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Basal and 3,3,'4,4',5-Pentachlorobiphenyl-Induced Expression of Cytochrome P450 1A, 1B and 1C Genes in Zebrafish

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Abstract

The cytochrome P4501C (*CYP1C*) gene subfamily was recently discovered in fish, and zebrafish (*Danio rerio*) *CYP1C1* transcript has been cloned. Here we cloned the paralogous *CYP1C2*, showing that the amino acid sequence is 78% identical to *CYP1C1*, and examined gene structure and expression of *CYP1A, CYP1B1, CYP1C1*, and *CYP1C2*. Xenobiotic response elements were observed upstream of the coding regions in all four genes. Zebrafish adults and embryos were exposed (24 hours) to 100 nM 3,3',4,4',5- polychlorinated biphenyl (PCB126) or 20 ppm acetone and subsequently held in clean water for 24 hours (adults) or 48 hours (embryos). All adult organs examined (eye, gill, heart, liver, kidney, brain, gut, and gonads) and embryos showed basal expression of the four genes. *CYP1A* was most strongly expressed in liver, whereas *CYP1B1*, *CYP1C1*, and *CYP1C2* were most strongly expressed in heart and eye. *CYP1B1* and the *CYP1C* genes showed an expression pattern similar to one another and to mammalian *CYP1B1*. In embryos *CYP1C1* and *CYP1C2* tended to have a higher basal expression than *CYP1A* and *CYP1B1*. PCB126 induced *CYP1A* in all organs, and *CYP1B1* and *CYP1C1* in all organs except gonads, or gonads and brain, respectively. *CYP1C2* induction was significant only in the liver. However, in embryos all four genes were induced strongly by PCB126. The results are consistent with *CYP1C1* and *CYP1C2*, as well as *CYP1A* and *CYP1B1*, being regulated by the aryl hydrocarbon receptor. While *CYP1A* may have a protective role against AHR agonists in liver and gut, *CYP1B1, CYP1C1*, and *CYP1C2* may also play endogenous roles in eye and heart and possibly other organs, as well as during development.

Keywords

cytochrome P4501 (CYP1); 3,3',4,4',5- polychlorinated biphenyl (PCB126); real time PCR; zebrafish

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Introduction

Cytochromes P450 (CYPs) are the principal enzymes catalyzing oxidative metabolism of toxicants, including important environmental chemicals. CYP1 family members (Nelson *et al.*, 1996) are prominent in oxidation of polycyclic aromatic hydrocarbons, aromatic amines, and a number of drugs. In mammals, the paralogous CYP1A1 and CYP1A2 and related CYP1B1 catalyze detoxication as well as activation of protoxicants and promutagens (Conney, 1982; Nebert *et al.*, 2004; Shimada and Fujii-Kuriyama, 2004). These CYP1s also catalyze oxidative steps in biosynthesis and degradation of endogenous regulatory molecules (*e.g.* steroids and eicosanoids; Nebert and Russell, 2002; Lewis, 2004). Exogenous chemicals can affect organisms by disrupting such endogenous processes directly, as well as after oxidation to more reactive metabolites. Differences in activity of the enzymes involved can influence susceptibility of organs, individuals, or species to toxicity, affecting the inference of mechanisms from experimental studies and possibly influencing clinical decisions.

Expression level is a major factor influencing the role of CYPs in substrate oxidation and effects. Knockout studies show that ligand-activated aryl hydrocarbon receptor (AHR) largely determines the induction of mammalian CYP1As and CYP1B1 (*e.g.*, Shimada *et al.*, 2002). AHR also regulates CYP1As in non-mammalian vertebrates; knock-down of AHR2 in zebrafish (*Danio rerio*) embryos abolishes induction of the single CYP1A by the potent AHR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Prasch *et al.*, 2003; Dong *et al.*, 2004). TCDD also induces zebrafish *CYP1B1* (Handley-Goldstone et al 2005), presumably via the AHR. Knockout and knockdown studies show that AHRs mediate TCDD toxicity in mammals and fish (Fernandez-Salguero *et al.*, 1996; Prasch *et al.*, 2003; Dong *et al.*, 2004).

Zebrafish are becoming an important model in developmental and adult toxicological studies (*e.g.*, teratogenesis and carcinogenesis). Developmental effects of TCDD have been studied extensively, and embryos exposed to TCDD show yolk sac and pericardial edema, slowed heart rate and blood flow, craniofacial malformations, swim bladder defects, neural degeneration, arrested gill development, and death (Henry *et al.*, 1997; Handley-Goldstone *et al.*, 2005; Carney *et al.*, 2006). One hypothesis is that toxicity involves reactive oxygen species produced by uncoupling of CYP1A by TCDD or coplanar polychlorinated biphenyls (PCBs) (Schlezinger *et al.*, 1999; Shertzer *et al.*, 2004; Schlezinger *et al.*, 2006). However, conflicting results regarding the role of CYP1A in TCDD toxicity (Teraoka *et al.*, 2003; Carney *et al.*, 2004), imply the involvement of other AHR regulated genes, possibly including other CYP1s. Understanding of the full complement of *CYP1* genes is required to discern their role in embryonic, systemic, and organ-specific effects of AHR agonists. Knowledge of *CYP1* genes is limited in zebrafish, especially in adults, where for many organs there is no information on any CYP1.

Recent discovery in fish of the new CYP1C subfamily (Godard *et al.*, 2005; Itakura *et al.*, 2005) highlights the lack of knowledge of CYP1s. There are two paralogs in fish, *CYP1C1* and *CYP1C2*, but the regulation, substrates, and biological functions of the *CYP1Cs* in the zebrafish model are unknown. Our aim was to determine the patterns of expression of *CYP1C1* and *CYP1C2* as compared to *CYP1A* and *CYP1B1* in adult organs and embryonic

zebrafish. As the other known CYP1s are in part AHR regulated, we investigated both basal expression and the responses to two potent AHR agonists, 3,3',4,4',5-PCB (PCB126), a major component of the AHR agonist activity in the environment, and β-naphthoflavone (βNF), a commonly studied agonist. We addressed the hypothesis that the *CYP1C* genes respond to AHR agonists as do *CYP1A* and *CYP1B1*. In order to accomplish these studies, we cloned and compared the sequence of *CYP1C2*, not previously cloned from zebrafish. We also compared gene structure of the *CYP1*s, including possible response element motifs that might support involvement of AHR in regulation of the new CYP1s, as well as of *CYP1B1* and *CYP1A* in zebrafish. The results indicate a broader AHR responsive set of CYP1s than previously known, and the expression patterns suggest that the new CYP1Cs may have physiological roles, apart from the involvement in xenobiotic responses.

Materials and methods

Animals

Sexually mature zebrafish of two wild-type varieties were used. Tubingen long fin (TL) zebrafish were progeny of TLs obtained from the laboratory of Mark Fishman, crossed with TLs raised from eggs obtained from the Zebrafish International Resource Center at the University of Oregon (Eugene, OR, USA). A second undefined variety was purchased at a local pet store (PS). Fish were maintained in the Woods Hole Oceanographic Institution zebrafish facility and the experimental procedures were approved by the Institutional Animal Care and Use Committee. The zebrafish were held in 2:1 female to male groups at a density of ≤5 fish/l in aerated, filtered and re-circulated system water (28.5 °C) in 3 or 10 l tanks in an Aquatic Habitat™ system (TL fish) or a 40-l glass tank (PS fish). The system water was composed of Instant Ocean™ (60 mg/l), sodium bicarbonate (50 mg/l), calcium sulfate (8.5 mg/l) and Kent's Freshwater Essentials™ (53 μl/l) in distilled water. Fish were fed 2× daily with brine shrimp (*Artemia salina*) and once daily with Omega One flakes (Omega Sea Ltd. Sitka, AK, USA). TL embryos were obtained from group breedings of 30 female and 15 male fish.

Cloning

CYP1C1 and *CYP1C2* transcripts were cloned from gill tissue of both the TL and PS zebrafish varieties. Gill arches were dissected and placed in RNAlater™ (Ambion, Austin, TX, USA). RNA was prepared using RNA stat-60 (Tel-Test Inc. Friendswood, TX, USA), mRNA was isolated from this RNA using the MicroPoly(A)Pure kit (Ambion), and cDNA was subsequently synthesized using the PowerScript™ Reverse Transcriptase (Clontech Laboratories Inc., Mountain View, CA, USA) and oligo dT primers (Operon Biotechnologies Inc., Huntsville, AL, USA). Full-length *CYP1C1* and *CYP1C2* cDNAs were amplified by using PCR primers to the 3' and 5' untranslated regions (UTRs; Table 1; Operon Biotechnologies Inc.) and Advantage™ cDNA PCR Kit (Clontech Laboratories Inc.). *CYP1C1* and *CYP1C2* 1600-base pair PCR products were resolved on a 1% agarose gel and then isolated, cloned, and sequenced using previously described procedures.

Exposure of adult zebrafish

In three sets of experiments, fish were exposed via ambient water to 1 μM βNF, 100 nM PCB126, or a dose range of PCB126 (between 0.3 and 100 nM). The concentrations given are nominal.

βNF

Mixed groups of male and female fish of the TL and PS varieties (6 fish, *i.e.*, 3.5–3.7 g biomass per liter) were placed in two cling-wrap-covered glass beakers filled with continuously aerated zebrafish system water (28 °C). Aliquots of βNF (Sigma-Aldrich Inc. St. Louis, MO, USA) dissolved in acetone or acetone only were added to the beakers, yielding 1 μM βNF and 20 ppm acetone. After 24 h of exposure, the dosing solutions were renewed, and 24 h later the fish were killed by decapitation. Sex was confirmed by gonad examination. Gill and liver were dissected and organs from individual fish were frozen in liquid nitrogen and stored at −80 °C.

PCB126

Male and female TL or PS zebrafish (6 fish, corresponding to 4.2–4.6 g and 1.6–2.5 g of biomass per liter, respectively) were exposed to PCB126 (Cambridge Laboratories Inc., Andover, MA, USA) as described above for βNF. Aliquots of PCB126 dissolved in acetone or acetone only were mixed into the water, yielding 100 nM PCB126 and 20 ppm acetone. Following 24 h of exposure, the dosing solutions were replaced with clean zebrafish system water, and 24 h later the fish were killed by decapitation and sex confirmed. Gill and liver from two similarly exposed TL fish of the same sex were pooled. Four pools were analyzed (2 male and 2 female). A wider range of organs was examined from PS fish, *i.e.*, liver, gut, kidney, ovary, testes, heart, brain, eye, and gill. In this experiment organs from multiple (3–10) fish of the same sex were pooled to obtain sufficient amounts of cDNA for the analyses. Four replicate exposures were performed resulting in four male and four female pooled samples for each organ. The dissected organs were preserved in RNAlater.

PCB126 concentration-response

PS zebrafish were exposed to doses of PCB126 ranging from 0.3 nM to 100 nM (and 20 ppm acetone), or 20 ppm acetone alone, as above (6 fish, 2.0–2.7 g biomass per liter). Expression of the suite of *CYP1* genes was analyzed in gills and livers from individual fish.

Exposure of zebrafish embryos

Fertilized TL zebrafish eggs (33±5) were placed in 10 cm glass Petri dishes containing 25 or 30 ml of 0.3× Danieau's solution. At 8 hours post-fertilization (hpf), aliquots (2.5 or 3.0 μl) of PCB126 in acetone, or acetone alone, were added to the dishes yielding 100 nM PCB126 and 100 ppm acetone (nominal concentrations). The embryos were incubated at 28 °C. At 32 hpf, the dosing solutions were replaced with fresh 0.3× Danieau's solution and any dead embryos were removed; mortality was normally 2 embryos or fewer per dish. No difference in mortality was observed between controls and exposed fish, and subsequent to 32 hpf no mortality was observed. The embryos were held for an additional 48 h with replacement of the Danieau's solution after 24 h. At 80 hpf the experiment was terminated. All embryos in a

dish were pooled, frozen in liquid nitrogen, and stored at −80 °C. Ten biological replicates were analyzed.

Quantification of CYP1 mRNA

RNA was isolated as described above and the isolates were DNase treated (TURBO DNA-free™ kit, Ambion). The quantity and quality of the RNA were determined spectrophotometically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using the Omniscript™ Reverse Transcriptase kit (Qiagen Inc., Valencia, CA, USA), random hexamer primers (Operon Biotechnologies Inc.) and the RNasin® RNase inhibitor (Promega, Madison, WI, USA).

Gene specific primers for *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, *ARNT2* and *β-actin* cDNA were synthesized by Operon Biotechnologies Inc. (Table 1). The ARNT2 primers could amplify identical sequences in *ARNT2a*, *ARNT2b*, and *ARNT2c*. Real time PCR was performed using the iQ SYBR Green Supermix (according to the manufacturers instructions) and an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR reaction mixtures consisted of iQ SYBR Green Supermix, primer (5 pmol each of forward and reverse primer) and cDNA (derived from 0.1 μg total RNA). In each sample the genes were analyzed in duplicate with the following protocol: 95°C for 4.5 min; 95°C for 15 s and 62 °C for 1 min (40 cycles). To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run.

Calculations and statistics

The cloned *CYP1* zebrafish cDNA sequences were aligned and compared to sequences obtained from the *Danio rerio* genome assembly version 6 (Zv6) in Ensembl (http://www.ensembl.org/Danio_rerio/index.html) using Sequecher 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA) and GCG 10 (Accelrys, San Diego, CA, USA). Long range genomic alignments were performed using Vista (Mayor *et al.*, 2000). Promoter region mapping was performed using GCG and MatInspector (Cartharius *et al.*, 2005) using the conservative consensus XRE (KNGCGTG) and the MatInspector database. Phylogenetic analyses were performed using MrBayes (Ronquist and Huelsenbeck, 2003).

No significant sex difference in *CYP1* gene expression was detected. Therefore the data for male and female fish were combined in the calculations, with the exception that the testes and ovary data were treated separately. For each reaction, relative mRNA expression of the *CYP1* genes was calculated according to the $E^{\Delta\Delta Ct}$-method (Livak and Schmittgen, 2001), using *β-actin* as a reference gene (unless otherwise indicated). PCR efficiency (E) was determined with the LinRegPCR program (Ramakers *et al.*, 2003). Outliers were excluded based on the Grubbs test. Data were log-transformed and statistical differences in gene expression between groups were determined by one-way ANOVA followed by Tukey's, Bonferroni's, or Dunnet's multiple comparison tests (Prism 4 by GraphPad Software Inc., San Diego, CA, USA). The post-hoc test used for a specific data set is given in the figure legends. Lowest observed effect concentration (LOEC) values for PCB126 to induce the *CYP1* genes were determined by one-way ANOVA followed by Dunnet's multiple

comparison test. Values for EC_{50}, *i.e.*, the PCB126 concentration causing half maximal *CYP1* gene induction, were calculated using the curve fitting routine of GraphPad Prism for nonlinear regression sigmoidal dose–response with variable slope.

Results

Cloned CYP1C1 and CYP1C2 transcripts

Using primers based on a predicted *CYP1C2* sequence derived from the zebrafish genome (Zv6), we were able to clone sequences from gill of both the TL and PS zebrafish. In parallel, we repeated the cloning and sequencing of *CYP1C1* from both TL and PS fish, using primers based on the *CYP1C1* sequence published by Godard et al. (2005). The aligned zebrafish TL CYP1C1 and CYP1C2 protein sequences (Fig 1) had a 78 % amino acid sequence identity. Two amino acid sequences, one 43 and one 124 residues long (*i.e.*, K45 to G87 and W327 to L450 in CYP1C2; Fig 1), and the heme binding signatures were identical in the two CYP1Cs.

Phylogenetic analysis showed that the zebrafish CYP1C2 is orthologous to CYP1C2 of other fish (pufferfish - *Takifugu rubripes* and scup - *Stenotomus chrysops*). The deduced TL CYP1C1 amino acid sequence differed from the Ensembl CYP1C1 sequence (ENSDARG00000058980) in four positions (E265, F266, P283, and D443), whereas the cloned TL *CYP1C2* differed from the predicted zebrafish CYP1C2 by one amino acid (S444; Fig 1). Slight differences between the TL and PS sequences also were observed in both CYP1C1 and CYP1C2. The differing amino acids in the CYP1C1 sequences were I/V29 and D/N443 (TL/PS respectively) and those in the CYP1C2 sequences were L/M74, K/R223 and S/N259. K223 is located in a putative substrate recognition site in the F/G loop (SRS2: Fig 1).

Gene structure

Analysis of the zebrafish genome shows that both *CYP1C1* and *CYP1C2* are single exon genes, located immediately adjacent to one another (4 kB apart) on chromosome 17 (Fig 2). Canonical xenobiotic response elements (XREs), which are binding sites for the AHR/ARNT heterodimer complex, were found in the promoter regions in all four of these zebrafish *CYP1* genes(Fig 2; Table 2). In the *CYP1C1* and *CYP1C2* genes, XREs are located within two regions (approximately 1 kb each) upstream of the start sites, one upstream of *CYP1C2*, and one between the coding regions of the two genes. Searching genome databases showed that both regions are conserved across four fish species (zebrafish, pufferfish, medaka - *Oryzias latipes*, and three-spined stickleback - *Gasterosteus aculeatus*; data not shown). In zebrafish these conserved regions are located approximately 1.5 kB upstream of *CYP1C1* and 8 kB upstream of *CYP1C2*. Other possible response elements in these conserved regions in *CYP1C1* and *CYP1C2*, or in the 1-kB region immediately adjacent to the transcription initiation sites, include those binding E2F, nuclear respiratory factor 1 (NRF1) and cytokine-induced nuclear factor-kappa B (NFκB). In addition, putative binding sites for peroxisome proliferator-activated receptor/retinoid X receptor (PPAR/RXR), estrogen related receptor (ERR), retinoic acid receptor (RAR), RAR-related orphan receptor (ROR), and 1.25-$(OH)_2$-vitamin D_3/retinoid X receptor (VDR/RXR) all were found

upstream of *CYP1C2*. Similar searches of the upstream proximal promoter regions of *CYP1B1* and *CYP1A* revealed other putative response elements, including in particular estrogen receptor (ER).

Basal and βNF-induced CYP1 expression in adult zebrafish

Initial studies examined expression of CYP1s in two organs (liver and gill) in TL and PS fish exposed to 1 μM βNF (or the carrier) via water. This βNF concentration has been shown to be high enough to induce ethoxyresorufin *O*-deethylase (EROD) activity in gills of various species of fish (Jönsson *et al.*, 2002; Jönsson *et al.*, 2003).

No difference was observed between the two zebrafish varieties in the basal expression of *CYP1* genes in gill or liver (Fig 3 a–h). Both varieties showed induction of *CYP1A* and *CYP1B1* in gills following exposure to βNF (Fig 3a and b). However, only the PS fish showed a significant induction of *CYP1C1* by βNF in gills (Fig 3 c), and neither PS nor TL fish showed induction of *CYP1C2* in gills (Fig 3 d). In the liver, none of the *CYP1* genes were significantly induced by this treatment with βNF, in either the TL or the PS variety (Fig 3 e–h).

Basal CYP1 gene expression in various tissues of zebrafish

Expression of *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* was detected in all organs examined in control zebrafish (PS), *i.e.*, brain, eye, gill, heart, liver, kidney, gut, testes, and ovary. Comparing the relative expression of the four *CYP1* genes in these nine organs requires a similar concentration of a reference transcript in all organs. The appropriateness of using *β-actin* as a reference gene in this way was assessed by comparing data normalized to *β-actin* with data normalized to *ARNT2*. This gave partially conflicting results, *e.g.*, compared to other organs brain appeared to have higher *CYP1C2* expression when *β-actin* was used as a reference gene and a lower expression when *ARNT2* was used (Fig 4). This indicates that the mRNA concentration of either *β-actin* or *ARNT2*, or both, can vary in different tissues. However, irrespective of which reference gene was used, liver and gut were among the organs having the highest basal expression of *CYP1A* (Fig 4 a) and heart and eye tended to have the highest basal expression of *CYP1B1* (Fig 4 b). Similarly, heart and eye were among the organs having the highest basal expression of both *CYP1C1* and *CYP1C2* (Fig 4 c and d). Gill exhibited a relatively high basal expression of *CYP1C1* and kidney a relatively high basal expression of *CYP1C2*. Ovary was among the organs having the lowest expression of all *CYP1* genes. The raw Ct values show higher transcript levels of *β-actin* than of *ARNT2* in all adult organs as well as in whole embryos.

When the basal levels of the four *CYP1* transcripts were compared within an organ in PS zebrafish, a higher level of *CYP1A* than of *CYP1B1*, *CYP1C1*, or *CYP1C2* was observed in the organs of the abdominal cavity, *i.e.*, liver, kidney, gut, and ovary (Fig 5 a–d). In liver the relative expression of *CYP1A* was more than 100-fold higher than the expression of the other *CYP1* genes. In gills and heart the *CYP1A* expression was higher than that of *CYP1B1* and *CYP1C2* and similar to that of *CYP1C1* (Fig 5 f and g), and in testes, eye, and brain no significant difference in basal level of the four *CYP1* genes was observed (Fig 5 e, h, and i).

TL zebrafish exhibited patterns of basal expression of *CYP1* genes in gill and liver that were similar to those in PS fish, *i.e.*, the expression of *CYP1B1*, *CYP1C1*, and *CYP1C2* was 19±8, 150±50, and 12±4 % of *CYP1A* in gills and 0.07±0.03, 1.5±1.0, and 0.3±0.1 % of *CYP1A* in liver (mean±SEM) in zebrafish TL.

PCB126-induced CYP1 gene expression in various organs of zebrafish

To assess the organ distribution of *CYP1* gene induction, fish were exposed to PCB126 at 100 nM in the water, a concentration we have seen to produce edema in embryos and thus likely high enough to ensure an effect on inducible genes. This concentration of PCB126 had no obvious effect on morphology or behavior of adult fish. *CYP1A* was induced by PCB126 (relative to the carrier control) in all organs examined (Fig 6), and *CYP1B1* was induced in all organs except gonads in PS fish (Fig 6 a–c and f– i). Induction of *CYP1C1* was observed in all organs except gonads and brain (Fig 6 a–c and f–h). In contrast, *CYP1C2* was significantly induced by PCB126 only in the liver, although a tendency for induction (not significant) was also observed in kidney and possibly gonads (Fig 6 a, b, d, and e). The patterns of PCB126-induced *CYP1* expression were basically similar in gills and liver of the two zebrafish varieties, except that the PS fish tended to show lower values for *CYP1A* and *CYP1B1* and higher values for *CYP1C1* and *CYP1C2* than was observed in TL fish. In TL fish the fold induction of *CYP1A*, *CYP1B1*, and *CYP1C1* was 160±50, 100±25, and 5±1 (all $p<0.001$), respectively, in gills, and 35±18, 35±4 (both $p<0.001$), and 6±1 ($p<0.05$) in liver (mean±SEM). In both gills and liver, *CYP1C2* tended to show a slight increase (*i.e.*, 2.0±0.5 fold of the control) after PCB126 exposure, although this was not significant in either organ ($p>0.05$).

We also performed a dose response experiment, to determine whether responsiveness to PCB126 varies among the four *CYP1* genes. The concentration-dependent effects of PCB126 on *CYP1* expression were examined in gill (*CYP1A* expression) and liver (expression of all four genes). Induction of *CYP1A* and *CYP1B1* showed similar concentration-dependence (Fig 7 a–b). *CYP1C1* was strongly and similarly induced at the three highest doses of PCB26, implying a dramatically increased expression between 3 and 10 nM PCB126. The EC_{50} values for PCB126 induction of *CYP1A*, *CYP1B1*, and *CYP1C1* in liver were 13, 14, and 7 nM, respectively. The EC_{50} for *CYP1A* induction in gill was 32 nM. The PCB126-EC_{50} values for *CYP1* gene induction in the liver were not statistically different from one another, nor were the EC_{50} values for *CYP1A* induction in gill and liver significantly different. In liver the LOEC for *CYP1A*, *CYP1B1* and *CYP1C1* induction were 30, 10 and 10 nM, and in the gill the LOEC for *CYP1A* was 10 nM PCB126.

Basal and PCB126-induced CYP1 gene expression in zebrafish embryos

In addition to *CYP1* gene expression in adult zebrafish, we also studied the expression of these genes in 80-hpf embryos exposed to the carrier (controls), or to 100 nM PCB126. In controls the *CYP1C1* expression was significantly higher than the expression of *CYP1A* and *CYP1B1* (Fig 8 a). *CYP1C2* expression also tended to be higher than expression of *CYP1A* or *CYP1B1*, although this was not significant. Following exposure to PCB126 a strong induction of all four *CYP1* genes was observed in the embryos (Fig 8b).

Discussion

With the discovery of the *CYP1Cs*, the suite of *CYP1* genes possibly involved in AHR agonist effects is expanding. Here we present a comprehensive analysis of the expression of the CYP1s in the zebrafish model, including the recently discovered CYP1Cs, showing that *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2* all are expressed in multiple organs of control adult zebrafish, and also appear to be constitutively expressed in zebrafish embryos. *CYP1A* has been widely studied in fish (e.g., Arinç *et al.*, 2000) but there are very few studies of fish *CYP1B* (Leaver and George, 2000; El-kady *et al.*, 2004b; El-kady *et al.*, 2004a; Willett *et al.*, 2006) or the *CYP1Cs* (Godard *et al.*, 2005; Itakura *et al.*, 2005; Wang *et al.*, 2006). Our results showing appreciable basal levels of expression of the *CYP1C*s and *CYP1B1* in some organs and in embryos, suggest that there are endogenous roles for the *CYP1B1* and *CYP1Cs*. Furthermore, all four CYP1s were induced by the potent AHR agonist PCB126. These results and the discovery of several putative XREs in the *cis*-regulatory regions of the *CYP1C* genes, suggest that they are AHR-regulated, as are the *CYP1A*s and *CYP1B1*. However, whereas *CYP1C2* showed a strong induction response in embryos, only a minor response was seen in adults, indicating that *CYP1C2* has a difference in regulation from the other *CYP1* genes.

Expression of *CYP1* genes was studied in two different varieties of adult zebrafish, *i.e.*, Tubingen Longfin (TL), which is widely used in developmental studies, and a more “common” zebrafish, purchased in a local pet store (PS). The two varieties exhibited largely similar patterns of basal and induced *CYP1* gene expression, suggesting that the current results reflect responses that can be expected in zebrafish generally. However, there were some CYP1C sequence differences between the TL and PS fish (Fig. 1).

Basal expression of CYP1 genes in adult zebrafish

CYP1 genes likely play physiological functions in zebrafish during their whole lifespan, as all four genes showed a basal expression in embryos as well as in all organs we examined in adult controls. However, it seems likely they have different roles. In particular, there seems to be a distinct difference between *CYP1A* on one hand and *CYP1B1* and the *CYP1C* genes on the other, as indicated by the difference in expression patterns and inducibility by PCB126. Thus, of the four *CYP1* transcripts analyzed, *CYP1A* was the most abundant in organs in the abdominal cavity (*i.e.* liver, kidney, and gut), whereas other organs exhibited more comparable levels of expression of the four genes. The marked *CYP1A* expression in liver, kidney, and gut may reflect functions associated with the role of these organs in nutrient uptake and processing of body waste products, *i.e.*, detoxification of endogenous metabolites and food-derived AHR agonists. Other studies suggest that CYP1A may play an important role in eliminating endogenous AHR ligands (Nebert *et al.*, 2000).

CYP1B1, *CYP1C1*, and *CYP1C2* all showed a marked similarity in organ distribution patterns. The similar expression patterns suggest that there may be functional similarities between the *CYP1B* and *CYP1C* genes. Phylogenetic analyses indicate that the *CYP1B* and *CYP1C* genes form a monophyletic clade, and thus are more closely related to each other than they are to the *CYP1As*. We observed a low coefficient of evolutionary functional divergence between the CYP1Bs and the CYP1Cs (Goldstone, unpublished) relative to the

comparison with the CYP1As, calculated with the site-specific rates of amino acid substitution using the method of Gu and Vander Velden (2002). *CYP1C1* and *CYP1C2* are located immediately adjacent to one another on zebrafish chromosome 17, suggesting that the fish-specific *CYP1C* duplication is independent from, and likely more recent than, the separate whole genome duplication event in the fish lineage (Goldstone et al, unpublished results). Thus, *CYP1C1* and *CYP1C2* may have similar functions due to a short divergence time.

The basal expression patterns for zebrafish *CYP1B1, CYP1C1*, and *CYP1C2* we observed are similar to that for *CYP1B1* in mammals. Thus, the expression levels of the three fish genes were relatively high in heart and relatively low in ovary, like the expression of *CYP1B1* in human (Choudhary *et al.*, 2005). The stronger expression of *CYP1A* than *CYP1B1, CYP1C1*, and *CYP1C2* in zebrafish liver also agrees with the relative expression of *CYP1A1* and *CYP1B1* observed in human liver (Choudhary *et al.*, 2005). *CYP1B1*, *CYP1C1*, and *CYP1C2* all showed high basal expression in zebrafish eye. *CYP1B1* is essential for normal development of the mammalian eye (Libby *et al.*, 2003). The cellular localization of *CYP1B1*, *CYP1C1*, or *CYP1C1* in the fish eye has not been studied, but in mammals CYP1B1 is present in corneal and ciliary epithelia and in retina (Choudhary *et al.*, 2006). The similar expression patterns of *CYP1B1/1C* genes in zebrafish and *CYP1B1* in mammals suggest that endogenous functions of these genes may be served similarly in different vertebrate groups. Given the absence of *CYP1C* genes in mammals, it is possible that some of the CYP1C functions have been adopted by mammalian CYP1B1. The fact that the mammalian and fish *CYP1B1s* appear to diverge from one another may imply that the mammalian and fish *CYP1B1s* have evolved different functions.

Induced expression of CYP1 genes in adult zebrafish

CYP1A transcript was induced by PCB126 in all organs that we studied in zebrafish. In TCDD-exposed zebrafish increased immuno-staining for CYP1A protein was observed only in liver and kidney (Buchmann *et al.*, 1993). However, although it is possible that some posttranscriptional regulation of *CYP1A* contributes to the differences between our findings and those of Buchmann et al, real time PCR is a more sensitive quantitative method than immunohistochemistry. Other studies using immunohistochemical detection have shown that *CYP1A* is induced at least in some cells (*e.g.*, endothelial cells) in all organs of fish (*e.g.*, Smolowitz *et al.*, 1991; Van Veld *et al.*, 1997). Our results thus support a ubiquitous expression of *CYP1A* but also indicate a large variation in the magnitude of induction among organs, *i.e.*, from 5-fold in gut to over 100-fold in brain and testes.

CYP1B1 and *CYP1C1* also were induced by PCB126 in most organs we studied in zebrafish. Previous studies have shown induction of *CYP1B1* in a variety of organs in fish and mammals exposed to AHR agonists (*e.g.*, Bhattacharyya *et al.*, 1995; El-kady *et al.*, 2004a; Willett *et al.*, 2006). More recently, *CYP1C1* was reported to be induced in many of the same organs we examined, in killifish exposed to benzo[a]pyrene (B[a]P) (Wang *et al.*, 2006). The magnitude of induction of zebrafish *CYP1B1* and *CYP1C1* was relatively low in most organs, although gill showed a 41-fold induction of *CYP1B1* and kidney a 27-fold induction of *CYP1C1*. To our knowledge, our results with *CYP1C2* are the first to

demonstrate induction of this new gene. However, in contrast to *CYP1C1*, which was strongly induced by PCB126 in many organs, only minor changes in the levels of expression of *CYP1C2* were seen following exposure either to PCB126 or βNF, indicating a low responsiveness of *CYP1C2* to AHR agonists in adult zebrafish. This difference in regulation suggests different metabolic roles for the two *CYP1Cs*.

CYP1 gene expression in zebrafish embryos

In control embryos *CYP1C1* and *CYP1C2* tended to have 2-fold higher basal expression than *CYP1A* and *CYP1B1*. Similar results showing higher expression for *CYP1C1* relative to *CYP1A* were observed in killifish embryos (Wang *et al.*, 2006). These results hint that the CYP1C genes play a role in fish embryo development. The finding that PCB126 caused a strong induction of *CYP1C2* in embryos but only a small effect on *CYP1C2* expression in adults suggests further that *CYP1C2* is regulated differently in embryos than in adults.

The low basal or constitutive expression of *CYP1A* transcript in control zebrafish embryos is similar to results obtained previously (Andreasen *et al.*, 2002). However, a remarkable induction (300-fold) was observed following exposure to PCB126 at 100 nM. In comparison, *CYP1B1*, *CYP1C1*, and *CYP1C2* increased less (18-, 25- and 41-fold, respectively) in embryos exposed to PCB126. A higher responsiveness of *CYP1A* than of *CYP1B1* was reported in a cDNA microarray study of zebrafish embryos exposed to TCDD, *i.e.*, *CYP1A* and *CYP1B1* were induced 63- and 5-fold, respectively (Handley-Goldstone *et al.*, 2005). Killifish embryos exposed to B[a]P exhibited a 330-fold induction of *CYP1A* and a 18-fold induction of *CYP1C1* (Wang et al., 2006). The large difference in relative induction between *CYP1A* and the *CYP1B1* and *CYP1C* genes is in part a reflection of the lower basal level of *CYP1A* in embryos. Nevertheless, in PCB126-exposed embryos the *CYP1A* transcript seems to be more abundant than the *CYP1C* transcripts and much more abundant than the *CYP1B1* transcript.

CYP1 induction by βNF and PCB126 in zebrafish liver

Somewhat surprisingly, *CYP1A*, *CYP1B1*, and *CYP1C1* transcripts were induced in gills but not in liver of PS zebrafish exposed to βNF in the water, while similar exposure to PCB126 induced these genes in both organs. Toxicokinetic processes could be involved in the difference and in the lack of effect of βNF. PCB126 is considered a very slowly metabolized PCB congener, while βNF is well metabolized by CYP1A in fish (unpublished results). βNF conceivably could have been metabolized by CYP1A induced in gill and other organs such that the concentrations of parent compound reaching the liver were too low to induce transcription of CYP1A there. If there were induction early during the exposure that declined by sampling time, it would imply a rapid decay of CYP1A mRNA; human *CYP1A1* transcript has a 7-hour half-life (Ciolino and Yeh, 1999). However, the 1 μM concentration of βNF we used (about 270 μg per liter ×2), with the 3.6 g of fish/liter would be equivalent to a dose of 150 mg/kg, assuming that all the βNF was accumulated. 100% accumulation is unlikely, yet even at 10 % accumulation, an extensive metabolism of βNF would be required to reduce the concentration to ineffective levels. By comparison, in studies with another rapidly metabolized inducer, B[a]P, Van Veld *et al*. (1997) found that *Fundulus heteroclitus* exposed to B[a]P in the water at a much lower dose (10 μg per liter = 40 nM) than our dose

of βNF, was able to induce CYP1A protein in the liver at four days, and in rainbow trout (*Oncorhynchus mykiss*) an even lower dose, 1 nM of waterborne B[a]P, induces liver EROD 8-fold (Jönsson *et al.*, 2006a). Troxel et al. (1997) observed rapid induction followed by a decline of EROD activity in the liver of zebrafish injected (ip) with 150 mg βNF/kg, *i.e.*, the magnitude of EROD activity was halved from day 1 to day 2. While this might be interpreted as reflecting a rapid metabolism of βNF by the liver and decline in CYP1A, it also is possible that increasing βNF doses may have been somehow inhibitory to CYP1A expression, as well as to CYP1A activity. Jönsson *et al.* (2006b) saw that 10 μM βNF strongly suppressed EROD induction in primary gill cells in culture. Moreover, βNF at 3.6 μM was reported not to induce CYP1A activity in ZF-L cells, a TCDD-responsive zebrafish liver cell line (Miranda *et al.*, 1993), while Evans *et al.* (2005), saw a strong induction of CYP1A transcript in ZF-L cells treated with 10 μM βNF. Determining actual rates of βNF metabolism, the content of βNF residues in liver of exposed fish, and whether βNF might suppress CYP1A transcript expression are required to resolve these issues.

CYP1 gene regulation

Consensus XRE sequences were identified upstream of the coding regions of both *CYP1C1* and *CYP1C2*. The number of potential XREs upstream of the *CYP1C* genes is comparable to that in the *CYP1B1* gene, but fewer than in the *CYP1A* gene, where there are a large number of upstream XREs (Fig 2; Table 2). Similar consensus XRE sequences have been shown to be functional in the eel (*Anguilla japonica*) *CYP1A1* gene (Ogino *et al.*, 1999). While the functionality of these XRE sequences in zebrafish needs to be confirmed, the number and position of the XREs in the *CYP1C* genes are consistent with regulation of these genes by the AHR. The fact that both *CYP1C* genes were induced in embryos by the potent AhR agonist PCB126 also indicates regulation via the AHR. Notably, the EC_{50} values we observed for PCB126 are of a similar order of magnitude as those reported for CYP1A mRNA induction in ZF-L cells (Henry *et al.*, 2001). Furthermore, the EC_{50} value for PCB126-induced *CYP1C1* expression in liver was similar to those of *CYP1A* and *CYP1B1* suggesting a similar mechanism for induction of the three *CYP1* genes, *i.e.*, via the AHR. The concentration-dependent induction of *CYP1C2* was not significant, although the calculated EC_{50} value for PCB126 was in the same range as those of the other three *CYP1* genes, indicating that *CYP1C2* also is AHR regulated. However it may also be controlled by additional factors.

In mammals, numerous CYP genes including CYP1s are expressed in embryos at various developmental stages (Choudhary *et al.*, 2004). Nuclear receptors are known to modulate transcription of CYPs through complex mechanisms involving both activation and repression. In mammals, CYP1 enzymes have been suggested to function in the synthesis and elimination of nuclear receptor ligands, thus serving to regulate signal transduction (Choudhary *et al.*, 2004). Changes in the level of various CYP1 enzymes could affect growth, morphogenesis, and homeostasis (Choudhary *et al.*, 2004). The presence of putative response elements for various transcription factors (including E2F, NRF1, NFκB, PPAR/RXR, ERR, RAR, ROR, and VDR/RXR) upstream of the coding start sites in the *CYP1C* genes support the notion that the CYP1Cs could participate in a wide range of endogenous functions. These might include cell proliferation, respiration, inflammation, apoptosis, and

fatty acid metabolism. Although the functionality of these possible response elements is not confirmed, they deserve further study.

Conclusion

Here we report the first comprehensive study of expression of the full suite of inducible CYP1 family genes, in zebrafish adults and embryos. *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2* transcripts were detected in all adult organs analyzed, as well as in embryos. Significant induction of *CYP1A*, *CYP1B1* and *CYP1C1* was detected in most adult organs studied, whereas *CYP1C2* was less inducible than the other genes. Clearly the *CYP1Cs* are regulated at least in part via the AHR. *CYP1C* genes occur in amphibians and birds, as well as fish, but have been lost in mammals (Goldstone, unpublished). Thus, an understanding the regulation and functions of *CYP1Cs* becomes important to a general understanding of the mechanisms of toxic action of AHR agonists, as well as to understanding of possible endogenous functions of the *CYP1* genes. Given their phylogenetic distribution, knowledge of the *CYP1Cs* in zebrafish or other non-mammalian models could point to physiological functions of the CYP1 family that may be more difficult to discern in mammals.

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Fig 1.

Alignment of *CYP1C1* and *CYP2C2* sequences derived from the *Danio rerio* genome assembly version 6 (Zv6; *ENS 1C1* and *ENS 1C2*), and cloned *CYP1C1* and *CYP2C2* cDNA sequences from zebrafish Tubingen Longfin (TL) and zebrafish purchased in a local pet store (pet store-obtained zebrafish, PS). Small rectangles, amino acids that are identical in sequences cloned from both TL and PS zebrafish but differ from the corresponding Zv6 sequence; pentagons, amino acids in sequences cloned from either TL or PS zebrafish that differ from the other strain and from the Zv6 sequence; “heme bind” means heme binding site; shaded areas labeled SRS1-6 = Substrate binding sites 1–6.

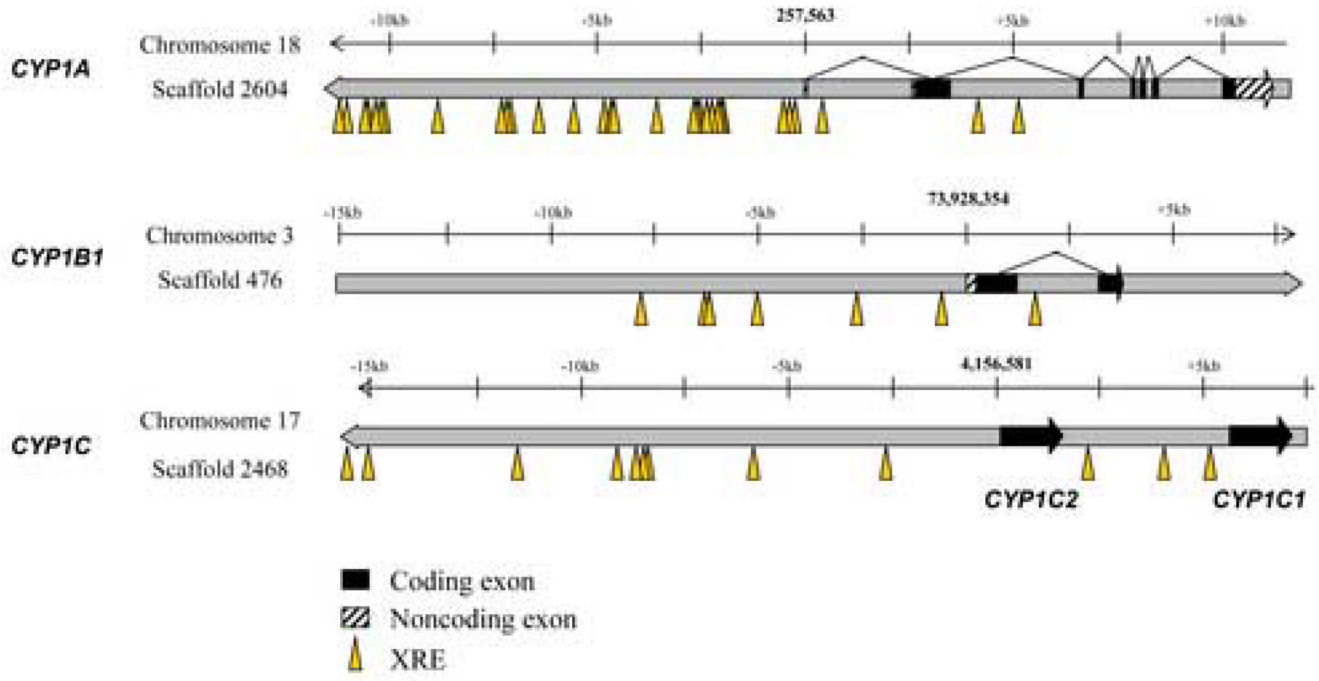

Fig 2.

Xenobiotic response elements (XRE) and gene structure in the *CYP1* gene promoter regions observed in the *Danio rerio* genome assembly version 6 (Zv6). The degenerate sequence KNCGGCGT was used to map the XREs.

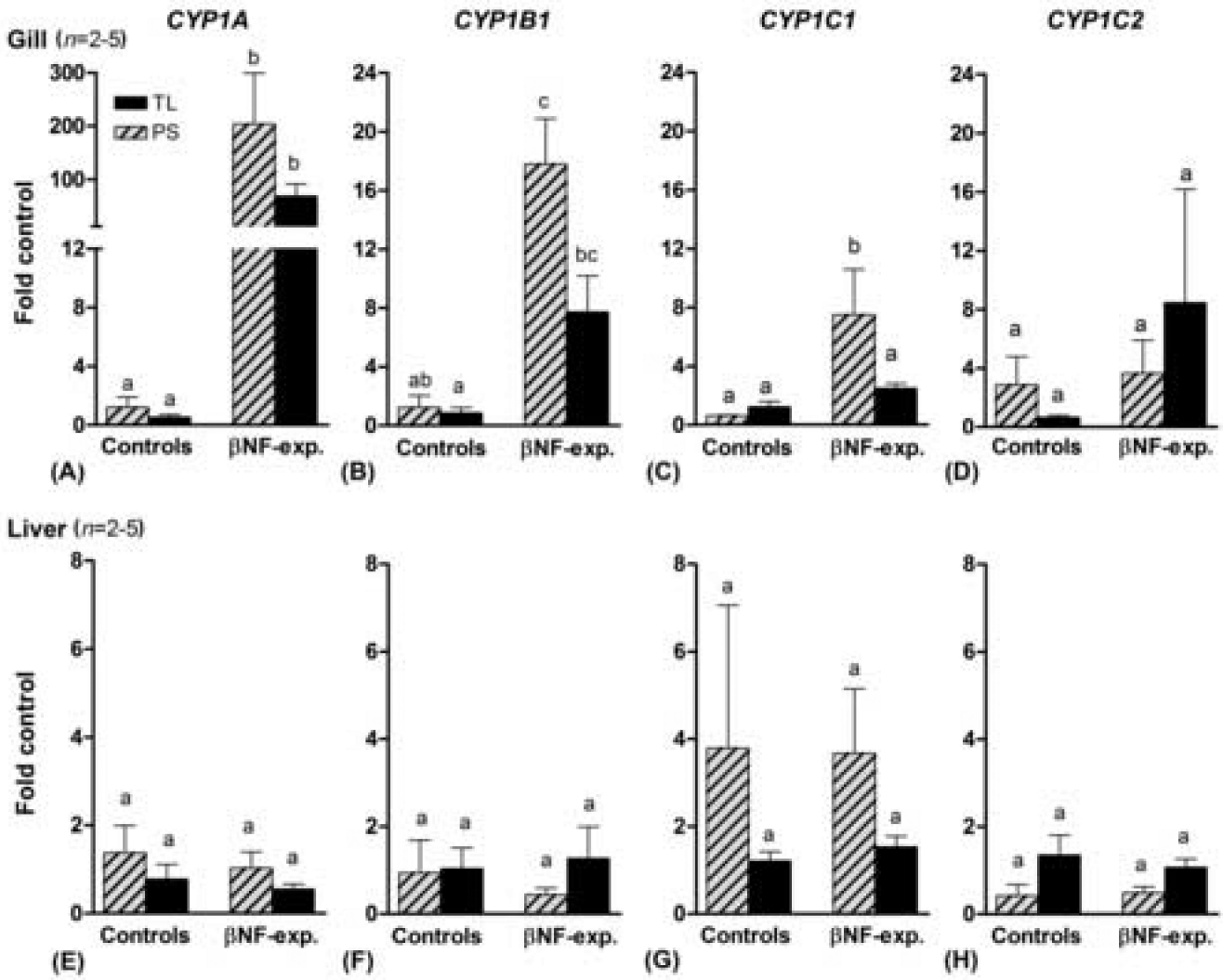

Fig 3.

CYP1A, *CYP1B1*, *CYP1C1*, and *CYP1C2* expression in gills and liver of zebrafish TL (black bars) and PS (hatched bars) following exposure to β-naphthoflavone (1 μM) or the carrier (20 ppm acetone; mean ± SEM). For each *CYP1* gene, basal and induced expression was calculated using the mean value of controls of both varieties as a calibrator. Differences between groups were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test. A statistical difference between groups at $p < 0.05$ is indicated by differences in the letters above the bars.

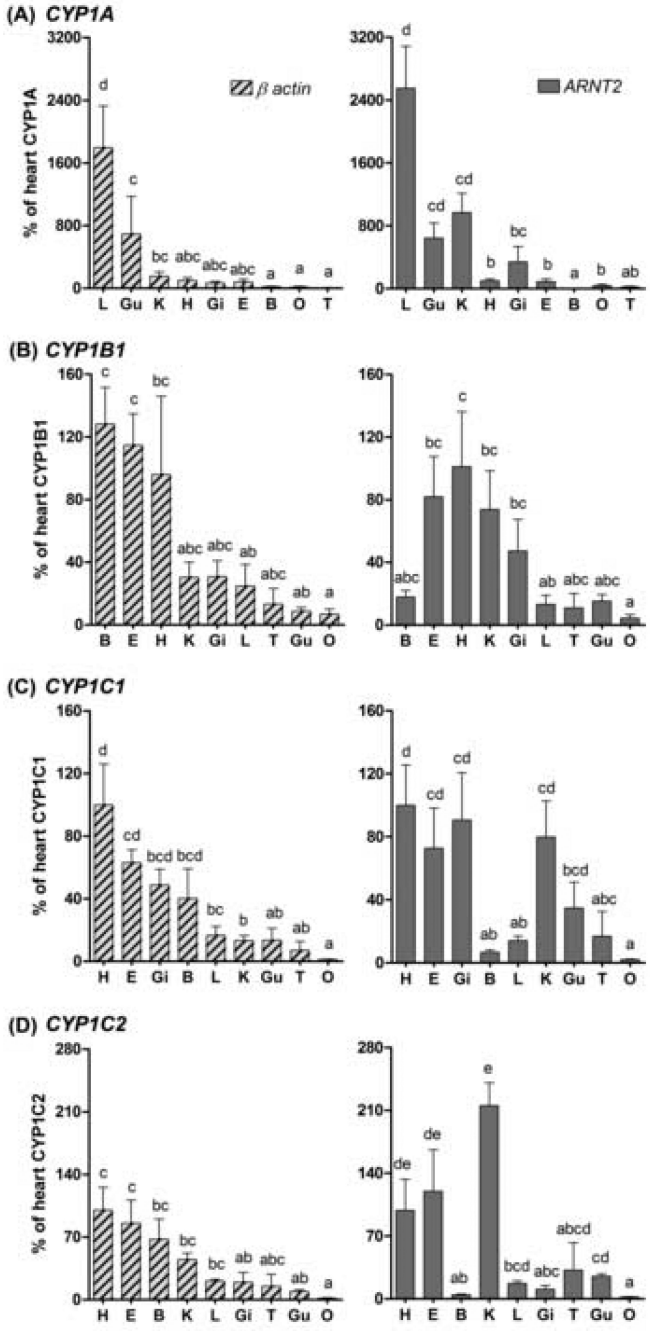

Fig 4.

Basal levels of *CYP1* transcripts in various organs of the zebrafish PS as a percentage of the expression level in heart (*i.e.*, heart was used as a calibrator organ). Data were normalized to both reference genes, *β-actin* and *ANRT2*. B, brain; E, eye; Gi, gill; Gu, gut; H, heart; K, kidney; L, liver; O, ovary; T, testes. Differences between groups were determined by one-way ANOVA followed by Tukey's Multiple Comparison Test after log-transformation. A statistical difference between groups at $p < 0.05$ is indicated by differences in the letters above the bars.

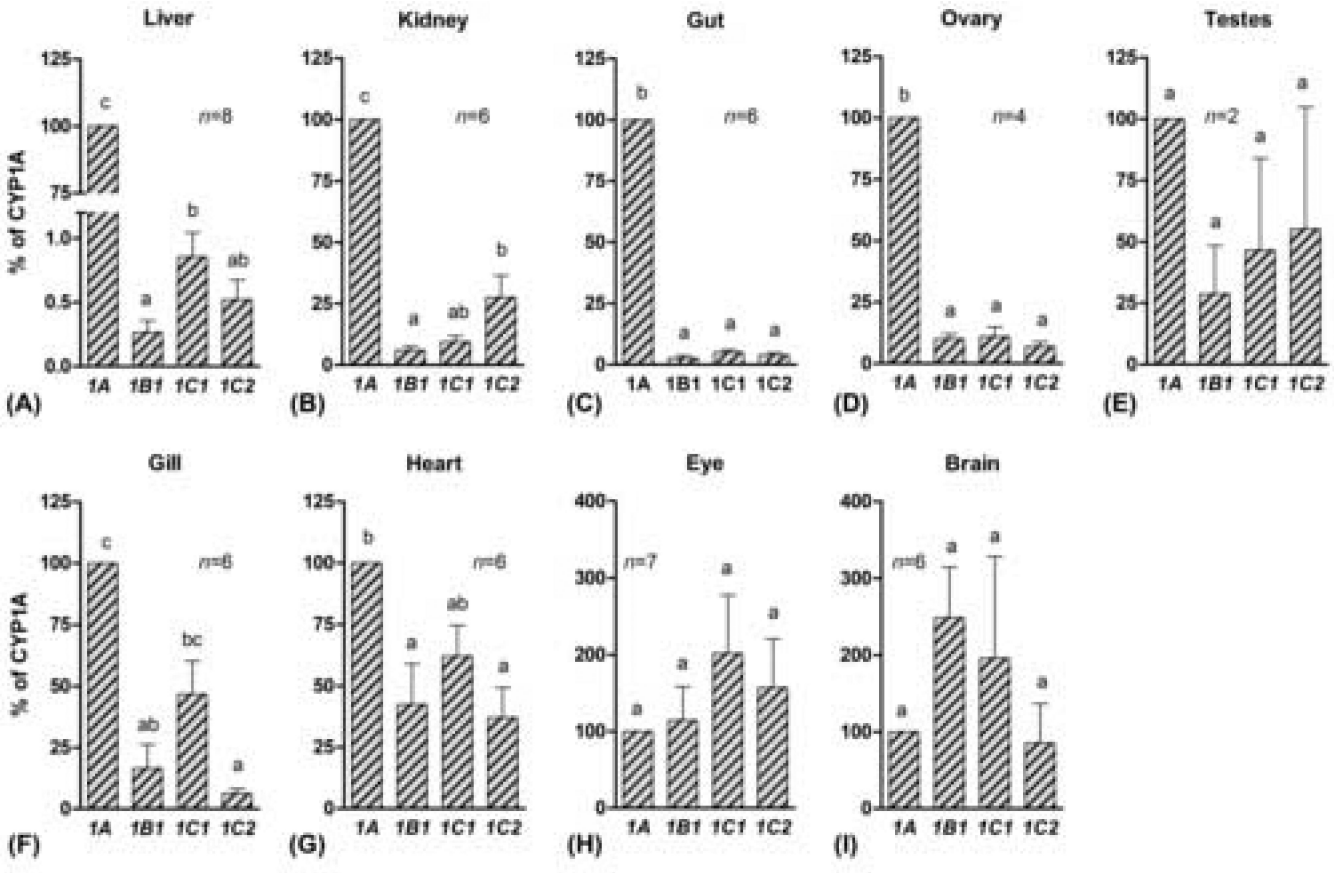

Fig 5.

Basal levels of *CYP1* transcript in various organs in zebrafish PS (mean ± SEM). The relative expression of *CYP1B1*, *CYP1C1*, and *CYP1C2* were calculated in each control sample using the mean values for *CYP1A* expression as a calibrator. Results are shown as a percentage of *CYP1A* expression. Differences in expression between *CYP1* genes in an organ were determined by repeated measures one-way ANOVA followed by Tukey's Multiple Comparison Test after log-transformation. A statistical difference between groups at $p < 0.05$ is indicated by differences in the letters above the bars.

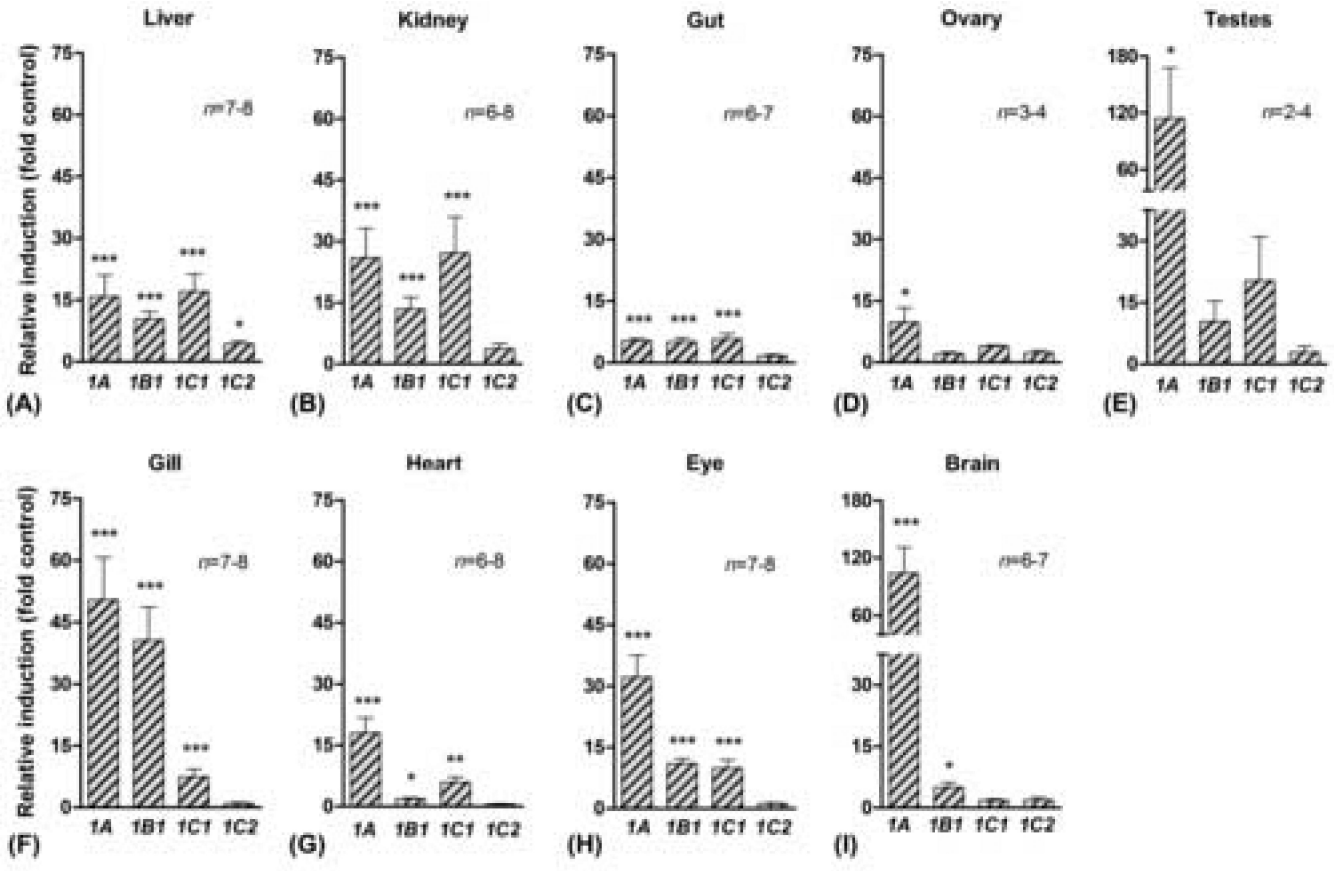

Fig 6.

Fold induction of *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* in various tissues in the zebrafish PS following exposure to PCB126 (100 nM; mean ± SEM). Mean values of control expression were used as calibrators (fold control). Significant differences as compared with the control (* = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$) were determined by one-way ANOVA followed by Bonferroni's multiple comparison of selected pairs.

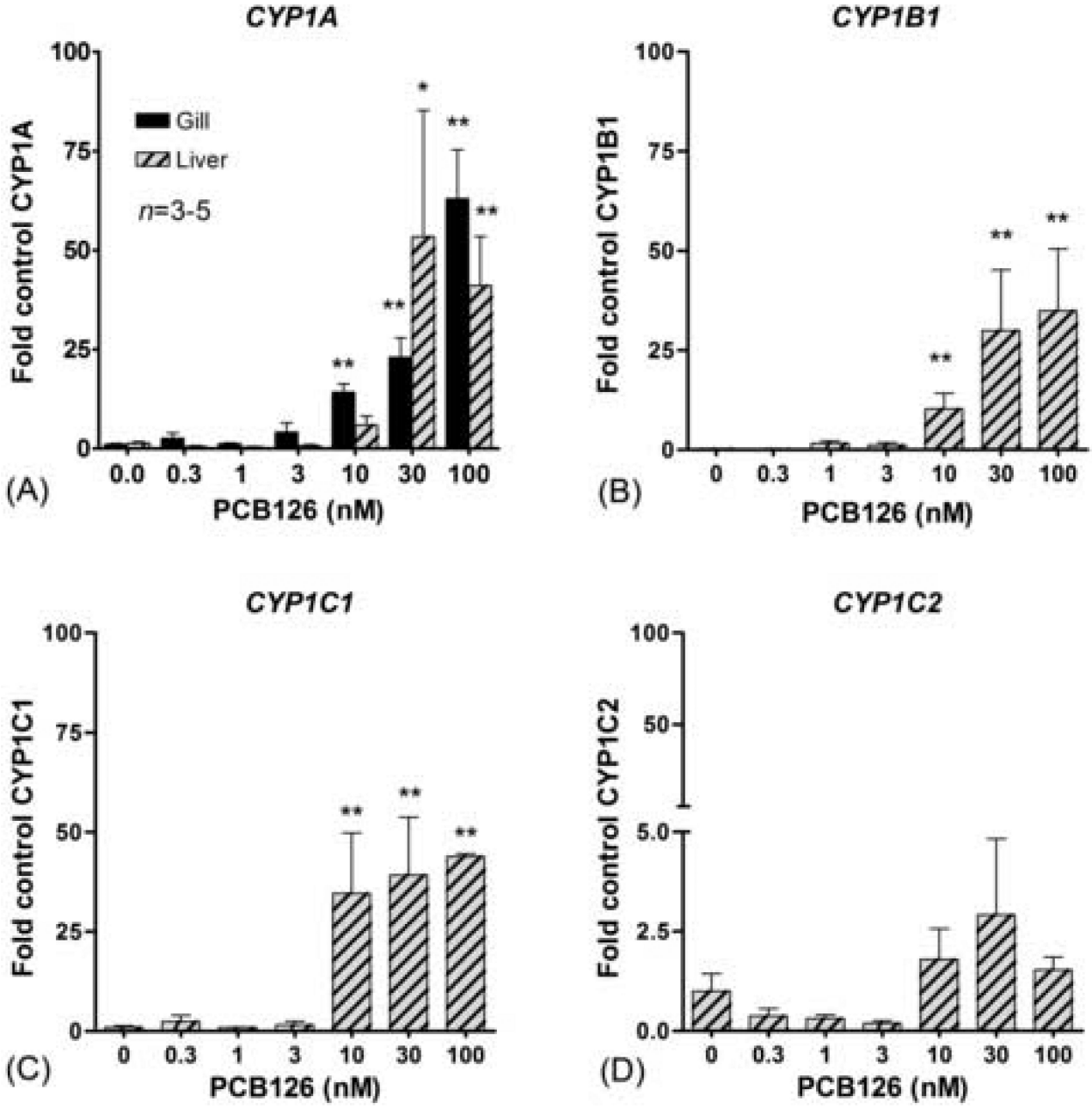

Fig 7.

PCB126 concentration-response of *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* expression (fold control) in gills and liver in the zebrafish PS. Significant differences as compared with the control (* = $p<0.05$ and ** = $p<0.01$) were determined by one-way ANOVA followed by Dunnett's Test.

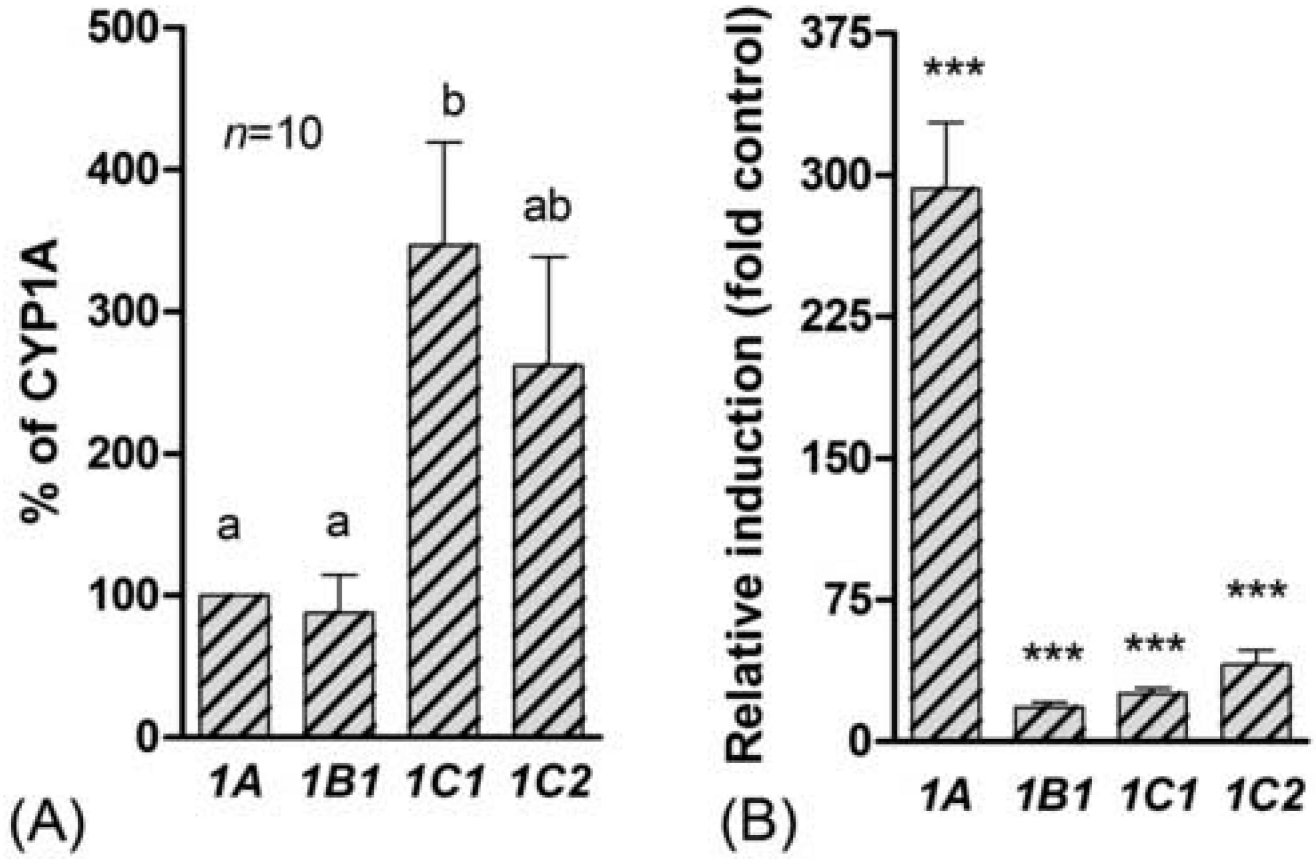

Fig 8.

Basal (A) and PCB126-induced (B) *CYP1* gene expression in zebrafish embryos. (A) Basal expression is shown as percentage of the *CYP1A* expression and significant differences in basal expression between genes were determined by one-way repeated measures one-way ANOVA and Tukey's Multiple Comparison Test. A statistical difference between groups at $p < 0.05$ is indicated by differences in the letters above the bars. (B) Induced expression is shown as the multiple of the control (fold control) significant differences compared with the control (*** = $p<0.001$) were determined by one-way ANOVA followed by Bonferroni's multiple comparison of selected pairs.

Table 1

Primer sequences used for cloning of *CYP1C1* and *CYP1C2* transcripts and quantification of *CYP1* gene expression by real time PCR.

Primer	Sequences
Cloning	
CYP1C1 Utr F	5'-ATCAGCACCGAACACCAGCG
CYP1C1 Utr R	5'-CCCCATTCGACTGGATGTTTTAAC
CYP1C2 Utr F	5'-ACCCTCCAACTGAAGAGGGCAGAAA
CYP1C2 Utr R	5'-TCATAGGCAGTGGGTTAGACAGCACA
Real time PCR	
ZFCYP1A F[1)]	5'-GCATTACGATACGTTCGATAAGGAC
ZFCYP1A R[1)]	5'-GCTCCGAATAGGTCATTGACGAT
ZFCYP1B1 F	5'-GCTCAGCTGGTCCATTGATACC
ZFCYP1B1 R	5'-CATCAGCGACAGCAACACAC
ZFCYP1C1 F	5'-AGTGGCACAGTCTACTTTGAGAG
ZFCYP1C1 R	5'-CCAAACAAGAAAGACTTTTGAGC
ZFCYP1C2 F	5'-GTGGTGGAGCACAGACTAAG
ZFCYP1C2 R	5'-TTCAGTATGAGCCTCAGTCAAAC
β-actin F[1)]	5'-CAACAGAGAGAAGATGACACAGATCA
β-actin R[1)]	5'-GTCACACCATCACCAGAGTCCATCAC
ARNT2 F	5'-CACCTTTGGATCACATCTCATTG
ARNT2 R	5'-TCACCCTCCTTAGACGGACC

[1)] Evans et al. 2005.

Table 2

Localization and number of putative xenobiotic response elements in the four *CYP1* genes in zebrafish (*Danio rerio*).

Gene	# exons	Number of consensus XRE's within				Notes
		−1kb	−2kb	−5kb	−10kb	
CYP1A	7	3	3	15	21	5' UTR length unknown intergenic region is 4kb
CYP1B1	2	0	1	2	6	
CYP1C1	1	1	2	3	4	
CYP1C2	1	0	0	2	6	