher in the ovary and lower in the kidney compared to the expression in the liver of female zebrafish (Figure 1; Supplementary Figure 1A); all other organs had statistically similar expression to the liver. The olfactory rosette was not included in the statistical analysis because of small sample size. In males, CYP3C1 was similar in the liver, eye, testis, kidney and olfactory rosette; the remaining organs had expression that was significantly lower than in the liver (Figure 1; Supplementary Figure 1B). There were significant gender differences in the

expression of CYP3C1 in individual tissues. CYP3C1 expression was significantly higher in liver, eye, and kidney from males compared to females. Thought not statistically tested, there was higher mean expression of CYP3C1 in the male olfactory rosette as opposed to the female. No test for significance could be preformed due to the small n (2) of the female olfactory rosette. The expression of CYP3C1 was higher in gonads and intestine in females than in males (Figure 1).

CYP3C2 was detected in all organs of both male and female zebrafish. CYP3C2 expression was not statistically different across organs in females, although female intestine had the highest mean CYP3C2 expression of any organ in males or females. Variation in many of the female organs, and in particular in intestine, was quite high for CYP3C2 suggesting high inter-pool variation in expression of CYP3C2. In males, the expression of CYP3C2 was higher in the gonads than in the liver and significantly lower expression was observed in the brain, eye, gill and kidney than in the liver (Figure 2, Supplementary Figure 2B). Expression of CYP3C2 in the female heart was significantly higher than CYP3C2 expression in the male heart (Figure 2).

CYP3C3 was detected in several organs of both male and female. Statistical analyses did not include female gonads, olfactory rosette and intestine due to low sample size (n=2). CYP3C3 expression in the other female organs were not statistically different from liver, however the mean CYP3C3 expression in the female appeared to be much higher in the brain and heart (Figure 3; Supplementary Figure 3A). Expression of CYP3C3 in the male intestine was significantly higher than expression in liver (Figure 3; Supplementary Figure 3B). The lowest expression of CYP3C3 in males was seen in the

brain, eye, gill, kidney and spleen (Figure 3; Supplementary Figure 3B). The olfactory rosette was not included in the statistical analyses because this organ had very high variation between samples and cycle threshold (CT) from 2 pools exceeded the working range of 35CTs. Comparing across genders, CYP3C3 expression was higher in the brain and eye from females than in males. Significance was not detected between the intestine of males and females due to the fact that the females intestine had only an n=2.

CYP3C4 expression was statistically higher than liver in the female gonads and heart (Figure 4; Supplementary Figure 4A). No significant differences were seen in the expression level of CYP3C4 between liver and extrahepatic organs in males but the mean expression appeared to be higher in the brain, eye and intestine (Figure 4; Supplementary Figure 4B). Interestingly, there was significantly higher expression in the female gonads than in the male gonads, and male intestine compared to female intestine (Figure 4).

CYP3C Gene Expression in Embryos

Expression of the CYP3C genes in each embryonic stage was relative to the expression of that gene in the 4-8 to cell stage for each isoform (Figure 5). CYP3C1 had a decrease in expression of the gene from that found at the 4-8 cell stage to all other stages. There was a decrease in CYP3C3 expression at the prim-6 stage; expression was similar at all other developmental time points measured. CYP3C2 expression was measurable but similar across developmental stages (Figure 5). CYP3C4 expression was not detectable at any developmental time point (data not shown).

CYP3B Gene Expression in Adult Organs

The primer for CYP3B4 always resulted in the appearance of a primer dimer when expression in a certain organ was low and typically in the no template controls. When template with higher expression of CYP3B4 was present, the primer dimer was not present in the reaction. Expression of CYP3B4 in male organs and female brain was not quantifiable; in all organs there was little amplification and primer dimers dominated the reaction. Expression was detectable and quantifiable in all of the other female organs. The organs with highest CYP3B4 expression appeared to be the liver, intestine and olfactory rosette (Figure 6). Expression of CYP3B4 in the female eye, gill and spleen was significantly lower than expression in the liver (Figure 6).

Within the female medaka organs, CYP3B5 was expressed predominately in the intestine, liver and olfactory rosette while the expression in the brain, gill, gonad, eye, and spleen were significantly lower than liver (Figure 7; Supplementary Figure 5A). The kidney was not included in the statistical analysis due to very high variation between pools. In the male organs, expression was high in the intestine and liver. The gonads had a high mean expression but there was low sample size for this organ (Figure 7; Supplementary Figure 5B). There was little expression detected in the brain, eye, gill, heart and spleen from males (Figure 7; Supplementary Figure 5B). In fact, expression was detectable but not quantifiable due to high CT values. CYP3B5 was expressed more highly in female liver, brain, eye, gill, heart, intestine and spleen than was found in males (Figure 7).

Significantly more CYP3B6 expression was detected in the intestine than in the female liver (Figure 8; Supplementary Figure 6A). Expression in the female olfactory rosette was variable between sampled pools and was not statistically different than in liver. Expression was significantly lower in the female brain, eye, gill, and spleen than the liver (Figure 8; Supplementary Figure 6A). The lowest expression of CYP3B6 in the males was seen in the brain and eye. The gonads and kidney were not statistically different than liver; the kidney had high variation in expression. In general, CYP3B6 was more highly expressed in the female organs than in the male organs. There was a significant gender difference in expression between the male and female liver, brain, eye, gill, heart and intestine (Figure 8).

Relative Expression of Each CYP Isoform

Differences in expression between each isoform were observed; the cycle threshold for each isoform in liver samples is summarized in Table 7. CYP3C1 had the lowest CT in both males and female zebrafish. CYP3C2 had the highest CT value in male and female zebrafish liver. For the CYP3Bs, CYP3B6 had the lowest CT in both the male and female liver.

DISCUSSION

Cytochrome P450 enzymes play important roles in the metabolism of many exogenous and endogenous compounds; CYP families 1-4 are thought to be particularly important in exogenous compound metabolism. The diversification of CYPs is credited to

the changing demands of the environment encountered during the evolutionary history of each species (Ingelman-Sundberg et al., 1999). Some CYP homologs are found across species and function in a similar way. However, some species or taxonomic lineages have unique CYPs that may mediate a novel function. The specific expression pattern of a given CYP may suggest its function. For example, human CYP3As metabolize many pharmaceuticals and, not surprisingly, are most highly expressed in the liver and the gut (Liu et al., 2007). Novel CYP genes have been identified in genome sequencing projects but the function typically remains uncharacterized. In these cases, expression studies may contribute to understanding the function of these novel CYPs.

The CYP3 family has long been known to contain CYP3A genes in teleost fish. Completion of teleost genomes have resulted in the discovery of unique CYP3B, CYP3C and CYP3D subfamilies that have so far only been characterized in teleost fish (Yan and Cai, 2010). Some functional, expression and regulation studies exist for fish CYP3A genes (Celander et al., 1996; Hasselberg et al., 2008; Husoy et al., 1994b; Pascussi et al., 2000; Scornaienchi et al., 2010; Tseng et al., 2005; Uno et al., 2004) but only one study exists for CYP3Cs, and in that study only CYP3C1 is characterized (Corley-Smith et al., 2006). Here we look at CYP3C1-4 expression in zebrafish organs and developmental time points and expression of CYP3B3-6 in medaka organs to determine the expression patterns of the CYP3B and CYP3C genes in teleost fish. To the best of our knowledge, ours is the first study that quantifies the expression of these novel CYP3s in zebrafish and medaka in multiple organs and throughout development.

Expressed Sequence Tags and RT-PCR

The zebrafish genome has some of the larger ESTs library sizes of the teleost fishes with a completed genome and EST data was mined for CYP3C1, CYP3C2, CYP3C3 and CYP3C4 sequences. Medaka, fugu and stickleback had limited EST libraries and no EST data was found for any CYP3B or CYP3D gene. EST data is considered reliable, providing a good insight on relative expression of certain genes, if the library has a minimum of 10 000 sequenced EST (Schmitt et al., 1999). The available CYP3C ESTs suggested that CYP3C1, CYP3C2, CYP3C3 and CYP3C4 were expressed in different organs in the zebrafish adult; with CYP3C1 having the broader expression in many organs (Table 5). EST library data suggested that CYP3C4 had limited expression in most tissues; indeed expression was found in the adult library but no specific organ (Table 5). Yet, RT-PCR suggested that CYP3Cs were not only present in the adult zebrafish but expression of all CYP3C genes was likely in multiple organs and in both males and female fish (Table 6). Many EST organ libraries included CYP3C1 transcripts, but no CYP3C1 expression was found in gill (13152 ESTs, Table 5) and heart (31170 ESTs, data not shown) libraries but expression has been found in both organs with PCR approaches in this study (Table 6, Figure 1, Supplemental Figure 1) and others (Corley-Smith et al., 2006). The conflict between the PCR and EST data suggests that the EST libraries may be too small to produce reliable expression data for this CYP subfamily and that the 10 000 transcript cut off might only apply for more highly expressed genes (Schmitt et al., 1999)

Primer Design and PCR Optimization

The amino acid similarity was high between all of the investigated isoforms (Table 4). We were not able to design unique primers to amplify CYP3B3 but all other primers were shown to amplify the expected target (data not shown). After optimization of quantitative PCR, primer dimers were found in some reactions although only in the no template controls or in organs with very low expression (e.g. CYP3B4). No primer dimers existed in the standard curve using cloned template or in female organs with high CYP3B4 expression, suggesting that the primer dimers were a result of lowly expressed genes and not a problem with primer design or optimization of PCR reaction.

Hepatic Expression of CYP3Cs and CYP3Bs Genes

The liver is one of the body's main detoxification organs and it is not surprising that most drug and other xenobiotic metabolizing CYPs such as CYP1A (Goksøyr and Husøy, 1998; Sarasquete and Segner, 2000) and CYP3As have high expression in the liver (Guengerich, 2008; Tseng et al., 2005; Wrighton and Stevens, 1992; Zanger and Schwab, 2013). The zebrafish EST library size for liver is moderate (17232) and CYP3C1 was sequenced at 116 transcripts per million (Table 5). Corley-Smith et al (2006) showed that CYP3C1 appeared mostly in the liver compared to the other studied organs. We hypothesized the liver would be the site of highest CYP3C and CYP3B expression and choose to show our data relative to hepatic expression. Expression of CYP3C1 (Figure 1) and CYP3C2 (Figure 2) was higher in the male liver, CYP3B4 (Figure 6) was higher in the female liver and CYP3B5 was higher in both male and female liver compared to other

organs (Figure 7). Similarly, studies on CYP3A show the gene to be highly expressed in liver from Atlantic cod (*Gadus Marhua*) (Hasselberg et al., 2008; Husoy et al., 1994b), zebrafish (Bresolin et al., 2005; Tseng et al., 2005), medaka (Kullman and Hinton, 2001) and several other fish species (Christen et al., 2010; Sun et al., 2013). Some functional studies suggest that fish CYP3A interact with and metabolize several xenobiotics (Christen et al., 2010; Hasselberg et al., 2008; Mortensen et al., 2011; Sun et al., 2013; Tseng et al., 2005; Wassmur et al., 2010). The higher expression of CYP3C and CYP3B genes in the zebrafish and medaka liver, like expression of CYP3As in other teleost livers, may be indicative of a role in xenobiotic metabolism. However, it is important to note that though there was higher expression in liver compared to the other organs, the overall expression of these genes was low - moderate with amplification becoming higher than background at an average of about 25 CTs (Table 7). By comparison, the reference gene, 18s RNA was above background at 8-9 CTs (data not shown).

Extrahepatic Expression of CYP3Cs and CYP3Bs

The intestine is another main site for xenobiotic metabolizing enzymes (Kaminsky and Fasco, 1992; Obach et al., 2001). The intestine is a major site of absorption of foodborne xenobiotics (Obach et al., 2001). Many xenobiotic metabolizing CYP enzymes are found in intestinal tissue such as CYP1A1, 1B1, 2C, 2D6, 2E1, 3A4, and 3A5 (Zhang et al., 1999). We hypothesized that the expression of some of these CYP3s would be higher in the intestine. In this study, expression of CYP3C2 (Figure 2) and CYP3B5 (Figure 7) was highest in both the male and female intestine and CYP3C3 (Figure 3),

CYP3C4 (Figure 4) were high in the male intestine, while CYP3B4 (Figure 6) and CYP3B6 (Figure 8) were high in female intestine. In mammals CYPs such as CYP3A and CYP1A are expressed in the intestine and are responsible for xenobiotic metabolism (Kaminsky and Fasco, 1992). CYP3A in fish have been detected in the gut in levels comparable to liver (Hegelund and Celander, 2003; Lee et al., 2012; Tseng et al., 2005; Vaccaro et al., 2007) and some functional studies suggest that intestinal CYP3As are involved in xenobiotic metabolism in fish (James et al., 2005; Lee and Buhler, 2002). CYP1A, an extensively studied CYP that is widely involved in xenobiotic metabolism, is expressed in the intestine of fish (Goksøyr and Husøy, 1998; Hegelund and Celander, 2003; Hong et al., 2007) and the ingestion of xenobiotic results in the induction of CYP1A in the intestine (Liu et al., 2013). The fact that CYP3C2, CYP3B5 and CYP3B6 were found most highly expressed in liver and intestine further supports the notion that these CYP isoforms are likely involved in xenobiotic metabolism.

Expression of xenobiotic metabolizing CYPs, such as CYP1A or CYP3As have been shown to be expressed in other extra hepatic organs including kidney (Ortiz-Delgado et al., 2008), gill (Hegelund and Celander, 2003; Husoy et al., 1994a; Stegeman et al., 1991; Tseng et al., 2005) and olfactory organs (Matsuo et al., 2008; Saucier et al., 1999; Stegeman et al., 1991). Most of these studies suggest that these CYPs are involved in controlling toxicity caused by contaminants such as PCBs, dioxins and others. Studies have shown morphological changes in the eyes, gill and olfactory rosettes of fish exposed to contaminants (Hawkes, 1980). It can be hypothesized that these organs would express cytochrome P450s specialized to combat these pollutants or are otherwise necessary for

adequate function. In this study, expression in these organs was detected above background for nearly all tested isoforms (Figures 1-4, 6-8).

The expression of these genes in the olfactory rosette is most interesting. The olfactory rosettes are small organs and very hard to dissect; several tissues were too small to get sufficient RNA for all assays. Thus, this tissue was not included in all statistical analyses. Yet, CYP3C1 (Figure 1), CYP3B4 (Figure 6) and CYP3B5 (Figure 7) expression in the male olfactory rosette was as high as expression in the liver. CYP3B6 seemed to be highly expressed in the olfactory rosette, though variation between sampled organs was large (Figure 8).

The olfactory rosettes of fish are located on top of the fish's snout, in the nostrils, and are a series of highly folded olfactory epithelia responsible for detecting odours. This mechanism is important for fish mating, feeding, migration and predator avoidance and other behaviours (Tierney et al., 2010). These organs are readily exposed to the environment, making them susceptible to waterborne contaminants (Klaprat et al., 1992; Matsuo et al., 2008). Matsuo et al. (2008) quantified CYPs expressed in the coho salmon (*Oncohynchus kisutch)* olfactory rosette and found a significant amount of CYP3A27 and suggested that the enzyme might be involved in xenobiotic metabolism in this organ. Similarly, CYP1A1 was also detected in the olfactory organ of rainbow trout (*Oncorhynchus mykiss*) and EROD activity was inducible by waterborne β-naphthoflavone and microsomes from the olfactory rosette organ were capable of testosterone hydroxylation (Monod et al., 1994). The authors concluded that the presence of CYPs in the olfactory rosette might be critical for quickly metabolizing and clearing

compounds for detoxification or for normal olfactory functions. Similarly, the presence of several CYPs in the insect antennae has been credited to the need for quick metabolism of both endogenous and exogenous compounds (Cano-Ramirez et al., 2013; Maibeche-Coisne et al., 2005). Like the olfactory rosettes, the antennae are important organs in pheromone sensing, feeding, and predator avoidance (Pottier et al., 2012) and quick cycling of compounds encountered by the antennae is very important to properly maintain those behaviours. Perhaps the CYP3C and CYP3B isoforms play a role in clearing odorants in water from the olfactory rosettes in order to insure adequate function and detoxification in case of environmental contamination.

Gender Differences in Isoform Expression

In this study, expression of CYP3Cs and CYP3Bs was investigated in both genders and there were gender differences in expression between each isoform (Figures 1-4, 6-8). Most notably, expression of CYP3C1 and CYP3C4 was significantly higher in ovary, compared to testis (Figure 1, Figure 4). We hypothesize that this sex difference may be due to the CYP3C's relevance in steroidal metabolism or regulation of the gene by sex steroids, specifically estrogen.

Many mammalian studies have shown a sex specific expression of certain CYPs such as the expression of CYP2C11 in male rats but not in female rats, or the expression of CYP2C12 increases in adult female rats but is suppressed in male rats (Waxman and Chang, 2005). Furthermore, expression of some mammalian CYPs is regulated by testosterone and estrogen (Waxman and Chang, 2005). CYPs that are regulated by

testosterone are predominately male specific CYPs and genes regulated by estrogen are predominately female-specific genes (Waxman and Chang, 2005). Mammalian CYP1A, CYP1B1, CYP3A and CYP19 play an important role in estrogen production and metabolism and the CYP1B1 gene contains EREs that make the gene responsive to estrogen (Tsuchiya et al., 2004). Zebrafish CYP1s and CYP3A65 are involved in estradiol metabolism (Scornaienchi et al., 2010) while CYP19 is responsible for estradiol production (Callard et al., 2001). CYP19 is expressed in the teleost brain and ovary. The brain variant, CYP19b, contains upstream EREs that are responsive to estrogen (Callard et al., 2001). There is evidence for a sex difference in expression of CYP19 in amphibians, where female undifferentiated organs have higher level of CYP19 than males (Oshima et al., 2006). EREs were detected in the 10 kb upstream region of all CYP3C genes using the FIMO algorithm with background nucleotide frequency corrections (A. McArthur, personal communication), supporting our suggestion that CYP3C isoforms may be transcriptionally regulated by estradiol. Investigating upstream factors such as EREs may provide a better idea of whether these CYPs are involved in estrogen metabolism and/or xenobiotic metabolism.

CYP3C Expression Throughout Development

Expression of CYP3Cs was investigated in five stages of zebrafish development. Each stage studied represented a benchmark in development, where important developmental events were represented and easily identified. Expression of CYP3C1, CYP3C2, and CYP3C3 were observed in all stages studied; CYP3C4 expression was

lacking in all developmental stages. Corely-Smith et al (2006) identified embryonic expression of CYP3C1 at 12 -120 hours post fertilization (hpf). Microarray data detected expression of CYP3C1, CYP3C2, and CYP3C3 from 3hpf to 48hpf (Goldstone et al 2010). Similar to our findings, expression was highest early on (3hpf) and fluctuated slightly throughout development for CYP3C1 and CYP3C3 (Figure 5). In the case of CYP3C2, expression levels seemed generally constant through developmental stages (Figure 5), as had been previously suggested (Goldstone et al., 2010). The presence of CYP3C1 and CYP3C3 in very early embryos suggests maternally deposited genes; expression was down regulated later in development. However, in the case of CYP3C2, it appears that there is no change or little change of expression from the 4-8cell stages. This suggests that the embryo begins to express and maintain gene expression throughout development. However, it is important to note that developmental expression of genes in whole embryos does not take into account organ localization of a gene and that dilution of a signal may occur (Goldstone et al., 2010). For example Corley-Smith et al. (2006) demonstrated localization of expression of CYP3C1: during 12 hpf, expression was detected thought-out the whole embryo but near 48 hpf, expression became predominately localized to the brain. Previous studies have provided evidence for the importance of CYP enzymes in development for both endogenous (Reijntjes et al., 2007) and exogenous compounds (Wang and Zhou, 2009). To date, it is unclear what roles CYP1-3 genes play in zebrafish development and further studies understanding the function and regulation of these important enzymes are clearly needed.

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TABLES

Table 1. Accession numbers and chromosome locations for zebrafish CYP3C and medaka CYP3B genes. All accession numbers were obtained from Genbank. Chromosome locations were obtained from Yan and Cai (2010), and confirmed on by BLAST search on ENSEMBL, respectively. Accession numbers for CYP3Bs were not found.

Gene	Accession Number	Chromosome Location
CYP3C1	BC052130.1	chr3: 40,964,024-40,971,364
CYP3C2	XM_681689.1	chr3: 40,953,676-40,960,305
CYP3C3	BC085438.1	chr3: 40,942,744-40,950,564
CYP3C4	BC124295.1	chr3: 40,930,879-40,940,506
CYP3B3		chr15: 14,900,678-14,906,428
CYP3B4		chr15: 14,910,849-14,918,611
CYP3B5		chr15: 14,921,023-14,926,010
CYP3B6		chr15: 14,927,785-14,936,321

Table 2. PCR and qPCR primers for CYP3 and housekeeping genes in zebrafish and medaka. CYP3C and CYP3B primers were designed based on coding sequences obtained from GenBank. Housekeeping gene sequences RPL-7 and 18S RNA were obtained from Zhang and Hu (2007); EF1-α was obtained from Craig et al., (2007) and β-actin was obtained from Alsop and Vijayan, (2008). All CYP3C, β-actin and EF1-α primers were specific for zebrafish. All CYP3B, RPL-7, and 18S RNA primers were specific for medaka. Primer sequences are shown for both forward (F) and reverse (R) strands. The optimal annealing temperatures (Temp) are given in degrees Celsius (^{o}C) and product length is given in base pairs (bp).

Gene	Primer Sequence (5'—3')	Temp (^{0}C)	Product length (bp)
CYP3C1	*F:* TCCAGACCTCTGGGAGTCTCCTAAT	60	100
	R: GCATGAAGGCACACTGGTTGATCT		
CYP3C2	*F:* TAAGCATGTTTTCAAAGCCGACTGTGG	62	152
	R: GGTTGTTCACTTGTAGTGTCCTTCAC		
CYP3C3	*F:* GGGAATAAGTGTGTTTTCAAGGTCAATTATGA	60	165
	R: GGTTGTTCACTTGTGGTGTCTTTCGC		
CYP3C4	*F:* TGG TCG CTG ACC TGG AAG TGA	62	89
	R: AAG GGG CCA GCC AGT CCT GT		
CYP3B4	*F:* GGTACCGCAACAAATGAAGG	62	211
	R: ACTGCTGCAACTCCATTGC		
CYP3B5	*F:* AATTGAAACCCTACCCTCAGC	62	140
	R: TCTCTGCGTCTGGCATCTC		
CYP3B6	*F:* GCCGTATCGGGTTTTCAGGA	64	89
	R: ATGTCCCTCCCCATTTGCAG		
β-actin	*F:* TGTCCCTGTATGCCTCTGGT	60	200
	R: AAG TCC AGA CGG AGG ATG G		
EF1-α	*F:* GTGCTGTGCTGATTGTTGCT	62	200
	R: TGTATGCGCTGACTT CCT TG		
RPL 7	*F:* CGCCAGATCTTCAACGGTGTAT	60	72
	R: AGGCTCAGC AATCCTCAGCAT		
18s RNA	*F:* TCCACCTTCCAGCAGATGTG-	63	76
	R: AGCATTTGCGGTGGACGAT		

Table 3. QPCR assay performance. All standard curves were optimized for annealing temperature, primer concentration and template volume. All curves included a minimum of 5 points or more. The equation, efficiency, and goodness of fit (Rsq) for the standard curves of each reaction are given below.

Gene	Equation	Effeciency (%)	Rsq
CYP3C1	Y = -3.283*LOG(X) + 23.23	101.7	0.974
CYP3C2	Y = -3.538*LOG(X) + 14.25	91.7	0.997
CYP3C3	Y = -3.330*LOG(x) + 14.23	99.7	0.989
CYP3C4	Y = -3.306*LOG(X) + 16.18	100.7	0.993
CYP3B4	Y = -3.409*LOG(X) + 9.98	96.7	0.966
CYP3B5	Y = -3.150*LOG(X) + 18.38	107.7	0.900
CYP3B6	Y = -3.683*LOG(X) + 18.67	96.9	0.976
β-actin	Y = -3.319*LOG(X) + 18.82	103.3	0.991
EF1-α	Y = -3.184*LOG(X) + 22.70	106.1	0.990
RPL-7	Y = -3.334*LOG(X) + 18.26	95.0	0.990
18s-RNA	Y = -3.376*LOG(X) + 9.83	97.8	0.977

Table 4. Amino acid sequence identity of CYP3Cs (A) and CYP3Bs (B). The identity matrix was constructed in BioEdit version 7.0.5 (Hall 1999) using an alignment generated in Clustal X (Thomson et al., 1997). Sequence identity is shown in percent.

A)

GENE	**CYP3C1**	**CYP3C2**	**CYP3C3**	**CYP3C4**
CYP3C1	100	70	72.2	76.3
CYP3C2		100	90.6	71.0
CYP3C3			100	72.3
CYP3C4				100

B)

GENE	**CYP3B3**	**CYP3B4**	**CYP3B5**	**CYP3B6**
CYP3B3	100	74.5	72.1	71.0
CYP3B4		100	74.9	75.8
CYP3B5			100	75.3
CYP3B6				100

Table 5. Zebrafish EST library expression of CYP3C1, CYP3C2, CYP3C3, and CYP3C4. Gene expression is provided for adult tissues (A) and through development (B). Data are normalized expression, in transcripts per million, as determined in UniGene (Pontius et al. 2003). No ESTs were found for CYP3Bs or CYP3Ds. The heart and muscle are not shown because there was no expression of any gene investigated.

A)

Gene	**Organ**										
	Bone	**Brain**	**Eye**	**Fin**	**Gills**	**Intestine**	**Kidney**	**Liver**	**Olfactory Rosette**	**Reproductive System**	**Skin**
CYP3C1	262	11	16	59	0	240	97	116	112	21	221
CYP3C2	0	0	0	0	76	0	0	0	0	0	0
CYP3C3	0	0	0	0	0	0	0	0	28	0	0
CYP3C4	0	0	0	0	0	0	0	0	0	0	0

B)

Gene	**Developmental Stage**							
	Egg	**Gastrula**	**Segmentation**	**Pharyngula**	**Hatching**	**Larval**	**Juvenile**	**Adult**
CYP3C1	0	278	0	0	45	99	0	67
CYP3C2	0	0	0	0	0	0	0	1
CYP3C3	0	0	0	0	0	49	0	17
CYP3C4	0	0	0	0	0	0	0	11

Table 6. Zebrafish CYP3C and medaka CYP3B gene expression in adult female (A) and male (B) organs by reverse transcriptase PCR. Expression was determined by reverse transcriptase PCR, using primers as indicated in Table 2. The symbol "+" indicates sites showing expression of the CYP3s and "-" indicate sites where expression was not seen. Int is intestine and Olf Ros are the olfactory rosettes.

A)

Gene	Organs									
	Brain	**Eye**	**Gill**	**Heart**	**Int**	**Kidney**	**Liver**	**Olf Ros**	**Spleen**	**Ovary**
CYP3C1	+	+	+	+	+	+	+	-	+	+
CYP3C2	+	+	+	+	+	+	+	-	+	+
CYP3C3	+	+	-	-	+	+	+	-	+	+
CYP3C4	+	+	+	+	+	+	+	-	+	+
CYP3B4	+	+	+	+	+	+	-	-	+	+
CYP3B5	+	+	-	-	+	+	-	+	+	+
CYP3B6	+	+	-	+	-	+	+	-	+	+

B)

Gene	Organs									
	Brain	**Eye**	**Gill**	**Heart**	**Int**	**Kidney**	**Liver**	**Olf Ros**	**Spleen**	**Testis**
CYP3C1	-	-	-	-	+	+	-	-	-	+
CYP3C2	+	+	+	+	+	+	+	+	+	+
CYP3C3	+	+	+	+	+	+	+	+	+	+
CYP3C4	-	+	+	+	-	+	-	+	+	+
CYP3B4	+	+	+	+	-	+	-	-	+	+
CYP3B5	+	+	-	+	+	+	+	+	+	+
CYP3B6	+	+	-	+	+	+	+	+	+	+

Table 7: Cycle threshold values of male and female liver for CYP3Cs and CYP3Bs. Reported values represent an average of four different cDNA samples for each gender. Expression of CYP3B4 was not quantifiable in male organs.

Gene	Male	Female
CYP3C1	25.06	29.02
CYP3C2	29.31	30.97
CYP3C3	27.97	29.84
CYP3C4	27.00	30.31
CYP3B4	N/A	26.06
CYP3B5	31.17	24.21
CYP3B6	27.70	23.59

FIGURES

Figure 1: Expression of CYP3C1 in adult male and female zebrafish organs. Expression of CYP3C1 in each organ is relative to the expression of the gene in the male liver. An asterisk is used to indicate statistical differences in expression between males and females at $p < 0.050$, as determined by a two-tailed t-test. Error bars depict standard error of mean. N = 3 or 4 except for the female olfactory rosette (Olf. Ros.; n = 2). Gene expression in female olfactory rosettes was not included in the statistical analysis.

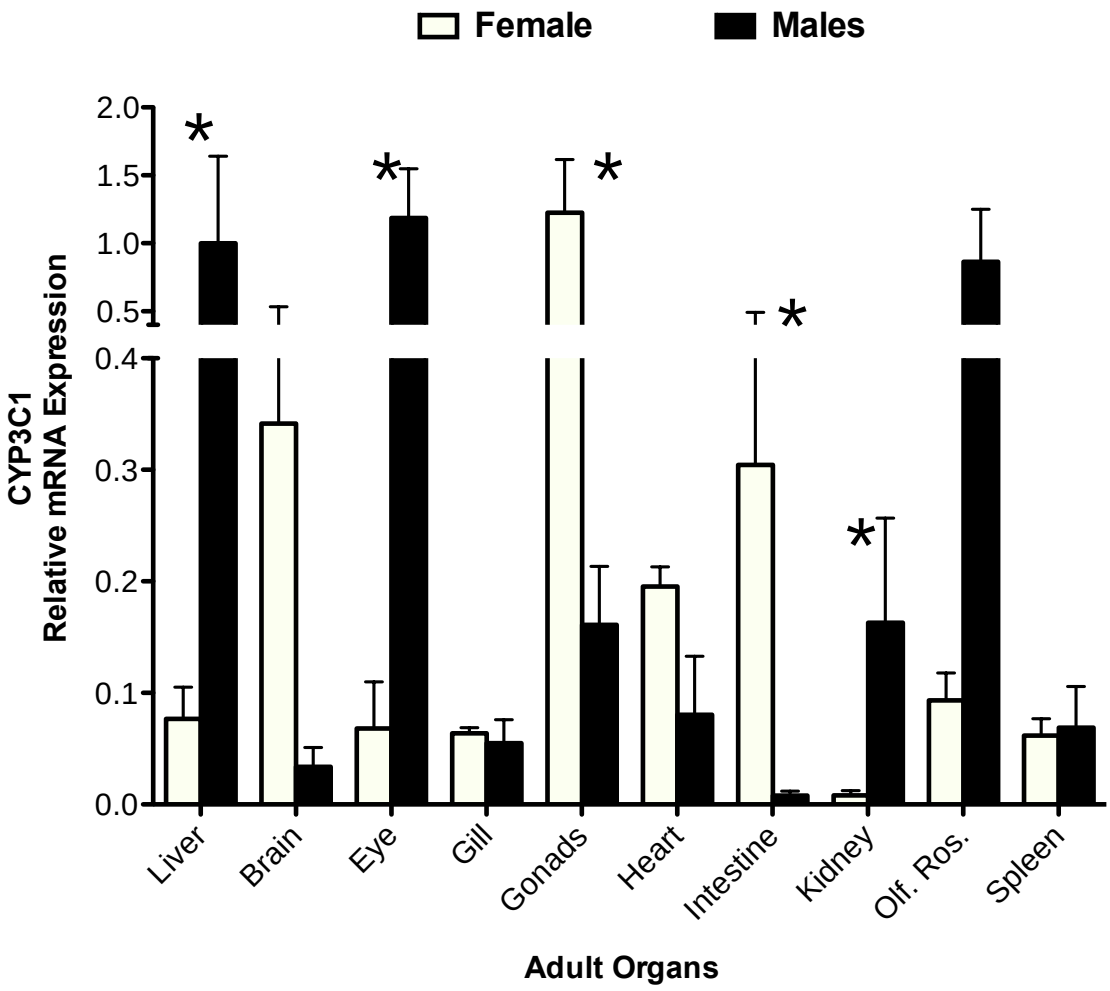


Figure 2: Expression of CYP3C2 in adult male and female zebrafish organs. Expression of CYP3C2 in each organ is relative to the expression of the gene in the male liver. An asterisk is used to indicate statistical differences in expression between males and females at $p < 0.050$, as determined by a two-tailed t-test. Error bars depict standard error of the mean. N=3-4.

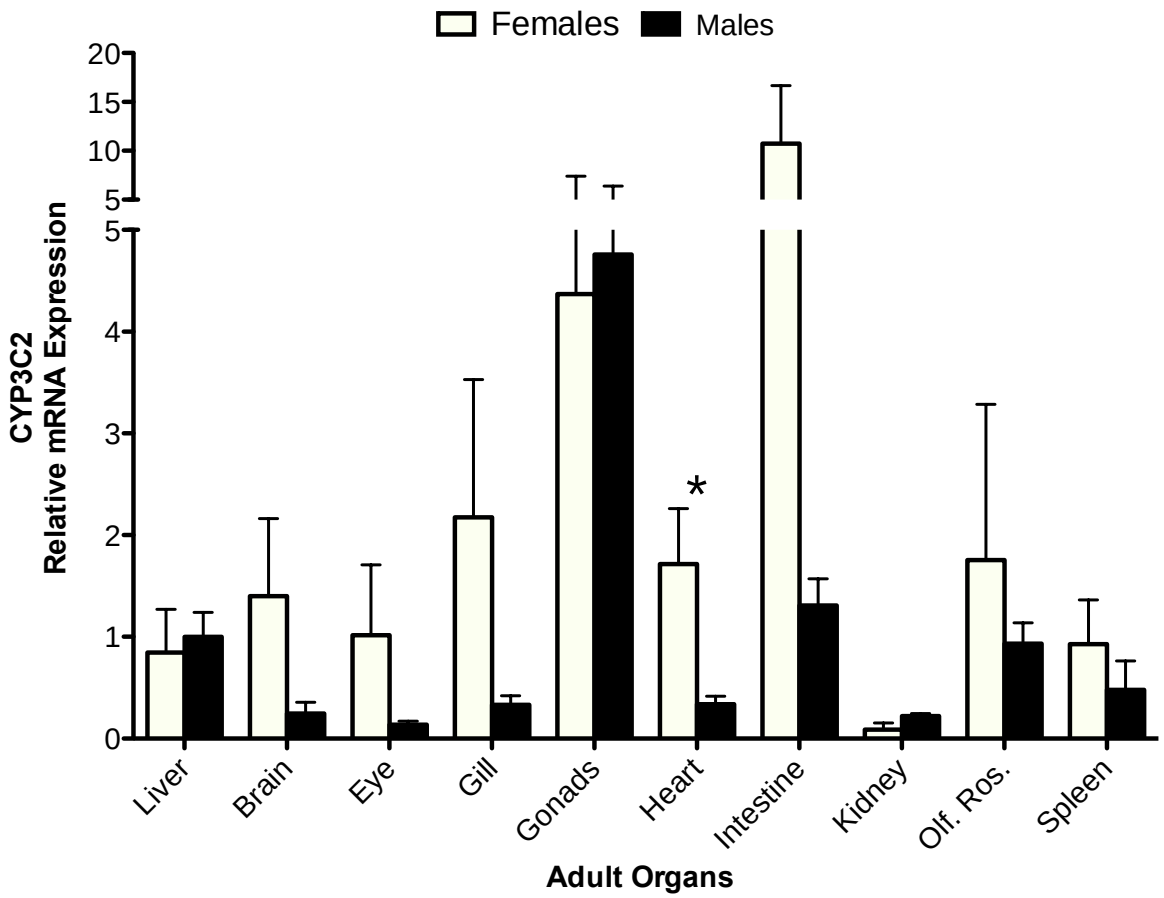


Figure 3: Expression of CYP3C3 in male and female zebrafish organs. Expression of CYP3C3 in each organ is relative to the expression of the gene in the male liver. An asterisk is used to indicate statistical differences in expression between males and females at $p < 0.050$, as determined by a two-tailed t-test. Error bars depict standard error of the mean. N=3 or 4 except for female intestine (n=2). Statistical analysis did not include female gonad, female and male olfactory rosette due to large variation between pools and female intestine due to a small n.

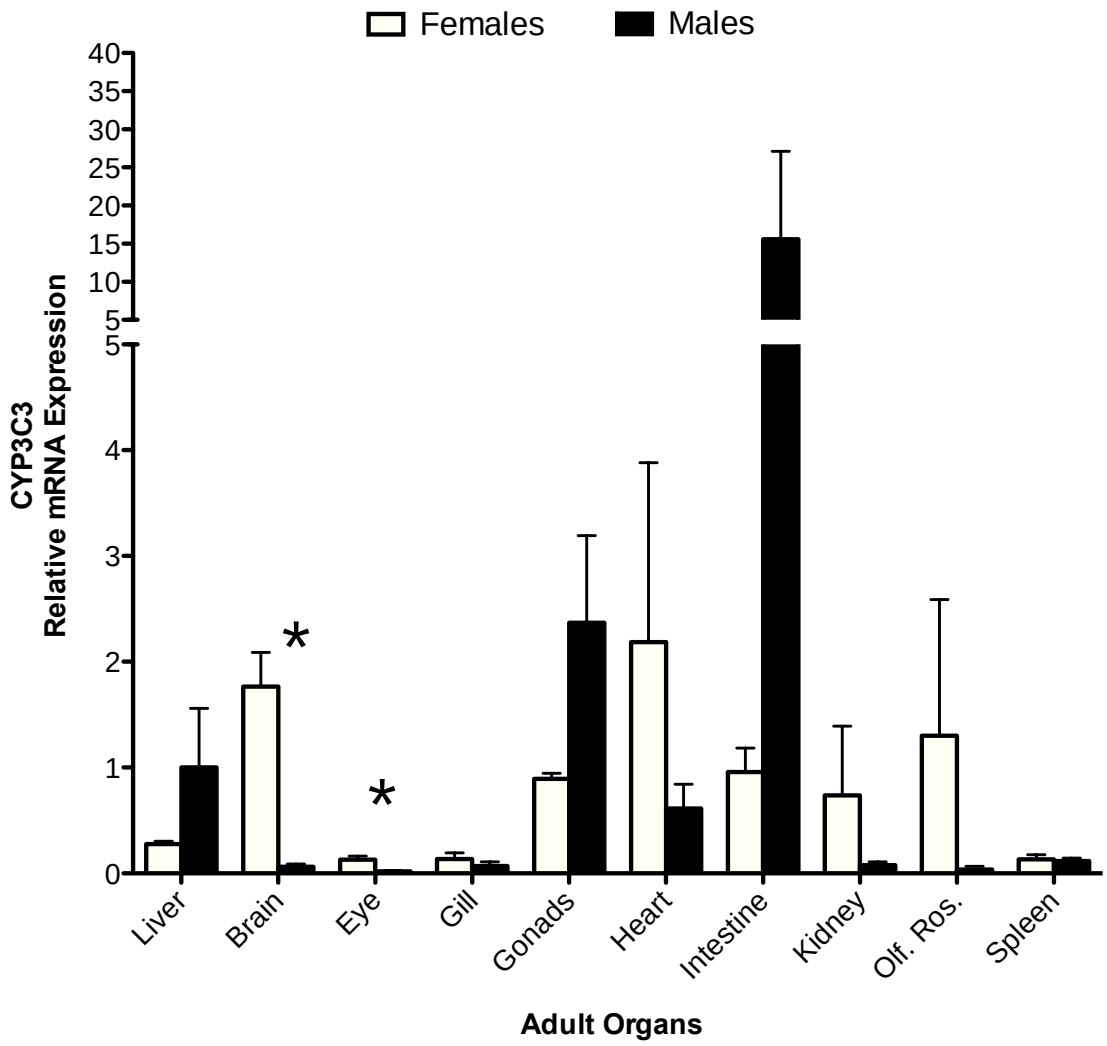


Figure 4: Expression of CYP3C4 in male and female zebrafish organs. Expression of CYP3C4 in each organ is relative to the expression of the gene in the male liver. An asterisk is used to indicate statistical differences in expression between males and females at $p < 0.050$, as determined by a two-tailed t-test. Error bars depict standard error of the mean. N = 3 or 4 except the female olfactory rosette (n = 2); female olfactory rosette was not included in statistical analyses.

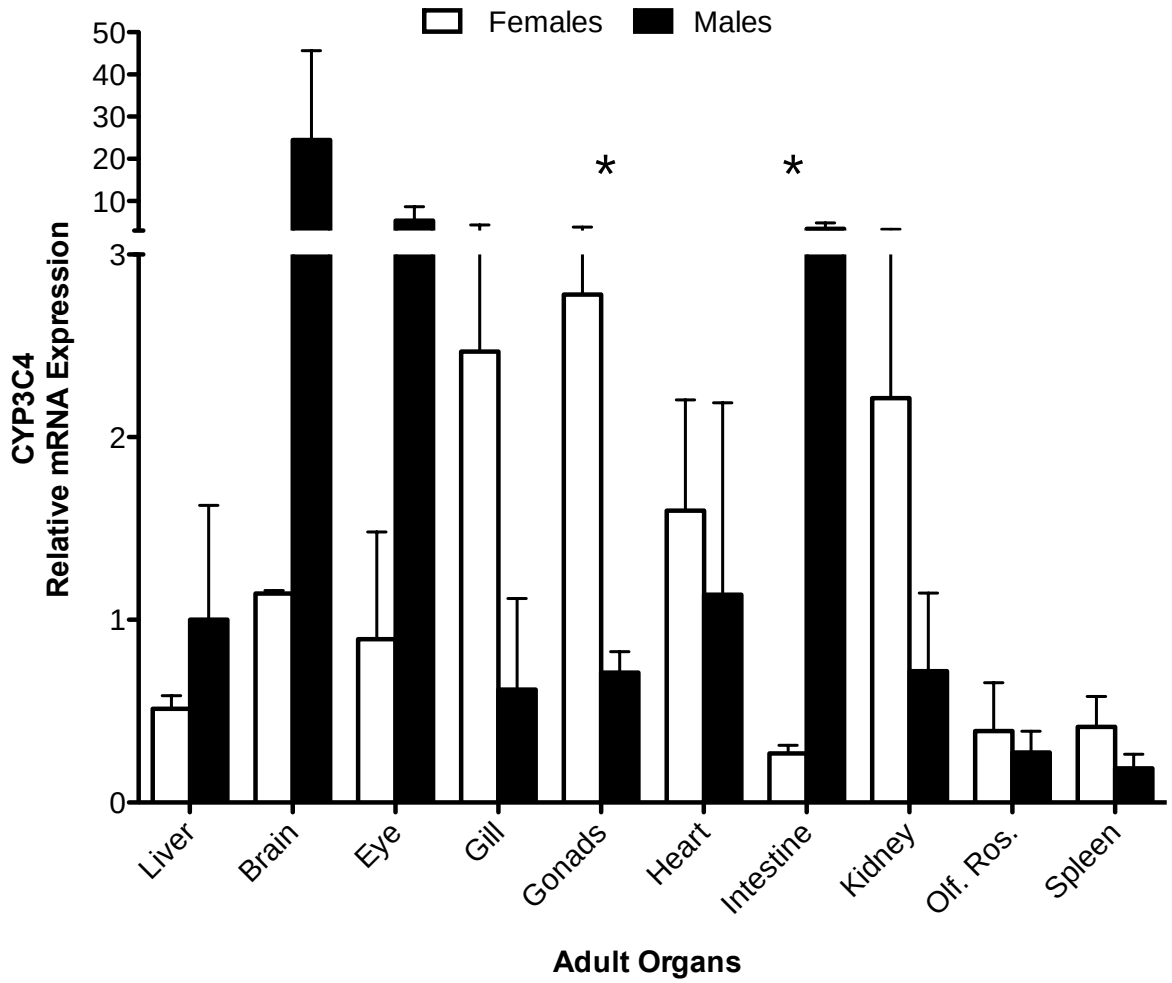


Figure 5: Expression of CYP3C1, CYP3C2 and CYP3C3 during development in zebrafish. All data is relative to expression of each gene during the 4-8 cell stage. Asterisk depict differences from the 4-8 cell stage for each gene, as determined by One-Way Anova ($P<0.001$). N=4, error bars depict standard error of the mean.

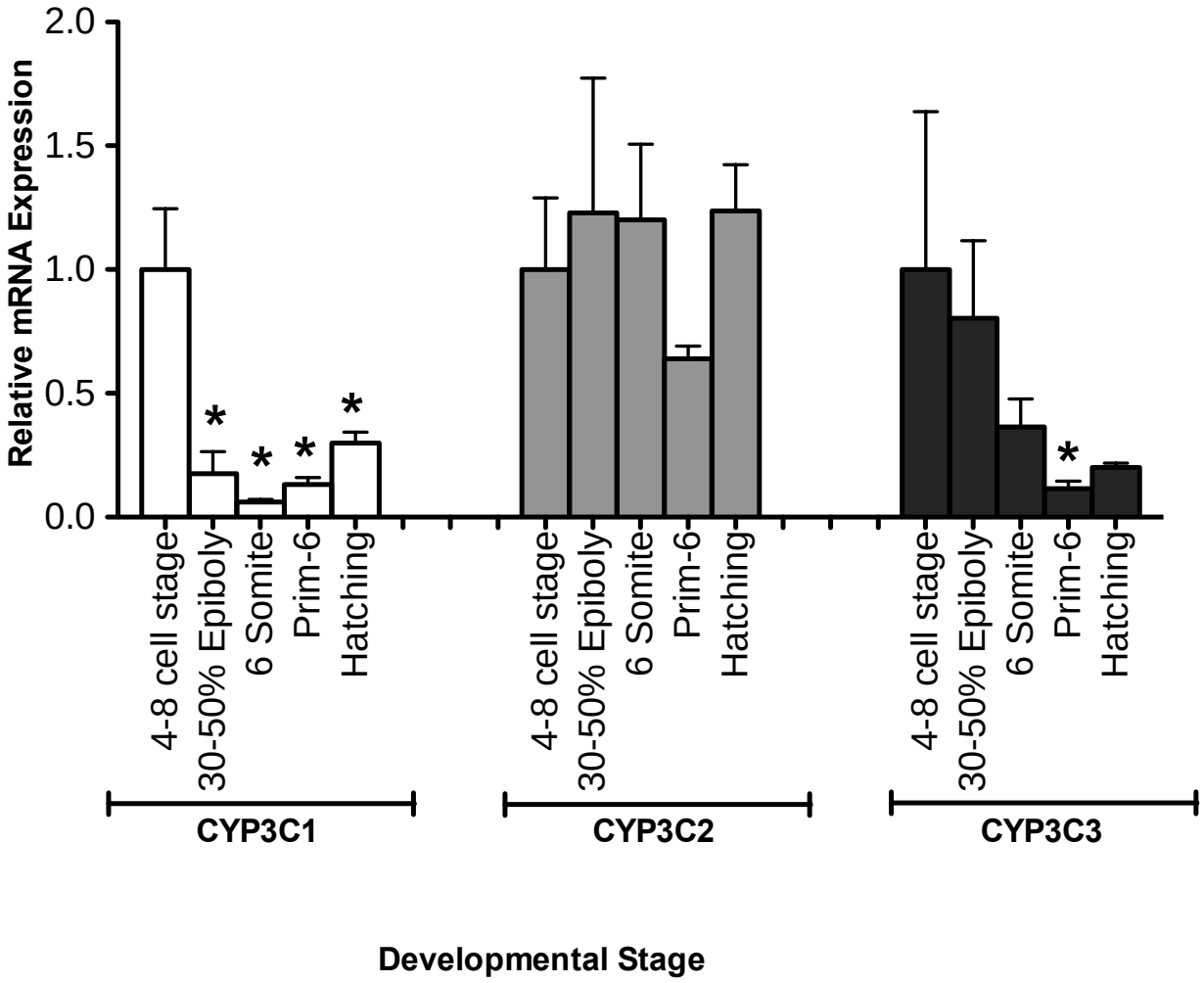


Figure 6: Expression of CYP3B4 in female medaka organs. Expression of CYP3B4 in each organ is relative to the expression of the gene in the liver. N = 3 or 4 for all organs except kidney (n=2). Brain not included due to the formation of primer dimer formation in reaction. An asterisk is used to indicate statistical of organs from liver at $p < 0.050$, as determined by a one-way anova. Error bars depict standard error of the mean

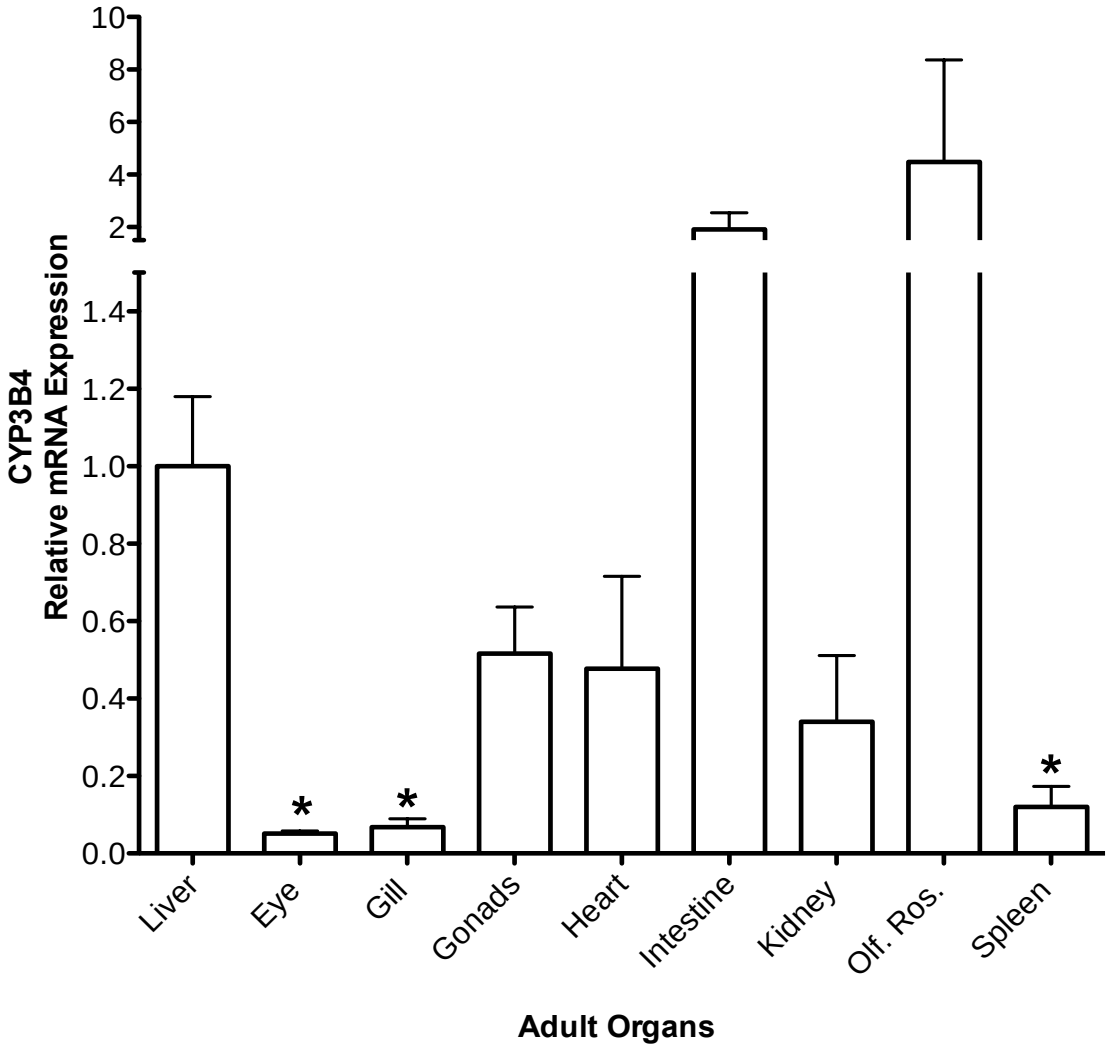


Figure 7: Expression of CYP3B5 in adult male and female medaka organs. Expression of CYP3CB5 in each organ is relative to the expression of the gene in the liver. N = 3 or 4 for all organs; male gonads and olfactory rosette had an n=2. An asterisk is used to indicate statistical differences between expression in males and females at p <0.050, as determined by a two-tailed t-test. Error bars depict standard error of the mean. Kidney of female medaka was not included in statistical analyses because of the large variation between samples.

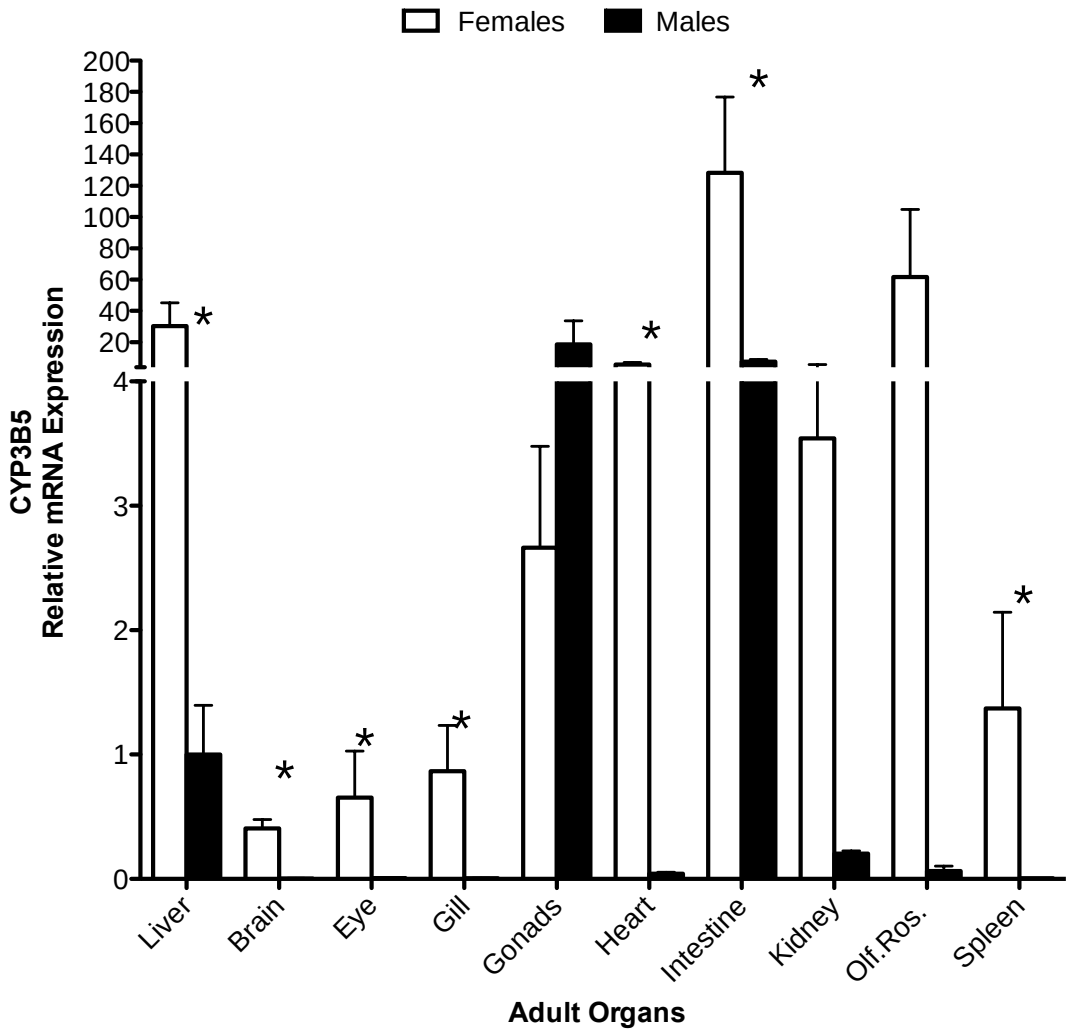
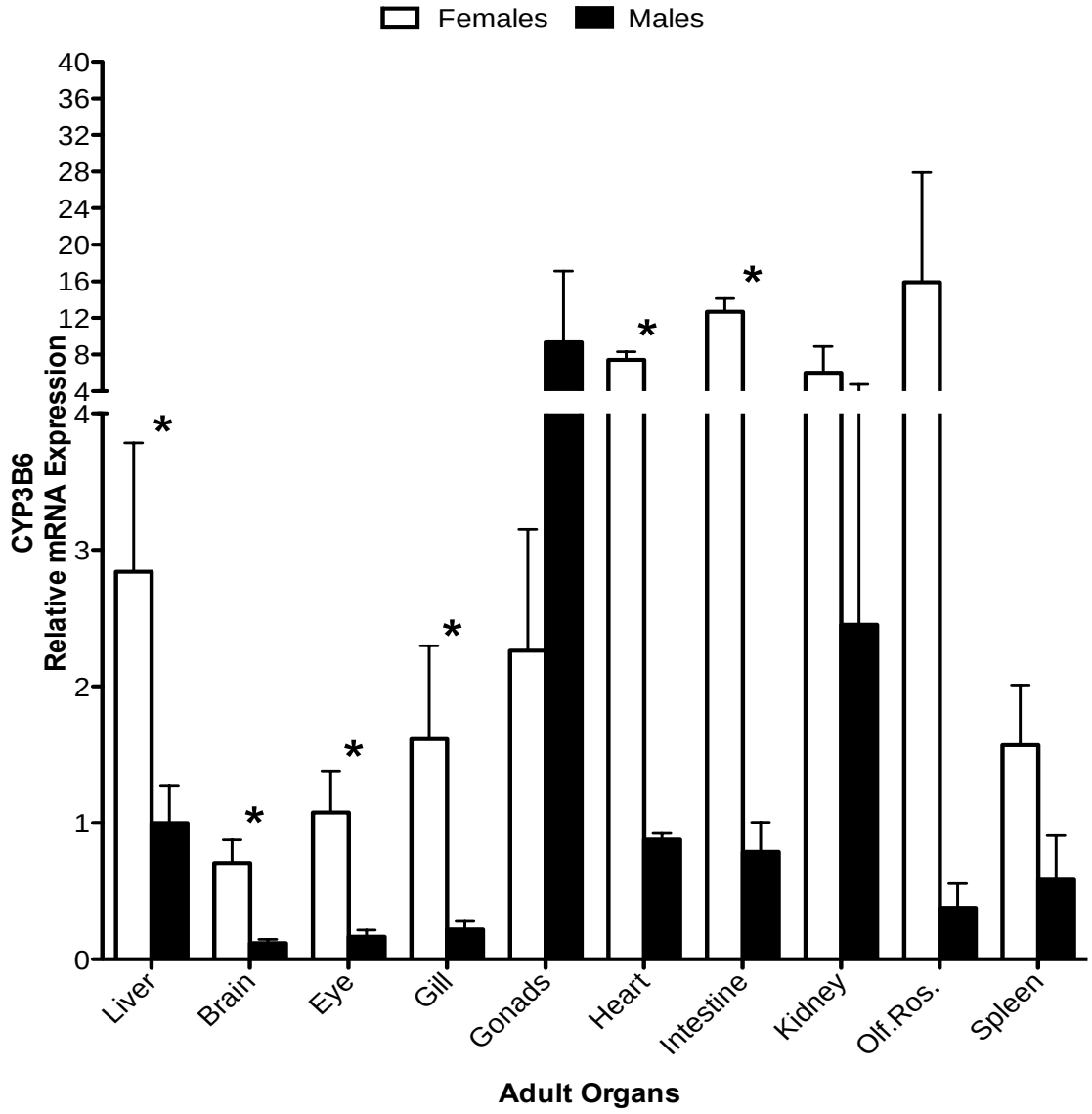


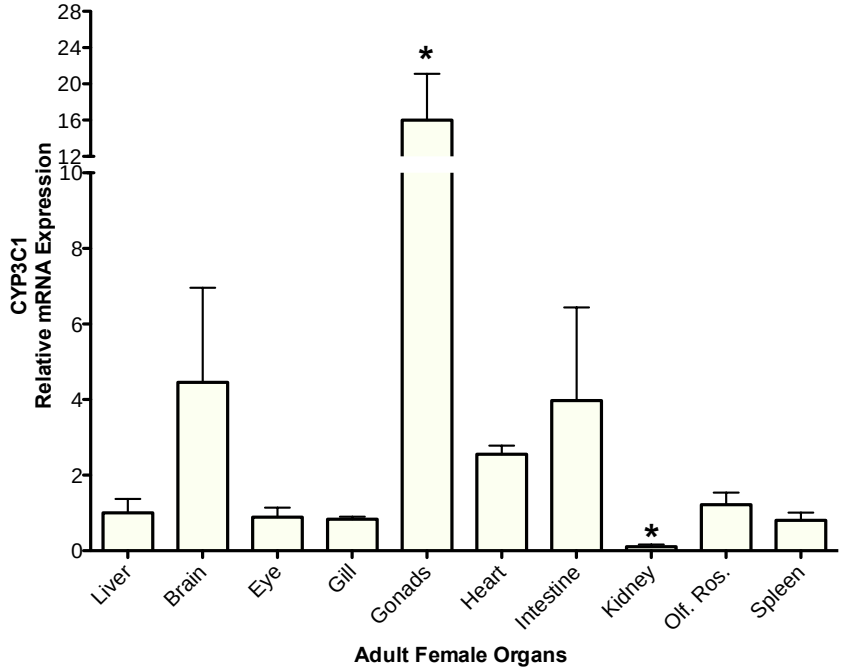
Figure 8: Expression of CYP3B6 in adult male and female medaka organs. Expression of CYP3B6 in each organ is relative to the expression of the gene in the liver. An asterisk is used to indicate statistical differences between expression in males and females at $p < 0.050$, as determined by a two-tailed t-test. N = 3 or 4. Error bars depict standard error of the mean.



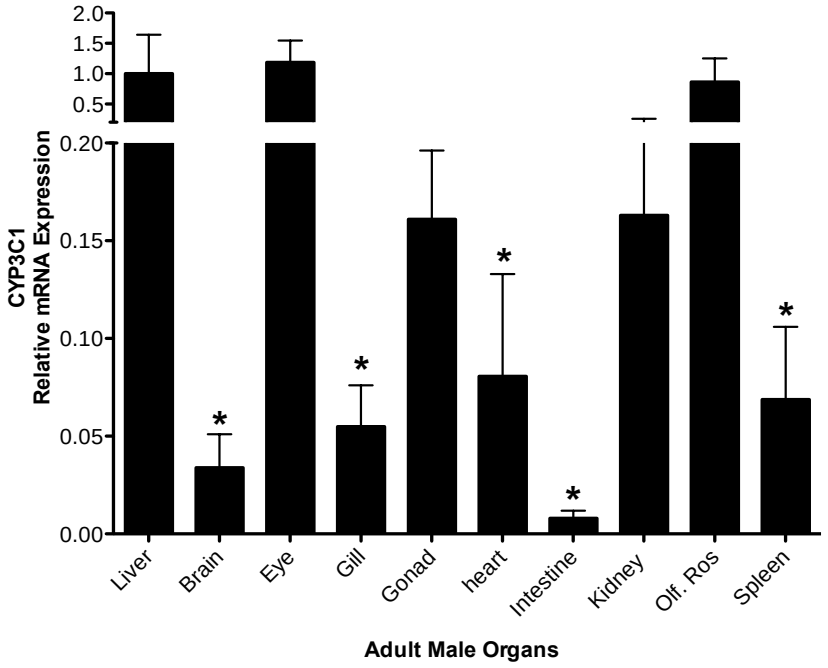
SUPPLEMENTARY FIGURES

Supplementary Figure 1. Expression of CYP3C1 in Female (A) and Male (B) organs. Expression is given relative to liver within each gender and organs that are statistically different than liver ($p<0.05$ by one-way anova) are denoted by an asterisk. N = 3-4 for all organs expect olfactory rosette of females (n=2). Error bars depict standard error of the mean.

A)

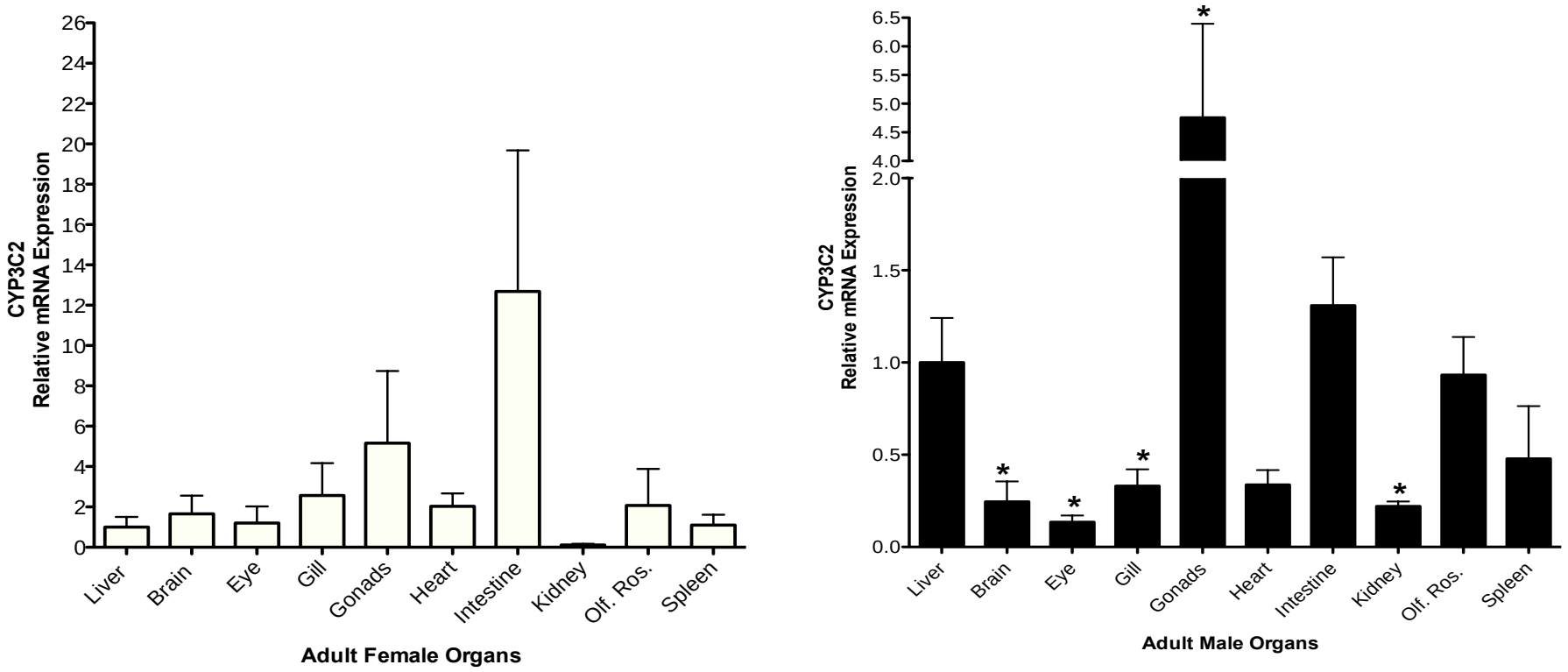


B)

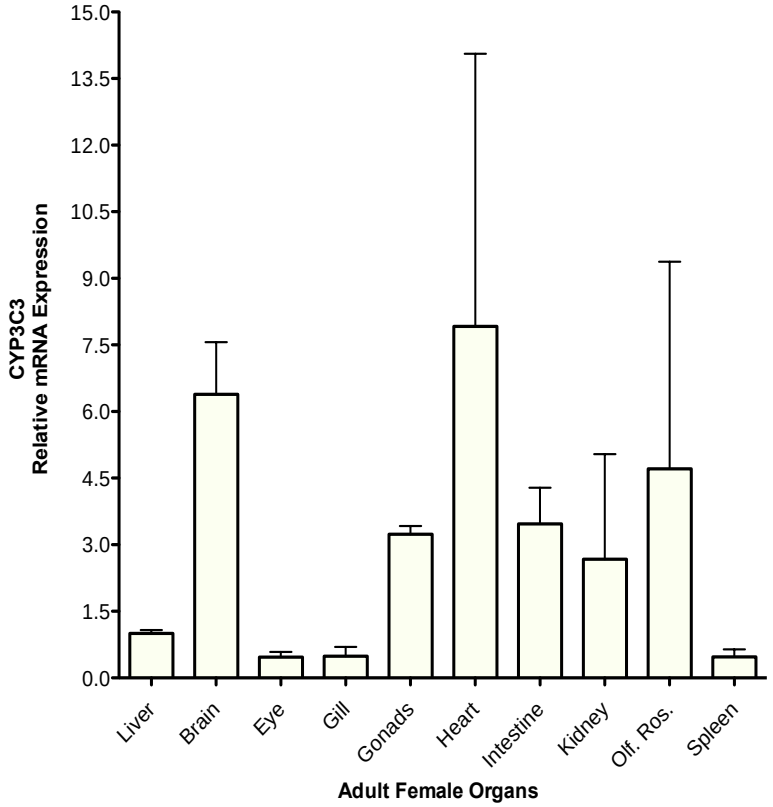


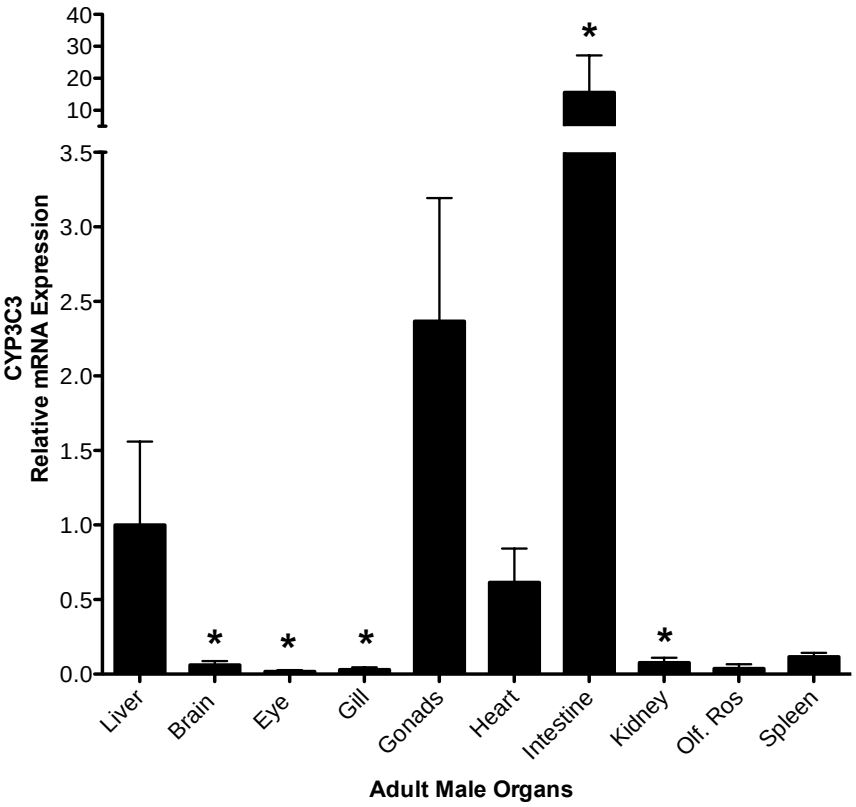
Supplementary Figure 2. Expression of CYP3C2 in Female (A) and Male (B) organs. Expression is shown relative to liver within each gender and organs that are statistically different than liver are denoted by an asterisk ($p<0.05$ by one-way anova). N = 3-4. Error bars depict standard error of the mean

A) B)

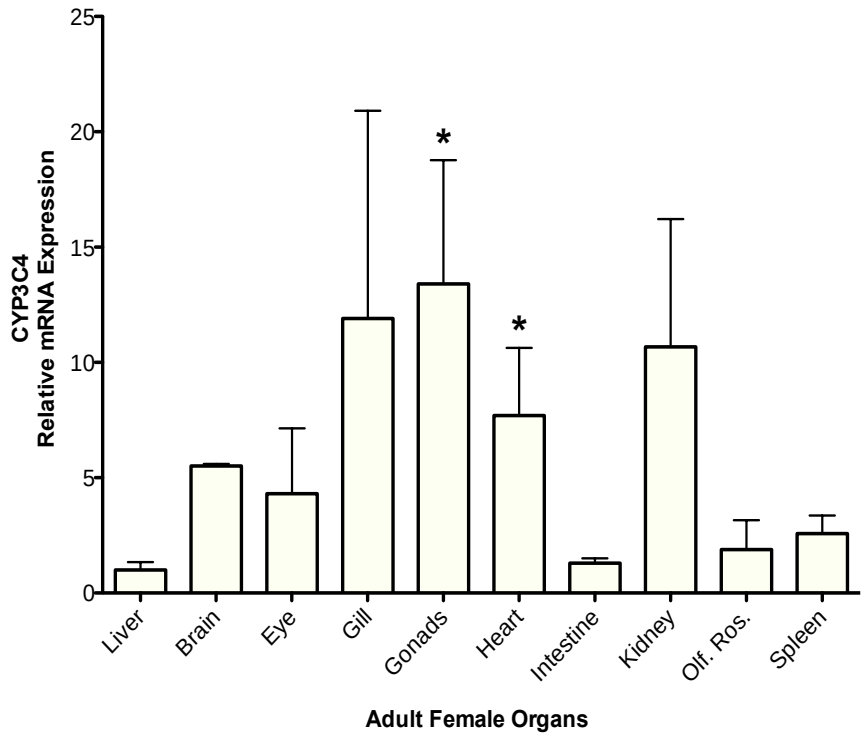


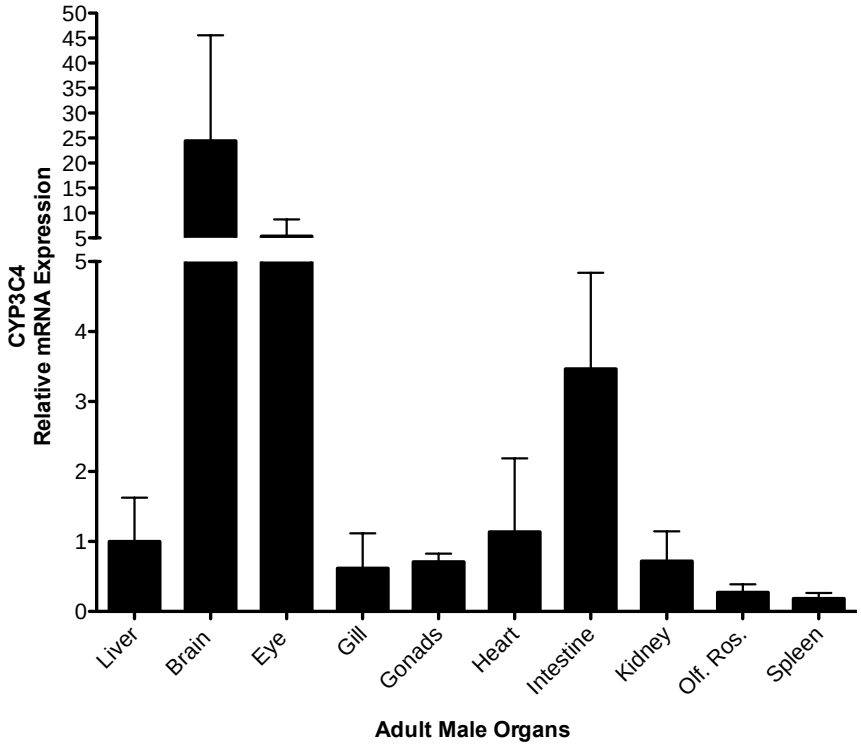
Supplementary Figure 3. Expression of CYP3C3 in Female (A) and Male (B) organs. Expression is shown relative to liver within each gender and organs that are statistically different than liver are denoted by an asterisk ($p<0.05$ by one-way anova). N = 3-4 except for female gonads and olfactory rosette (n=2). Error bars depict standard error of the mean



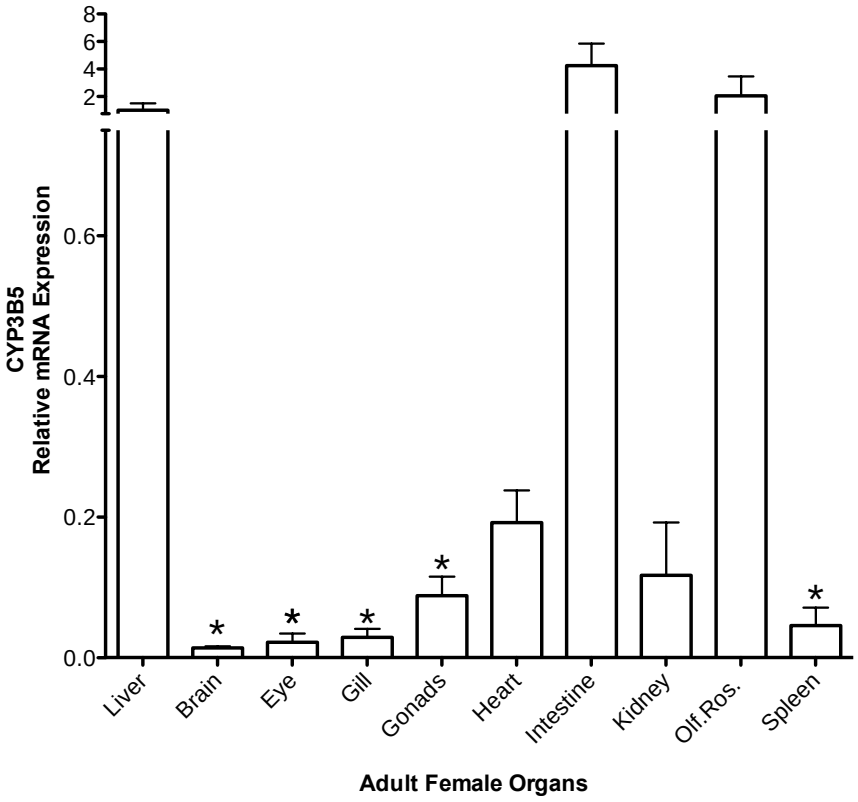


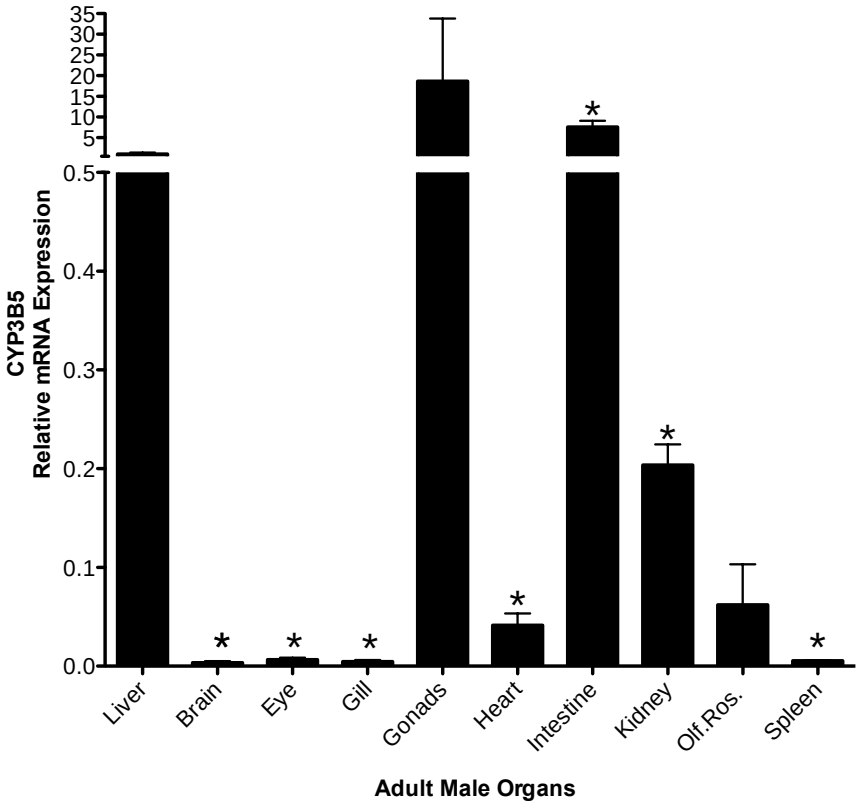
Supplementary Figure 4. Expression of CYP3C4 in Female (A) and Male (B) organs. Expression is shown relative to liver within each gender and organs that are statistically different than liver are denoted by an asterisk ($p<0.05$ by one-way anova). N = 3-4 except for female gonads and olfactory rosette (n=2). Error bars depict standard error of the mean



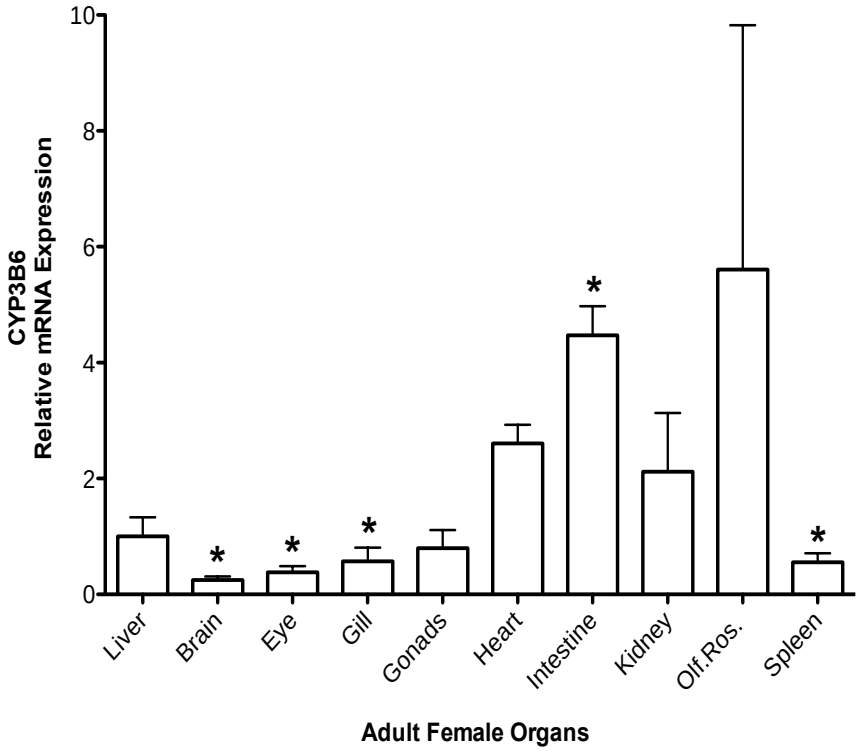


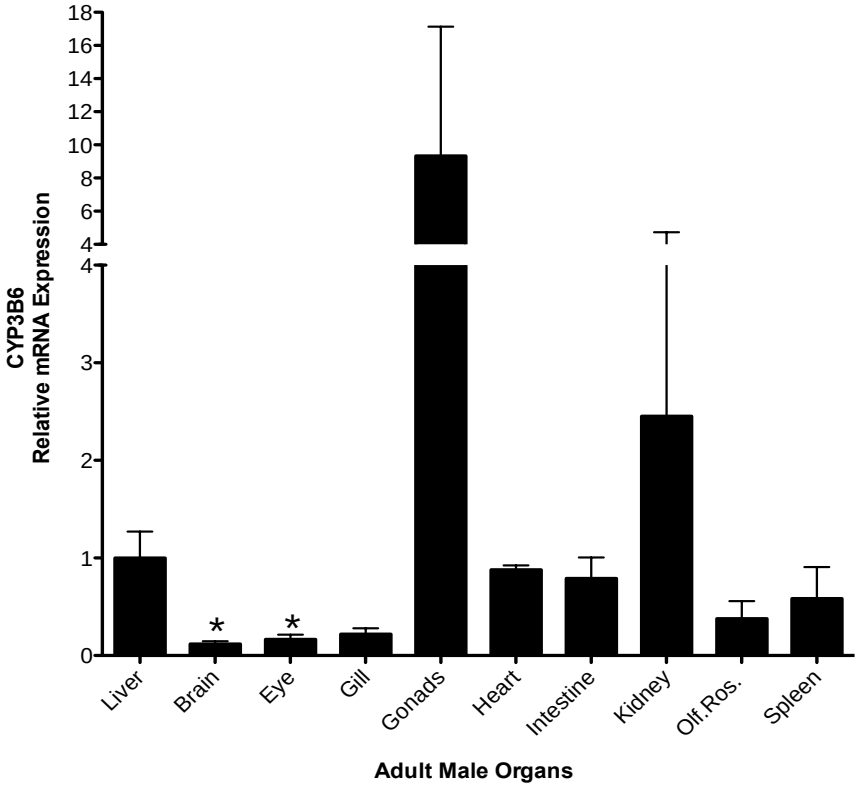
Supplementary Figure 5. Expression of CYP3B5 in Female (A) and Male (B) organs. Expression is shown relative to liver within each gender and organs that are statistically different than liver are denoted by an asterisk ($p<0.05$ by one-way anova). N = 3-4 except in testis and male olfactory rosettes (n=2). Error bars depict standard error of the mean





Supplementary Figure 6. Expression of CYP3B6 in Female (A) and Male (B) organs. Expression is shown relative to liver within each gender and organs that are statistically different than liver are denoted by an asterisk ($p<0.05$ by one-way anova). N = 3-4 except in testis and male olfactory rosettes (n=2). Error bars depict standard error of the mean





CHAPTER 3

GENERAL DISCUSSION

Teleost fish have the most diversified CYP3 clade, which includes CYP3As, CYP3Bs, CYP3Cs, and CYP3Ds; the latter three subfamilies have only been found in fish species (Yan and Cai, 2010). Intriguingly, only CYP3As are found in all fish species; the remaining CYP3 subfamilies appear to be lineage specific (Goldstone et al., 2010; Yan and Cai, 2010). Only CYP3C1 has been studied for expression and function (Corley-Smith et al., 2006).

It is important to understand the expression patterns of these novel CYPs in order to understand their function and why fish species have such a diverse CYP3 clade. Generally, xenobiotic relevant CYPs are expressed predominately in organs like the liver, kidney, intestine and gill (Ding and Kaminsky, 2003; Qiu et al., 2008; Shimada et al., 1994). However, expression may be seen in other organs that are constantly exposed to the environment such as the eye and olfactory rosette (Saucier et al., 1999; Choudhary et al., 2006; Matsuo et al., 2008). CYPs responsible for metabolism of endogenous compounds are more typically found in internal organs not involved in absorption, metabolism and elimination such as the brain and gonads (Callard et al., 2001).

Reliability of Expressed Sequence Tag (EST) Data

Genomic data, including EST expression library data, is becoming more widely available in multiple teleost species. However, EST library size in most teleost species is rather small and may not provide adequate information in terms of relative gene

expression. In this study, ESTs for zebrafish CYP3C, medaka CYP3B, and stickleback and pufferfish CYP3D genes were investigated to determine likely sites of expression of the novel CYP3 subfamilies. However, no expression of CYP3Bs and CYP3Ds were found in any library from medaka, stickleback or pufferfish, possibly due to the small EST libraries available in these species. The zebrafish's completed genome has resulted in well developed EST libraries. CYP3Cs appeared in many zebrafish organs and developmental stages (Chapter 2, Table 5). Yet, the expression pattern suggested by the EST library data was not similar to patterns established by RT-PCR (Chapter 2, Table 6), nor the quantitative results from real-time PCR (Chapter 2, Figure 1-4; 6-8).

The reliability of EST depends on how many total ESTs have been sequenced for each library (Schmitt et al., 1999). Schmitt et al., (1999) suggest that most cells have approximately 10 000 – 30 000 genes and when the amount of genes sequenced for each library is small, EST mining has not been exhaustive and lowly expressed genes are not well represented. As such, finding no transcripts of your gene of interest in a library containing 1000 total EST should not be interpreted similarly as with a library containing 100 000 total ESTs. Usually, 10 000 sequenced transcripts per library is thought to be needed to establish reliable expression patterns with EST library data (Schmitt et al., 1999). However, the zebrafish gill and heart EST libraries were greater than 10 000 yet no CYP3C1 ESTs were found; RT-PCR (Chapter 2, Table 6) and quantitative real-time PCR (Chapter 2, Figures 1) confirmed the expression of CYP3C1 in these two organs. It has been suggested that this cut off is only reliable for highly expressed genes (Schmitt et al 1999) and it would appear that CYP3Cs and CYP3Bs are generally not highly

expressed in most organs. The EST sequencing in most teleost fish is likely not exhaustive enough to find more lowly expressed genes. Thus, the use of RT-PCR in this study was necessary to understand where each isoform was expressed and in which organs to focus for quantification in real time PCR. The CYP3Bs and CYP3Cs appear to be more lowly expressed compared to expression of other families of CYPs. Expression above background of CYP3B and CYP3C was only detected at an average of 25 CT in liver samples (Chapter 2; Table 7), while the house keeping genes CT for liver were an average of approximately 19 CT for β-actin and 9 for 18s RNA (data not shown).

PCR Optimization and Analyses

Due to the high amino acid similarity between CYP3 isoforms, there was concern that the PCR reactions may amplify multiple isoforms. Many primers were designed and tested before a working pair was obtained. Some designed primers failed to amplify the gene of interest and would at times amplify another isoform. For example, I was unsuccessful at designing primers for CYP3B3; some primers amplified unknown products of wrong size or would amplify CYP3B5. Due to this issue, it was important to insure that all primers were amplifying the appropriate gene by sequencing the product produced by RT-PCR. In some cases, primers were not optimal for qPCR reactions. For example, CYP3C2 and CYP3C3 primers are longer than the recommended length for qPCR primers and the product length of the CYP3B4 reaction exceeded the recommended length. However, the primers used (Chapter 2, Table 2) were optimized and validated to obtain efficient amplification of the product of interest. In the case of

CYP3B4, primer dimers were consistently found in the no template control reactions and in the no reverse transcriptase controls. Yet, primer dimers were not found in standard curves based on cloned template nor were they found in female organs with high CYP3B4 expression, suggesting this was an issue with low gene expression and not an issue with primer design or optimization of the PCR reaction. Expression of CYP3B4 was not quantifiable for male organs; all reactions had a high CT and primer dimers developed in most wells.

In most cases, a standard curve with good efficiency was not obtained when pooling cDNA from the organs investigated. CYP3C1, CYP3C2, CYP3C3, CYP3C4, CYP3B4, CYP3B5, and CYP3B6 required amplified product to create a standard curve with a good range.

Qualitative real time PCR is a very sensitive and reliable technique that is able to give an adequate quantification of mRNA levels in experimental treatments. However, the accuracy of qPCR is dependent on many factors such as RNA quality, primer quality, and assay optimization. Samples are prone to contaminates acquired through extractions or loss of RNA integrity and the amount of contaminants, inhibitors or other factors between one sample to the next may not always be the same (Exposito-Rodriguez et al., 2008). House keeping genes must be used in order to correct for all of the issues mentioned above. β-actin is a popular housekeeping gene used for both adult and developmental research (Tang et al., 2007). However, some studies suggest that β-actin varies too much during development, between sex and/or across experimental treatments (Kouadjo et al., 2007; Warrington et al., 2000). These reference genes appear to be most

variable in the dynamic, developing organism (Fernandes et al., 2008; McCurley and Callard, 2008). It is recommended that multiple reference genes be investigated and that to insure optimal reliability of data, samples should be normalized to two or more internal controls (Vandesompele et al., 2002). Data can also be normalized to the total RNA of each sample, but this does not take into account the RNA quality and in some cases it is is not a good representation of the total mRNA in a sample (Vandesompele et al., 2002). In this study, multiple house keeping genes were investigated and used in order to choose one with the least variability. β-actin and RPL-7 showed the least variability within organs of zebrafish and medaka, respectively. In the case of the developmental data, samples were normalized to EF1-α and the total RNA of each sample. However, the results were similar and the data shown is normalized to EF1-α (Chapter 2, Figure 5).

Expressional data was collected for multiple organs in both sexes (CYP3C and CYP3B genes) and in different embryonic stages (CYP3C genes). Statistical analyses were based on comparisons across gender for an individual organ (i.e. male liver versus female liver) or by comparing liver to all other organs for a single gender (i.e. male liver versus male gill, male intestine, etc). Sample sizes were small (N=3-4) and prevented a comparison amongst all organs. Furthermore, some tissues were dropped from the statistical analyses. Both zebrafish and medaka are small animals that seldom grow bigger than 6cm. Dissecting small organs, such as the olfactory rosette, kidney, spleen and others was very difficult to do and was not possible from all individuals or the total sample size was not sufficient for all genes to be analyzed if reactions needed to be replicated. Sampling more organisms for a larger tissue mass, adding more pools of tissue to increase

replication and perhaps controlling for tissue weight contributing to each pool would increase the robustness of these findings.

Hepatic and Extrahepatic Expression as an Indication of Function

In this study, CYP3B and CYP3C isoforms were present in the main detoxifying organs of zebrafish and medaka (i.e. liver, intestine, kidney). The appearance of CYP1-4 family enzymes in these organs has been linked with their ability to metabolize exogenous compounds in order to detoxify compounds and reduce toxicity (Timbrell and Marrs, 2009). Intensive research on mammalian systems identified the importance of CYP3A isoforms in xenobiotic (mainly drug) metabolism in the liver (Guengerich, 2008) and intestine (Ding and Kaminsky, 2003; Guengrich, 1999). The little research available on fish CYP3As suggests that CYP3A genes are expressed in the main detoxifying organs and also plays a role in xenobiotic metabolism (Husoy et al., 1994; Sun et al., 2013; Tseng et al., 2005). The appearance of CYP3B and CYP3C isoforms in the major detoxification organs is indicative that they maybe involved in xenobiotic metabolism.

Transcriptional Elements as an Indication of Regulation

For many CYPs involved in xenobiotic metabolism, their expression is inducible. Typically, the substrates of a given inducible CYP gene are capable of upregulating the expression of the gene. Thus, knowledge of the substrates of each CYP may give insight on their regulatory mechanisms and vice versa. Xenobiotic compounds are ligands for nuclear receptors like the pregnane x receptor (PXR) (Kliewer et al., 2002), aryl

hydrocarbon receptor (AHR) (Denison and Nagy, 2003), constitutive androstane receptor (CAR) (Wei et al., 2000), and vitamin D receptor (VDR) (Matsunawa et al., 2012). These nuclear receptors are involved in regulating expression of several mammalian xenobiotic metabolizing CYPs but their role in fish xenobiotic metabolizing CYPs is less clear. Fish don't have a CAR; PXR, AHR and VDR are present in teleosts. In these cases it may be beneficial to investigate nuclear receptors on genes of interest to get a better understanding of the regulatory mechanism each gene.

The upstream region of the mammalian CYP3A gene contains a PXR/retinoid X receptor (RXR) heterodimer transcriptional response element; PXR is activated by a number of xenobiotics that result in increased expression of CYP3A4 (Kliewer et al., 2002). The mechanisms of CYP3A gene regulation in fish is poorly understood but expression of CYP3As in fish can be up regulated by some mammalian PXR agonists (Corcoran et al., 2012; Tseng et al., 2005; Wassmur et al., 2010). The available research suggests that expression of fish CYP3As is not as highly inducible as mammalian CYP3As by the PXR pathway (Wassmur et al., 2010). Yet, zebrafish CYP3C1 expression was not inducible by dexamethasone or rifampicin, two mammalian PXR agonists, suggesting that these don't function as PXR agonists in fish or fish CYP3C1 have a different regulatory mechanism (Corley-Smith et al., 2006). The mammalian PXR transcriptional response element consists of 2 half sites with a 6 nucleotide spacer; one half site is more highly conserved (Kliewer et al., 2002). Interestingly, neither half site are found upstream of some fish CYP3A genes and only the more conserved of the mammalian half site is found upstream of other fish CYP3A genes (Wassmur et al.,

2010). This data suggests that PXR may not function to regulate fish CYP3A as it does in mammals. More studies are required in order to understand if fish CYP3 expression is regulated by PXR but this is difficult due to the differences in response to PXR ligands by different fish species and the lack of a conserved PXR transcriptional response element upstream of CYP3A genes in fish.

There is some evidence that CYP3A in fish are regulated by the AHR (Chang et al., 2013; Tseng et al., 2005). The research on AHRs in fish is more clear than the PXR; it is a well studied nuclear receptor with conservation across vertebrates and is activated by planar, aromatic xenobiotics to induce transcription of xenobiotic metabolizing enzymes such as CYP1A in fish (Jönsson et al., 2010). The AHR is activated by PAHs, PCBs and other xenobiotics (Denison and Nagy, 2003). Observing how the fish CYP3s genes respond to a selection of AHR ligands may provide insight on whether the AHR regulates their expression. CYP3C1 was not responsive to TCDD suggesting a lack of role for the AHR in regulating this isoform (Corley-Smith et al., 2006).

In this study, there were sex difference in the expression of CYP3C and CYP3B isoforms (Figure 1-4, 6-8). Most notably, CYP3C1 and CYP3C4 expression was higher in the female gonads than in the male gonads (Figure 1; Figure 4). Sex difference in CYP content have been seen in rats where sex hormones contribute to the regulation of the CYPs (Waxman and Chang, 2005). Sex hormones, like estrogen, are cable of interacting with their receptors that then bind to nuclear receptors on the gene to promote transcription. Estrogen receptors bind estrogen responsive elements (EREs) whose sequences are well conserved and characterized in vertebrates (Gruber et al., 2004).

Indeed, the CYP3C genes appear to contain an upstream estrogen response element (ERE) in the 10kb upstream region of the gene indicating that that CYP3C expression could be estrogen regulated (McArthur, A. Personal communication). ERE response elements have been previously characterized upstream of CYPs that are estrogen responsive, such as CYP19 (Callard et al., 2001) and human CYP1B1 (Tsuchiya et al., 2004). This study measured mRNA levels of CYP3Cs and CYP3Bs in adult, reproducing fish and differences in expression across genders suggest that estrogen is contributing to the regulation of mRNA levels but this should be empirically tested.

Exposing fish to known activators of these nuclear receptors will provide insight on which ones are regulating the expression of each CYP3 in fish. Mining the 5' flanking region of the CYP3s for putative EREs, AHRs and PXRs would direct these in vivo exposure studies and provide clues as to which compounds may induce expression of fish CYP3s and may be metabolized by these enzymes.

FUTURE DIRECTIONS

This study resulted in the development of unique primers for the quantification of CYP3C1, CYP3C2, CYP3C3 and CYP3C4 in zebrafish and CYP3B4, CYP3B5 and CYP3B6 in medaka by quantitative real-time PCR. The expression of medaka CYP3Bs and zebrafish CYP3Cs was quantified in multiple adult organs of both male and female fish and CYP3Cs were quantified in multiple developmental stages. This study will contribute to the understanding of functions of CYP3s in fish. Some of the CYPs were

found in primary detoxifying organs such as the liver and intestine and their high expression in these organs implicates their function in xenobiotic metabolism.

Understanding if ligands of the PXR, AHR, ERE or other receptors are able to activate expression of CYP3s genes in fish will contribute to understanding the function of each fish CYP3. Current research on CYP3 regulation in fish is lacking but suggests that the regulation of CYP3s in fish varies from mammalian CYP3s (Celander et al., 1996; Wassmur et al., 2010) and varies between fish subfamilies. For example, CYP3C1 expression was not induced by AHR or PXR ligands (Corley-Smith et al., 2006), while fish CYP3As have been shown to be activated by both (Chang et al., 2013). Gender differences in expression of CYP3s suggest a role for estrogens and the ER in transcriptional regulation. Studying the upstream EREs in in vitro reporter assays, quantifying expression levels of the CYP3s in juvenile fish, and in vivo exposures of fish to estradiol could contribute to our understanding of how steroids may play a part in the regulation of CYP3A, 3B, 3C and 3D in fish.

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