Published in final edited form as:

Aquat Toxicol. 2012 November 15; 0: 106–113. doi:10.1016/j.aquatox.2012.08.006.

Basal and 3-methylcholanthrene-induced expression of cytochrome P450 1A, 1B and 1C genes in the Brazilian guppy, *Poecilia vivipara*

Tarquin Dorrington^{a,*}, Juliano Zanette^b, Flávia L. Zacchi^a, John J. Stegeman^c, and Afonso C.D. Bainy^a

^a Biochemistry Department, Universidade Federal de Santa Catarina, SC, 88034-257, Brazil

^b Universidade Federal do Rio Grande, Instituto de Ciências Biológicas, Rio Grande, RS, Brazil

^c Biology Department, MS 32, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

Abstract

In fish there are four cytochrome P450 (*CYP1*) subfamilies: *CYP1A*, *CYP1B*, *CYP1C*, and *CYP1D*. Here we cloned *Poecilia vivipara* *CYP1A*, with an inferred amino acid sequence 91% identical to *CYP1A* from the killifish *Fundulus heteroclitus*, another member of the Cypriniformes, and an important model in ecotoxicology. In addition, we examined the expression of *CYP1A*, *CYP1B1*, and *CYP1C1* by qPCR in liver, gill, and intestine of adult *P. vivipara* injected with 3-methylcholanthrene (3-MC) or held in clean water (control group) for 24 h. All three tissues examined showed basal expression of the three *CYP1* genes. *CYP1A* was most strongly expressed in the liver, while *CYP1B1* and *CYP1C1* were most strongly expressed in the gill and intestine respectively. 3-MC induced *CYP1A*, *CYP1B1*, and *CYP1C1* significantly (20–120-fold) in the three organs, consistent with the regulation of *CYP1A*, *CYP1B1* and *CYP1C1* via the aryl hydrocarbon receptor. Validation of *CYP1* gene biomarkers in fish collected from a contaminated urban mangrove environment was confirmed with significant induction of *CYP1A* and *CYP1C1* in gills (10–15-fold) and *CYP1B1* in liver (23-fold), relative to fish from a control site. The responsiveness of these *CYP1* genes indicates *P. vivipara* is suitable as a model for environmental toxicology studies and environmental assessment in Brazil.

Keywords

P450; CYP1A; Fish; *Poecilia vivipara*; PAH; Pollution

1. Introduction

Within the next decade Brazil is predicted to become one of the worlds largest oil producers. The discovery of large oil reserves and their subsequent production, combined with an

expanding population and industry has put coastal areas under threat from marine pollution events. As such there is a need for bio-monitoring programs using Brazilian species as sentinels and as models in mechanistic toxicology. Both directions need reliable biomarkers of contamination.

Among the most established biomarkers of environmental contamination are the cytochrome P450 enzymes. These enzymes are responsible for the metabolism of many toxicants, especially polycyclic aromatic hydrocarbons (PAH) found in petroleum. In mammals they are involved in the degradation of endogenous molecules such as steroids (Nebert and Russell, 2002). However, it is well known that in the process of detoxification of environmental contaminants, metabolites with greater toxicity can be generated, leading to an increased chance of sub-lethal effects (Shimada and Fujii-Kuriyama, 2004). The induction of CYP1As in both mammals and non-mammalian vertebrates is determined by the aryl hydrocarbon receptor (AHR) as demonstrated through knockdown studies (Fernandez-Salguero et al., 1996; Dong et al., 2004). The activation of AHR by PAHs, planar polychlorinated biphenyl (PCB) congeners, and dioxins has led to the adoption of the *CYP1A* gene as an established biomarker of environmental exposure and effect (Lambert et al., 2006; Jönsson et al., 2010).

There are currently a total of four CYP1 subfamilies known in fish. In addition to CYP1A, there is CYP1B1 (Leaver and George, 2000), two forms of CYP1Cs (Godard et al., 2005; Jönsson et al., 2007b), and CYP1D1 (Goldstone and Stegeman, 2008). The characterization and induction patterns of these genes have already been investigated in several species of fish. Both zebrafish (*Danio rerio*) and the Atlantic killifish (*Fundulus heteroclitus*) have become important models in non-mammalian vertebrate toxicological studies to date. In zebrafish, both PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) induces the expression of *CYP1A*, *CYP1B*, and *CYP1C* genes (Handley-Goldstone et al., 2005; Jönsson et al., 2007a,b), but not that of *CYP1D* (Goldstone et al., 2009). Similarly in *F. heteroclitus*, *CYP1A* (Morrison et al., 1998) *CYP1C1* (Wang et al., 2006), and *CYP1B1*, *CYP1C2*, and *CYP1D1* (Zanette et al., 2009), have all been identified, with PCB126 inducing an increase in all genes except that of *CYP1D1* (Zanette et al., 2009).

One of the most common fish species used in toxicological studies in the United States is *F. heteroclitus* due to its features such as small size, abundance, and adaptation to high contaminant levels (Burnett et al., 2007), however its distribution only reaches that of the southern USA. The guppy, *Poecilia vivipara*, Bloch and Schneider, 1801, is one of the most common fish species found in small ponds, rivers, and coastal lagoon ecosystems of Brazil. It is widely distributed along the coastline of South America from Venezuela to northern Argentina (Lee et al., 1983). It can tolerate a wide degree of salinity, and its small size (5 cm max) together with its rapid development, large population, low vulnerability and small home range (Wischnath, 1993) make it an ideal candidate as a toxicological model species for this region.

The aim of this study was to characterise and determine the patterns of expression of CYP1s in *P. vivipara* with the hypothesis that these genes are similarly expressed in the guppy as

they are in *F. heteroclitus* and the zebrafish in response to AHR agonists. We identified, cloned and compared the full-length sequence of *CYP1A* from *P. vivipara*. We also studied both the basal expression of *CYP1A*, *CYP1B1* and *CYP1C1*, and their responses to experimental exposure to the PAH 3-methylcholanthrene, and exposure in a contaminated field environment. The results show that the three *CYP1* genes in *P. vivipara*, have different organ distributions for basal expression, and differing induction levels following both laboratory and field exposure. Demonstrating induction of *CYP1* genes after PAH contamination helps to validate this fish model species for future bio-monitoring studies, and adds to the foundation for mechanistic toxicology studies in this alternative model.

2. Materials and methods

2.1. Animal collection

Twenty adult *P. vivipara* (1.0–1.5 g whole body weight) were obtained from Lagoa da Conceição, Florianópolis, SC, and transported to the aquarium facility at the Laboratório de Biomarcadores de Contaminação Aquática e Imunoquímica at the Federal University of Santa Catarina (Florianópolis). The fish were acclimated for two weeks in tanks containing sterile seawater at 19 °C (salinity 25). The fish were fed daily with fish flakes, and the water changed every 48 h. The university committee for research on animals approved all experiments.

2.2. Cloning and sequence analysis

Liver, intestine and gill were dissected from three randomly selected fish collected from the Lagoa da Conceição, Florianópolis, SC. Total RNA was isolated from pooled organs (0.1–0.2 g) using Aurum™ RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories Inc. Hercules, CA). The RNA quality was determined spectrophotometrically (NanoDrop ND1000; NanoDrop Technologies, Wilmington, DE). cDNA was then synthesised from 2 [H9262]g of RNA using the Omniscript Reverse Transcriptase Kit (Qiagen Inc., Valencia, CA) anchored primer oligo (dT) (MWG Biotech Inc., High Point, NC) and RNase inhibitor (Promega Corp., Madison, WI). Sense and antisense degenerate primers were designed using conserved regions of *CYP1A*, *CYP1B1* and *CYP1C1* from other fish species, avoiding conserved regions present in other subfamilies of CYP (Table 1). PCR products were visualised on agarose gels, purified, and ligated into the pGEM-T Easy vector (Promega) and transformed into *E. coli* (TOP10 Kit, Invitrogen). Plasmids were purified from positive colonies (QiaPrep, Qiagen) and sequenced. Nucleotide sequences were obtained using Big Dye 3.1 chemistry and an ABI 3130 sequencer (Applied Biosystems, Carlsbad, CA). Sequences were translated to the predicted amino acid sequences and aligned with other *CYP1* family members using ClustalW (Thompson et al., 1994). Calculation of identities among *P. vivipara* nucleotide and amino acid predicted *CYP1* sequences were performed using the Sequence Identity Matrix tool, Bioedit Sequence Alignment Editor Software (Hall, 1999). Gene specific primers were subsequently designed based on the identified *P. vivipara* *CYP1A* sequence, for RACE PCR determination of the 5' and 3' ends of the *CYP1A* gene (Table 1). The SMARTer™ RACE cDNA Amplification Kit (Clontech) was used to synthesise cDNA, and generate 5' and 3' products, which were gel-purified and cloned into the pGEM-T Easy Vector (Promega) and transformed into *E. coli* (DH5α).

Clones were sequenced using Big Dye 3.1 chemistry and an ABI 3130 sequencer (Applied Biosystems, Carlsbad, CA). Sequences were visualised for quality using the 4 Peaks Sequence Viewer (Mekentosj Software, Cambridge, UK). Sequences were assembled using CAP3 assembly and subjected to homology search through the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Molecular phylo-genetic analysis was performed with the CLC Sequence Viewer (Version 6.6.2) software package (CLC bio, Aarhus, Denmark). The alignments were performed using the Clustal W algorithm with very accurate alignment option, with a gap open cost of 10, gap extension cost of 1, and the end gap cost set to cheap. The phylogenetic trees were constructed using the UPGMA model algorithm with bootstrap analysis of 100 replicates, leading to the values shown for each node in Fig. 2. Branches with bootstrap values > 70% were considered resolved (Hillis and Bull, 1993). Substrate recognition sites were located referring to those indicated previously (Lewis et al., 2003).

2.3. Exposures

3-Methylcholanthrene (98%) (3-MC; CAS 56-49-5) was purchased from Sigma–Aldrich (USA) and dissolved in corn oil at a concentration of 10 mg/mL. Twenty-eight fish (0.5–1.5 g whole body weight) were acclimated for 24 h in two 40 L aquaria (14 fish per tank) at 19 °C. After this time 14 fish were individually weighed and injected intraperitoneally with 3-methylcholanthrene in corn oil at a concentration of 30 µg/g fish. Fourteen fish were also injected with an identical volume of corn oil as a control. No mortalities were observed during exposure. Both sets of fish were not anesthetized during injection due to the possibility of chemical interaction with detoxification activities (Fabacher, 1982). Twenty four hours after injection, control and 3-methylcholanthrene injected fish were killed by decapitation and liver, intestine, and gill from each fish dissected and immediately preserved in RNA later (Ambion), and stored at –20 °C.

CYP1 gene expression was also determined in field-exposed fish. Fifteen animals were collected from the polluted Itacorubi Man-grove. This mangrove area is situated in the middle of the city of Florianópolis and has undergone a 75% habitat loss over a 30-year period. Untreated sewage is discharged directly into the area from the large number of high-rise apartment buildings, and from runoff originating from a large landfill site situated on the edge of the mangrove (Mater et al., 2004). In addition a large highway cuts through the middle of the area with substantial runoff from vehicular traffic. Fifteen fish were collected from the mangrove using minnow traps, and immediately transported to the lab where they were killed and dissected as described above. Only liver and gill were removed for analysis. Sixteen control fish were also obtained from a clean site at Barra da Lagoa, SC. Control animals were killed and dissected at the same time as those from the Itacorubi Mangrove.

2.4. Real-time PCR

Total RNA was isolated as described above. The quantity and quality of the RNA was determined spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen), which includes an integrated genomic DNA elimination step. cDNA was subsequently quantified using the NanoDrop ND-1000. Gene specific primers for *CYP1A*, *CYP1B1*, *CYP1C1* and β -

actin cDNA were synthesised by Integrated DNA Technologies (Coralville, IA) and are shown in Table 1. Real-time PCR was performed using the Rotor-Gene 6000 real-time amplification system (Qiagen). The PCR reaction mixtures (25 µL) consisted of Rotor-Gene™ SYBR® Green (Qiagen), 1 µM each of forward and reverse primer, and 100 ng cDNA. Each group (control or exposed) consisted of seven or eight pools (depending on number of fish) of two fish, and were analysed in triplicate for each gene using the following protocol: 95 °C for 5 min; 95 °C for 5 s and 60 °C for 10 s (40 cycles). In order to confirm the amplification of a single product per primer set, a melt curve analysis was performed at the end of each PCR run using the integrated Rotor-Gene Software (Qiagen).

The basal mRNA expression of the CYP1 genes was calculated using the 2^{-C_T} method (Schmittgen and Livak, 2008) using β -actin as the reference gene. For fold-change in expression after exposure the comparative 2^{-C_T} method was used. The efficiency of the PCR reaction for each primer pair combination was calculated from a standard curve of control group cDNA and the Rotor-Gene 6000 analysis software (Qiagen). The comparative method is only used if the PCR efficiency of the target gene and endogenous reference gene are the same, or if the difference in expression levels is sufficiently high to tolerate the resulting error. Even though all gene efficiencies were identical, we validated all results using efficiency-corrected calculation program REST 2009 (Relative Expression Software Tool) from Qiagen.

Statistical determination of differences in gene expression between groups was carried out using one-way ANOVA followed by the Tukey's post hoc test or using Students *t*-test to compare control versus exposed groups. Data were logarithmically transformed. All statistical analysis was carried out using JMP9 software (SAS Software, USA). Specific details and methods are given in the figure captions.

3. Results

3.1. Cloned CYP1 transcripts

Using degenerate primers we were able to amplify partial *CYP1A*, *CYP1B1*, and *CYP1C1* fragments in *P. vivipara* with 653, 587 and 1047 nucleotides, respectively. The predicted amino acid sequences obtained from those fragments shared high identities, 89, 84 and 93% with the homologous CYP1A, CYP1B1 and CYP1C1, sequences respectively, from *F. heteroclitus*. Amplification using RACE PCR generated a cDNA fragment that was sufficient to encode the full *CYP1A* predicted sequence of 521 amino acids (GenBank accession number JX027071). The full nucleotide sequence obtained with amino acid translation is available in Supplemental Fig. 1. Alignment of the full-length *P. vivipara* CYP1A protein sequence (inferred) with other fish CYP1As (Fig. 1) showed a 91% amino acid sequence identity between *P. vivipara* and *F. heteroclitus*, and a 90% identity for the nucleotide sequence. For *P. vivipara* and *D. rerio* the CYP1A protein and nucleotide sequence identities were 70% and 67%, respectively. In addition to *CYP1A*, we amplified approximately 57% of a *CYP1B1* gene with 79% identity to *F. heteroclitus* CYP1B1, and approximately 78% of a *CYP1C1* gene with 93% identity to *F. heteroclitus* CYP1C1. Amino acid sequences were identical in *P. vivipara* and *F. heteroclitus* for SRS 2, SRS 5 and the heme binding site. For SRS 6 all six species had an identical protein sequence (Fig.

1). The annotation of these genes is in agreement with the classification of CYPs as established previously (Nelson et al., 1996), with identities higher than 55% as belonging to the same sub-family.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2012.08.006>.

Phylogenetic analysis was carried out with CYP1As from a variety of other species (Fig. 2). This shows that *P. vivipara* CYP1A is positioned closest to CYP1A from *F. heteroclitus* and medaka. As expected, the fish CYP1As form a separate clade from those of the mammalian species. Nucleotide and amino acid similarities between *P. vivipara* CYP1A, and *F. heteroclitus* CYP1A, CYP1B1, and CYP1C1 are summarized in Table 2.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2012.08.006>.

3.2. Basal CYP1 gene expression in various tissues of *P. vivipara*

The expression of *CYP1A*, *CYP1B1* and *CYP1C1* was observed in all three organs examined in the control *P. vivipara*, and levels of expression are presented as a percentage of the highest transcript level (Fig. 3). When the data are presented this way within each tissue, higher levels of *CYP1A* were observed in the liver, gill and intestine (Fig. 3A–C) compared to *CYP1B1* and *CYP1C1*. In both organs of the abdominal cavity (the liver and intestine), *CYP1A* levels were over 100-fold higher than the expression of *CYP1B1* and *CYP1C1* (Fig. 3A and C). When the organ distribution of individual *CYP1* genes was observed, *CYP1A* had the highest level in the liver, followed by the intestine and then the gill (Fig. 3D). *CYP1B1* expression was highest in the gill and lowest in the liver (gill > intestine > liver; Fig. 3E). *CYP1C1* however was highest in the intestine and lowest in the liver (intestine > gill > liver; Fig. 3F).

3.3. 3-MC-induced CYP1 gene expression in various organs of *P. vivipara*

The injection of *P. vivipara* with 3-MC (or the carrier oil) produced no mortalities in our experiments. All *CYP1* genes examined were induced in all three organs (liver, gill, intestine) as a result of 3-MC injection (Fig. 4). The highest changes were seen in the liver where *CYP1B1* was induced ~120-fold and *CYP1A* 75-fold (Fig. 4A). In the intestine *CYP1A* and *CYP1C1* also were induced strongly (~90-fold and ~50-fold respectively; Fig. 4C). Although levels of expression of all three genes were significantly induced, in gill the response of the *CYP1* genes in were much lower than in the other organs. *CYP1C1* showed the highest response in gill, with an increase of ~20-fold after injection with 3MC (Fig. 4B).

3.4. Induced CYP1 gene expression in gills and liver of *P. vivipara* from a contaminated environment

In order to evaluate if we could detect any response from environmentally exposed fish, we collected *P. vivipara* from a clean site and also from a mangrove area that is heavily contaminated from human activities (Mater et al., 2004). *CYP1A*, *CYP1B1* and *CYP1C1*

were all significantly induced in gills of *P. vivipara* from the contaminated site (Fig. 5B). The most strongly induced were *CYP1A* and *CYP1C1* (15-fold and 10-fold respectively), with *CYP1B1* weakly induced at ~3-fold (Fig. 5B). However, the *CYP1* response was considerably weaker in the liver than in the gills of fish from the contaminated site, with *CYP1B1* showing the only significant response at ~23-fold above the levels in reference site fish (Fig. 5A).

4. Discussion

4.1. Cloned CYP1 transcripts

CYP1s are established biomarkers used for the detection of chemical pollutants that activate the AHR. Here we present an analysis of CYP1s newly identified in the Brazilian guppy *P. vivipara*, demonstrating that *CYP1A*, *CYP1B1* and *CYP1C1* are all constitutively expressed in multiple organs of adult fish. In addition, all three of these CYP1s were induced by the AHR agonist 3-methylcholanthrene, and also in fish from a contaminated aquatic environment. *CYP1A* is an established biomarker in fish (Arinc et al., 2000) and although there are fewer studies for *CYP1B1* and *CYP1C1*, we conclude that this fish is an appropriate model species for future monitoring studies in Brazil. The three *P. vivipara* CYP1 genes are apparent orthologues of those from *F. heteroclitus*, another established biomonitoring model species, with *CYP1A* grouped closest together with those from similar species in the phylogenetic analysis. This is not unexpected as *P. vivipara*, *Fundulus* and the medaka are all from the superorder Acanthopterygii, whereas zebrafish (*Ostariophysi*) and rainbow trout and salmon (*Protacanthopterygii*) are from different superorders.

The use of South American cyprinodontiform fish as model species in environmental biomonitoring programs is a valid one given their characteristics and distribution. *P. vivipara* (Poeciliidae) is found along the entire coastline of Brazil, from Venezuela to Argentina, and *Jenynsia multidentata* (Anablepidae) is found from southern Brazil to Argentina (Froese and Pauly, 2012; Ferreira et al., 2012). This wide distribution shows their capacity to live under a range of stressful conditions, including pollution and other physical characteristics. Indeed, *P. vivipara* have been found to inhabit a single lagoon ecosystem containing a large range of salinities (Gomes and Monteiro, 2008) demonstrating the variation present among a single population. In addition there are other cyprinodontiform fishes, that are from the same family (Poeciliidae), such as the *Phalloceros*, *Cnesterodon* and *Phallotorynus* genus, which possess many species that are widely distributed in South America and could serve as surrogate models for toxicology studies (Lucinda, 2005, 2008; Lucinda et al., 2005).

4.2. Basal CYP1 gene expression in various tissues of *P. vivipara*

Our results show basal levels of expression of all three CYP1 genes in *P. vivipara*, and indicate that there are possibly different roles of these genes. *CYP1A* was much more highly expressed in the liver than *CYP1B1* and *CYP1C1* which matches with the observations of previous studies in fish (Jönsson et al., 2007b, 2010; Zanette et al., 2009; Gao et al., 2011). This pattern has also been observed with *CYP1A* and *CYP1B1* in mouse and human liver (Choudhary et al., 2005). Basal *CYP1A* levels were also very high in the intestine, which

seems logical given the role that both the liver and intestine play in the uptake and processing of chemicals and metabolites in the diet, which can include AHR agonists (Van Veld et al., 1997; Nebert et al., 2000). *CYP1A* basal levels were also highest in the gill, which has been observed previously in zebrafish (Jönsson et al., 2007b), killifish (Zanette et al., 2009), rainbow trout (Jönsson et al., 2010), and stickleback (Gao et al., 2011).

CYP1B1 is essential for the development of the human eye (Libby et al., 2003). Previous studies have shown the high levels of basal expression of *CYP1B1* in the eye and brain of various fish species (Jönsson et al., 2007b; Goldstone et al., 2009; Zanette et al., 2009), however in this study these organs were not tested so a comparison is not possible. *CYP1B1* basal levels were high in the gill of *P. vivipara* which was one of the organs, after the eye and heart, where *CYP1B1* was most abundant in *F. heteroclitus* (Zanette et al., 2009). *CYP1C* genes were not studied until very recently and their functions are not completely understood (Gao et al., 2011). They are present in fish, frogs and birds, but not in mammals (Godard et al., 2005; Goldstone et al., 2007; Zanette et al., 2009; Jönsson et al., 2011). They are also more closely related to *CYP1B1* than to *CYP1As* and demonstrate a similar expression pattern in previous studies (Jönsson et al., 2007b; Zanette et al., 2009). With *CYP1C1*, the higher levels are generally observed in the kidney, brain and testis, with low levels in the liver and gill. The results from this study also showed lower levels in liver and gill when compared to the highest basal *CYP1C1* level in the intestine.

4.3. 3-MC-induced CYP1 gene expression in various organs of *P. vivipara*

The inducibility of the three *CYP1* genes was evaluated in *P. vivipara* injected with 3-MC. A significant induction by 3-MC of all three *P. vivipara* *CYP1* genes suggests that they are all regulated by the AhR. Indeed all *CYP1A*, *CYP1B*, and *CYP1C* genes that are known in zebrafish and killifish are induced by AhR agonists (Jönsson et al., 2007b; Zanette et al., 2009). As expected, the induction of *CYP1A* in all organs was observed following injection, especially in the liver and intestine, which are the main detoxification organs. Many previous studies have demonstrated that *CYP1A* levels are induced in the related fish species of *F. heteroclitus* exposed to AhR agonists (e.g., Van Veld et al., 1997).

CYP1B1 also has been shown to be induced in a variety of tissues in both fish and mammals in response to exposure to AhR agonists (El-kady et al., 2004; Willett et al., 2006). In a previous study with carp treated with 3-MC, *CYP1B1* expression was observed in the liver, gill and intestine, with only the liver and intestine showing a significant increase. Although the increase in gill was not significant, the basal level of *CYP1B1* was quite high in the gills of carp (El-kady et al., 2004). Our results with 3-MC injected *P. vivipara* also show a similar pattern, with *CYP1B1* being significantly induced in liver, gill and intestine, but only a 3-fold increase in the gill (Fig. 4), perhaps due to a higher gill basal level (Fig. 3E). *CYP1C1* also showed a significant response in all three organs tested in our study, similar to studies in killifish where *CYP1C1* was induced by benzo[a]pyrene (B[a]P) (Wang et al., 2006).

Following injection with 3-MC we saw a significant induction in all *CYP1s* in the gill although *CYP1B1* was much lower in response. A low response in the gill of *CYP1B1*, with higher induction of *CYP1A* and *CYP1C1* has previously been observed in rainbow trout

exposed to PCB126 (Jönsson et al., 2010). It should also be noted that the relative induction level after exposure appears to be related to the initial basal level, which has been observed before in the case of *CYP1B1* and *CYP1C1* (Gao et al., 2011).

4.4. CYP1 gene expression in gills and liver of *P. vivipara* from a contaminated environment

The levels of *CYP1* gene expression were determined also in fish collected from a contaminated environment, with the expression profiles probably reflecting the local pollution load of this particular mangrove environment. This would indicate the presence of AhR agonists in this area. Globally there are increasing levels of PAHs in many urban areas, and Brazil is undergoing a rapid phase of urban development at this time. The Itacorubi mangrove is surrounded by high-rise residential buildings, a main highway and the municipal landfill site for the city of Florianopolis.

In our study, the largest response in the liver was seen with *CYP1B1*, whereas the gill shows a significant response for all three *CYP1* genes (although as with 3MC treatment, the fold increase of *CYP1B1* was lower). The weaker response in liver versus gill may suggest perhaps that the inducers are in fact more readily metabolized compounds such as PAHs (Jönsson et al., 2010), or that the source of PAH is waterborne exposure causing induction in organs relating to the route of exposure (Smolowitz et al., 1992). Many PAHs are both inducers and substrates for both CYP1 enzymes (Shimada and Fujii-Kuriyama, 2004), with the liver requiring higher waterborne concentrations to induce a response than for gills (Jönsson et al., 2006). The lower response in liver may therefore be the result of “first pass metabolism” in the gill (Stegeman et al., 1984), with the concentrations that reach the liver too low to induce transcription there (Jönsson et al., 2006). This is probably the case in our study for *CYP1A* and *CYP1C1* (Fig. 5A). The field results profile also may reflect the nature of the exposure, possibly indicating whether the inducers in the field were predominantly in the diet or in environmental matrices favouring direct exposure. The 3-MC injections could yield pharmacokinetic distributions more like dietary exposure, and possibly impact the liver more than the gills, while in the field-exposed fish the gills could be the first point of contact with some contaminants. However, injection of the PAH-like inducer BNF also strongly induced *CYP1A* protein expression in gill (Miller et al., 1989). The extent of effect in the different organs as related to route of exposure requires further experimentation.

As mentioned above, the relative induction level also appears to be related to the basal level, which has been observed before (Gao et al., 2011). We also observed *CYP1B1* showing a high basal level in the gill, but a low induction response in the same tissue following injection with 3MC, and also in the gill from the environmentally exposed fish. Interestingly the weaker response in liver compared to gills was also observed in environmentally exposed rainbow trout as well (Jönsson et al., 2010). It was also noted in that study that exposure at several sites appeared to suppress the induction of *CYP1C1* in liver. In our study *CYP1C1* expression levels were lowest in the liver and showed no significant induction (~1.5-fold compared to control) in field-exposed fish.

To conclude, we cloned the complete *CYP1A* gene, and obtained the sequences of *CYP1B1* and *CYP1C1* in the Brazilian guppy, *P. vivipara*. We demonstrated that they are

constitutively expressed in several organs and that the *CYP1s* differ in their response to the PAH 3-methylcholanthrene. The induction of these *CYP1* genes by 3-MC together with the results from fish from a contaminated environment, provide a new suite of biomarkers in *P. vivipara*, a vertebrate model with the potential for use in future environmental toxico-logical studies in Brazil, and which may provide a valuable model for comparing to other fishes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank C. Piazza and J. Cunha for help with the collection and dissection of the fish. TD was supported by the CAPES PNPd fellowship, Brazil. ACDB is a recipient of the CNPq Productivity Fellowship, Brazil. This study was supported in part by NIH grants to JJS (the Superfund Research Program 5P42ES007381 and R01ES015912), and by Brazilian INCT-TA to JZ (CNPq 573949/2008-5).

References

- Arinc E, Sen A, Bozcaarmutlu A. Cytochrome P4501A and associated mixed-function oxidase induction in fish as a biomarker for toxic carcinogenic pollutants in the aquatic environment. *Pure and Applied Chemistry*. 2000; 72:985–994.
- Burnett KG, Bain LJ, Baldwin WS, Callard GV, Cohen S, Di Giulio RT, Evans DH, Gómez-Chiari M, Hahn ME, Hoover CA, Karchner SI, Katoh F, Maclatchy DL, Marshall WS, Meyer JN, Nacci DE, Oleksiak MF, Rees BB, Singer TD, Stegeman JJ, Towle DW, Van Veld PA, Vogelbein WK, Whitehead A, Winn RN, Crawford DL. *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. *Comparative Biochemistry and Physiology. Part D*. 2007; 2:257–286.
- Choudhary D, Jansson I, Stoilov I, Sarfarazi M, Schenkman JB. Expression patterns of mouse and human CYP orthologs (families 1–4) during development and in different adult tissues. *Archives of Biochemistry and Biophysics*. 2005; 436:50–61. [PubMed: 15752708]
- Dong W, Teraoka H, Tsujimoto Y, Stegeman JJ, Hiraga T. Role of aryl hydrocarbon receptor in mesencephalic circulation failure and apoptosis in zebrafish embryos exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicological Sciences*. 2004; 77:109–116. [PubMed: 14657521]
- El-kady MAH, Mitsuo R, Kaminishi Y, Itakura T. cDNA cloning, sequence analysis and expression of 3-methylcholanthrene-inducible cytochrome P450 1B1 in carp (*Cyprinus carpio*). *Environmental Science*. 2004; 11:231–240.
- Fabacher DL. Hepatic microsomes from freshwater fish 2. Reduction of benzo(a)pyrene metabolism by the fish anesthetics quinaldine sulfate and tri-caine. *Comparative Biochemistry and Physiology. Part C*. 1982; 73:285–288.
- Fernandez-Salguero PM, Hilbert DM, Rudikoff S, Ward JM, Gonzalez FJ. Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. *Toxicology and Applied Pharmacology*. 1996; 140:173–179. [PubMed: 8806883]
- Ferreira RS, Monserrat JM, Ferreira JLR, Kalb AC, Stegeman JJ, Bainy ACD, Zanette J. Biomarkers of organic contamination in the south american fish *Poecilia vivipara* and *Jenynsia multidentata*. *Journal of Toxicology & Environmental Health, Part A*. 2012; 75(16–17):1023–1034. [PubMed: 22852852]
- Froese, R.; Pauly, D., editors. FishBase.. World Wide Web electronic publication. 2012. www.fishbase.org. Version (06/2012)
- Gao K, Brandt I, Goldstone JV, Jönsson ME. Cytochrome P450 1A, 1B, and 1C mRNA induction patterns in three-spined stickleback exposed to a transient and a persistent inducer. *Comparative Biochemistry and Physiology. Part C*. 2011; 154:42–55. [PubMed: 21354474]

- Godard CAJ, Goldstone JV, Said MR, Dickerson RL, Woodin BR, Stegeman JJ. The new vertebrate CYP1C family: cloning of new subfamily members and phylogenetic analysis. *Biochemical and Biophysical Research Communications*. 2005; 331:1016–1024. [PubMed: 15882979]
- Goldstone JV, Goldstone HMH, Morrison AM, Tarrant A, Kern SE, Woodin BR, Stegeman JJ. Cytochrome P450 1 genes in early deuterostomes (tunicates and sea urchins) and vertebrates (chicken and frog): origin and diversification of the CYP1 gene family. *Molecular Biology and Evolution*. 2007; 24:2619–2631. [PubMed: 17916789]
- Goldstone JV, Jönsson ME, Behrendt L, Woodin BR, Jenny MJ, Nelson DR, Stegeman JJ. Cytochrome P450 1D1: a novel CYP1A-related gene that is not transcriptionally activated by PCB126 or TCDD. *Archives of Biochemistry and Biophysics*. 2009; 482:7–16. [PubMed: 19103147]
- Goldstone JV, Stegeman JJ. Gene structure of the novel cytochrome P4501D1 genes in stickleback (*Gasterosteus aculeatus*) and medaka (*Oryzias latipes*). *Marine Environment Research*. 2008; 66:19–20.
- Gomes JL, Monteiro LR. Morphological divergence patterns among populations of *Poecilia vivipara* (Teleostei Poeciliidae): test of an ecomorphological paradigm. *Biological Journal of the Linnean Society*. 2008; 93:799–812.
- Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. 1999; 41:95–98.
- Handley-Goldstone HM, Grow MW, Stegeman JJ. Cardiovascular gene expression profiles of dioxin exposure in zebrafish embryos. *Toxicological Sciences*. 2005; 85:683–693. [PubMed: 15716485]
- Hillis DM, Bull JJ. An empirical test of bootstrapping method for assessing confidence in phylogenetic analysis. *Systematic Biology*. 1993; 42:182–192.
- Jönsson EME, Abrahamson AA, Brunström BB, Brandt II. Cytochrome P4501A induction in rainbow trout gills and liver following exposure to water-borne indigo, benzo[a]pyrene and 3,3", 4,4", 5-pentachlorobiphenyl. *Aquatic Toxicology*. 2006; 79:226–232. [PubMed: 16872689]
- Jönsson ME, Berg C, Goldstone JV, Stegeman JJ. New CYP1 genes in the frog *Xenopus (Silurana) tropicalis*: induction patterns and effects of AHR agonists during development. *Toxicology and Applied Pharmacology*. 2011; 250:170–183. [PubMed: 20965207]
- Jönsson ME, Gao K, Olsson JA, Goldstone JV, Brandt I. Induction patterns of new CYP1 genes in environmentally exposed rainbow trout. *Aquatic Toxicology*. 2010; 98:311–321. [PubMed: 20371123]
- Jönsson ME, Jenny MJ, Woodin BR, Hahn ME, Stegeman JJ. Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3", 4,4", 5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicological Sciences*. 2007a; 100:180–193. [PubMed: 17686920]
- Jönsson ME, Orrego R, Woodin BR, Goldstone JV, Stegeman JJ. Basal and 3,3", 4,4", 5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. *Toxicology and Applied Pharmacology*. 2007b; 221:29–41. [PubMed: 17445853]
- Lambert GH, Needham LL, Turner W, Lai TJ, Patterson DG, Guo YL. Induced CYP1A2 activity as a phenotypic biomarker in humans highly exposed to certain PCBs/PCDFs. *Environmental Science and Technology*. 2006; 40:6176–6180. [PubMed: 17051818]
- Leaver MJ, George SG. A cytochrome P4501B gene from a fish, *Pleuronectes platessa*. *Gene*. 2000; 256:83–91. [PubMed: 11054538]
- Lee, DS.; Platania, SP.; Burgess, GH. North Carolina State Museum of Natural History. Raleigh, NC.: 1983. Atlas of North American freshwater fishes. In: Occasional Papers of the North Carolina Biological Survey no. 1983–6..
- Lewis DFV, Gillam EMJ, Everett SA, Shimada T. Molecular modelling of human CYP1B1 substrate interactions and investigation of allelic variant effects on metabolism. *Chemico-Biological Interactions*. 2003; 145:281–295. [PubMed: 12732455]
- Libby RT, Smith RS, Savinova OV, Zabaleta A, Martin JE, Gonzalez FJ, John SWM. Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science*. 2003; 299:1578–1581. [PubMed: 12624268]

- Lucinda PHF. Systematics of the genus *Cnesterodon* Garman, 1895 (Cyprinodontiformes: Poeciliidae: Poeciliinae). *Neotropical Ichthyology*. 2005; 3(2):259–270.
- Lucinda PHF, Rosa RD, Reis RE. Systematics and biogeography of the genus *Phallotorynus* Henn, 1916 (Cyprinodontiformes: Poeciliidae: Poeciliinae), with description of three new species. *Copeia*. 2005; 2005(3):609–631.
- Lucinda PHF. Systematics and biogeography of the genus *Phalloceros* Eigenmann, 1907 (Cyprinodontiformes: Poeciliidae: Poeciliinae), with the description of twenty-one new species. *Neotropical Ichthyology*. 2008; 6(2):113–158.
- Mater L, Alexandre M, Hansel F, Madureira L. Assessment of lipid compounds and phosphorus in mangrove sediments of Santa Catarina Island, SC, Brazil. *The Journal of the Brazilian Chemical Society*. 2004; 15:725–734.
- Miller MR, Hinton DE, Stegeman JJ. Cytochrome P-450E induction and localization in gill pillar (endothelial) cells of scup and rainbow trout. *Aquatic Toxicology*. 1989; 14(4):307–322.
- Morrison HG, Weil EJ, Karchner SI, Sogin ML, Stegeman JJ. Molecular cloning of CYP1A from the estuarine fish *Fundulus heteroclitus* and phylogenetic analysis of CYP1 genes: update with new sequences. *Comparative Biochemistry and Physiology. Part C*. 1998; 121:231–240.
- Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, Dalton TP. Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochemical Pharmacology*. 2000; 59:65–85. [PubMed: 10605936]
- Nebert DW, Russell DW. Clinical importance of the cytochromes P450. *Lancet*. 2002; 360:1155–1162. [PubMed: 12387968]
- Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*. 1996; 6:1–42. [PubMed: 8845856]
- Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*. 2008; 3:1101–1108.
- Shimada T, Fujii-Kuriyama Y. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Science*. 2004; 95:1–6. [PubMed: 14720319]
- Smolowitz RM, Schultz ME, Stegeman JJ. Cytochrome P4501A induction in tissues, including olfactory epithelium, of topminnows (*Poeciliopsis* spp.) by waterborne benzo[a]pyrene. *Carcinogenesis*. 1992; 13(12):2395–2402. [PubMed: 1473249]
- Stegeman JJ, Woodin BR, Binder RL. Patterns of benzo[a]pyrene metabolism by varied species, organs, and developmental stages of fish. *National Cancer Institute Monographs*. 1984; 65:371–377.
- Thompson JD, Higgins DGD, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*. 1994; 22:4673–4680. [PubMed: 7984417]
- Van Veld PA, Vogelbein WK, Cochran MK, Goksøyr A, Stegeman JJ. Route-specific cellular expression of cytochrome P4501A (CYP1A) in fish (*Fundulus heteroclitus*) following exposure to aqueous and dietary benzo[a]pyrene. *Toxicology and Applied Pharmacology*. 1997; 142:348–359. [PubMed: 9070358]
- Wang L, Scheffler BE, Willett KL. CYP1C1 messenger RNA expression is inducible by benzo[a]pyrene in *Fundulus heteroclitus* embryos and adults. *Toxicological Sciences*. 2006; 93:331–340. [PubMed: 16873418]
- Willett KL, Ganesan S, Patel M, Metzger C, Quiniou S, Waldbieser G, Scheffler B. In vivo and in vitro CYP1B mRNA expression in channel catfish. *Marine Environmental Research*. 2006; 62(Suppl.):S332–S336. [PubMed: 16697458]
- Wischnath, L. Atlas of Livebearers of the World. T.F.H. Publications, Inc.; USA: 1993.
- Zanette J, Jenny MJ, Goldstone JV, Woodin BR, Watka L, Bainy ACD, Stegeman JJ. New cytochrome P450 1B1, 1C2 and 1D1 genes in the killifish *Fundulus heteroclitus*: basal expression and response of five killifish CYP1s to the AHR agonist PCB126. *Aquatic Toxicology*. 2009; 93:234–243. [PubMed: 19515436]

Fig. 1.

Alignment of the deduced amino acid sequence of the cloned *CYP1A* transcript in *Poecilia vivipara* with orthologous *CYP1A* sequences in the Atlantic killifish (*Fundulus heteroclitus*) (AAD01809.1), zebrafish (*Danio rerio*) (AAH94977.1), three-spined stickleback (*Gasterosteus aculeatus*) (ADO15701.1), Atlantic salmon (*Salmo salar*) (NP 001117159.1), and rainbow trout (*Oncorhynchus mykiss*) (AAB69383.1). Residues that are identical to *P. vivipara* *CYP1A* are indicated by a dot. "Heme bind" = heme binding site; "SRS" = substrate binding site. The SRS positions were marked according to Lewis et al. (2003). Accession numbers are provided in parenthesis.

Fig. 2.

Phylogenetic tree of *Poecilia vivipara* CYP1A deduced amino acid sequence with other selected *CYP1A* sequences from GenBank. Sequences presented are for trout (TROUT), salmon (SALMON), zebrafish (DANIO), killifish (FUNDULUS), medaka (MEDAKA), Brazilian guppy (POECILIA), mouse (MOUSE), and human (HUMAN). Tree generated using the UPGMA method. Node values represent the maximum likelihood bootstrap analysis calculated from 100 replicates. Accession numbers are available in the supplemental information table.

Fig. 3.

Relative basal expression of *CYP1A*, *CYP1B1*, and *CYP1C1* in control *Poecilia vivipara* shown as comparison of genes within an organ (A–C), and as genes among organs (D–F). Relative levels were determined by qPCR using the 2^{-C_T} method. Data are presented as a percentage of the highest *CYP1* transcript in each tissue (A–C), or of the tissue with the highest level of a particular *CYP1* transcript (D–F). The dotted lines indicate 100%. Equal letters indicate an absence of difference among groups (determined by one-way ANOVA and Tukey's post hoc test, $p < 0.05$; $n = 7-8$). Data are presented in a logarithmic scale.

Fig. 4.

Relative *CYP1* mRNA expression levels in the liver, gill, and intestine of *Poecilia vivipara* exposed to 3-methylcholanthrene (30 µg/g fish) and then kept in clean water for 24 h. The results shown are as comparisons among genes within an organ. Statistically significant differences versus the carrier control using Students *t*-test (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; $n = 7-8$). The dotted line (1-fold) represents expression of the control groups.

Fig. 5. Relative *CYP1* mRNA expression levels in the liver and gill of *Poecilia vivipara* from a contaminated mangrove site. The results shown are as comparisons among genes within an organ. Statistically significant differences versus fish from an unexposed control site using Students *t*-test (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; $n = 7-8$). The dotted line (1-fold) represents expression of the unexposed control groups.

Table 1

Primer sequences used for cloning of *CYP1A*, *CYP1B1*, and *CYP1C1*, and quantification of *CYP1* gene expression by real-time PCR in *P. vivipara*.

Primer	Sequence (5'-3')	Comment
Cloning		
PVCYP1A F	YCCTATCATTGGAATYTWCTGGAG	Degenerate
PvCYP1A R	TCTGTRATGTCACGGATGTTGTCC	Degenerate
PVCYP1B1 F	GCBGGSAGCATCGTGGACGTG	Degenerate
PvCYP1B1 R	TTGGACAGCTCCTCDDCRATGCA	Degenerate
PvCYP1C1 F	CAGCTGGGYCAGATGCCTCAC	Degenerate
PvCYP1C1 R	TCTGTCCTCSACWGACGGCAGYCG	Degenerate
Pv1A F	TTCATCCCAGACCGCTTCCTCAAT	RACE
Pv1A R	GGACAACATTCGTGACATCACAGA	RACE
Pv1B1 F	AGTGGTCCATCAACCACGACCCGGCCA	RACE
Pv1B1 R	CCCAAAGTCCCGGTTGAGGCCCTTGAA	RACE
Pv1C1 F	GCCAAGACCGGCTGCCGTCAGTCGAGGACA	RACE
Pv1C1 R	CCAAGGACACGGTGGTCTTCATCAA	RACE
Real-time PCR		
Pv1A _{per} -F2	CCGGAAGCTGGATGAGAACTCCAA	CYP1A
Pv1A _{per} -R2	TCACTGCCCATGACAGACCAGTAG	CYP 1A
Pv1B1 _{per} -F1	GAGCATCGTGGACGTGATGCCGTA	CYP 1B1
Pv1B1 _{per} -R1	TCGACTCGACGGACTTCCTGTGCTC	CYP 1B1
Pv1C1 _{per} -F2	GACATGATGACCAAGAGTTCAG	CYP 1C1
Pv1C1 _{per} -R2	ATGACATCTACCAAGCTACCTG	CYP 1C1
PvACT _{per} -F	TCCCTCTCAGCTGTGGTGGTGAA	β-actin
PvACT _{per} -R	ATGTACGTTGCCATCCAGGCCG	β-actin

Table 2

Identities between nucleotide sequences (presented in the right-top) and amino acid sequences (left-bottom) among *P. vivipara* (POEVI) and *F. heteroclitus* (FUNHE) full-length *cyp1* sequences. The full *F. heteroclitus* *Cyp1A*, *Cyp1B1*, *Cyp1C1*, *Cyp1C2* and *Cyp1D1* sequences were obtained from GenBank (accession numbers AF026800, FJ786959, DQ133570, FJ786960 and FJ786961, respectively).

	POEVI_1A	FUNHE_1A	FUNHE_1B1	FUNHE_1C1	FUNHE_1C2	FUNHE_1D1
POEVI_1A		0.90	0.45	0.46	0.46	0.53
FUNHE_1A	0.91		0.46	0.45	0.46	0.53
FUNHE_1B1	0.36	0.35		0.57	0.54	0.44
FUNHE_1C1	0.37	0.36	0.48		0.70	0.44
FUNHE_1C2	0.36	0.36	0.46	0.68		0.46
FUNHE_1D1	0.42	0.42	0.33	0.33	0.35	