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Cytochrome P450 1A, 1B, and 1C mRNA induction patterns in three-spined stickleback exposed to a transient and a persistent inducer

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Abstract

Cytochrome P450 1 (*CYP1*) mRNA induction patterns in three-spined stickleback (*Gasterosteus aculeatus*) were explored for use in environmental monitoring of aryl hydrocarbon receptor (AHR) agonists. The cDNAs of stickleback *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* were cloned and their basal and induced expression patterns were determined in brain, gill, liver and kidney. Also, their induction time courses were compared after waterborne exposure to a transient (indigo) or a persistent (3,3',4,4',5-pentachlorobiphenyl PCB 126) AHR agonist. The cloned stickleback *CYP1*s exhibited a high amino acid sequence identity compared with their zebrafish orthologs and their constitutive tissue distribution patterns largely agree with those reported in other species. PCB 126 (100 nM) induced different *CYP1* expression patterns in the four tissues, suggesting tissue-specific regulation. Both indigo (1 nM) and PCB 126 (10 nM) induced a strong *CYP1* expression in gills. However, while PCB 126 gave rise to a high and persistent induction in gills and liver, induction by indigo was transient in both organs. The number of putative dioxin response elements found in each *CYP1* gene promoter roughly reflected the induction levels of the genes. The high responsiveness of *CYP1A*, *CYP1B1*, and *CYP1C1* observed in several organs suggests that three-spined stickleback is suitable for monitoring of pollution with AHR agonists.

Keywords

Three-spined stickleback; cytochrome P450 1A, 1B and 1C (*CYP1A*, *CYP1B*, *CYP1C*) genes; gill EROD activity; indigo; PCB 126; biomonitoring

1. Introduction

The three-spined stickleback (*Gasterosteus aculeatus* L.) is a euryhaline species, found in streams, rivers, estuaries and marine coastal areas throughout Europe and northern parts of Asia and America (Sanchez et al., 2007). Because of its geographical distribution, robustness, and sensitivity to androgen and estrogen exposure, stickleback is an attractive model in environmental monitoring of hormonally active pollutants (Andersson et al., 2007,

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Björkblom et al., 2007, Geoghegan et al., 2008, Katsiadaki et al., 2002). Stickleback responds to aryl hydrocarbon receptor (AHR) agonists with induction of cytochrome P4501A (CYP1A) mRNA and the CYP1A-catalyzed ethoxyresorufin-*O*-deethylase (EROD) activity (Andersson et al., 2007, Sanchez et al., 2007, Williams et al., 2009). The usefulness of stickleback as a monitoring species for AHR agonists has not been systematically explored, however: the induction response has not been studied for other *CYP1* genes than *CYP1A*.

The regulation of CYP1A by the AHR is well established, and many organic pollutants are potent AHR agonists, including coplanar polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and dibenzofurans, and polycyclic aromatic hydrocarbons (*e.g.*, 3,3',4,4',5-pentachlorobiphenyl, PCB 126; 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD; and benzo[a]pyrene, BaP) (Denison et al., 2002, Williams et al., 2009). A variety of other compounds, including indigoids and tryptophan oxidation products (*e.g.*, 6-formylindolo[3,2-b]carbazole, FICZ) also activate the AHR (Adachi et al., 2001, Denison et al., 2002, Guengerich et al., 2004, Jönsson et al., 2009, Rannug et al., 1987). The natural product indigo is an efficient AHR agonist in various systems (Adachi et al., 2001, Guengerich et al., 2004, Jönsson et al., 2006). Indigo is a widely used dye in fabrics and has been used as an ingredient in Chinese traditional medicine, "Danggui Longhui Wan", intended for treatment of chronic myelocytic leukemia (Hoessel et al., 1999, Xiao et al., 2002). Several studies show that CYP1A induction by indigo is transient, suggesting that it is rapidly metabolized and/or eliminated (Jönsson et al., 2006, Sugihara et al., 2004). The AHR is a transcription factor that following agonistic binding translocates to the nucleus where it dimerizes with the AHR nuclear translocator (ARNT). The subsequent binding of AHR-ARNT complexes to dioxin-responsive elements (DREs) in the *cis*-regulatory regions of *CYP1* genes and other genes leads to transcriptional induction of these genes (Denison et al., 2002, Mandal, 2005, Whitlock, 1999). Numerous chemicals, including pharmaceuticals, have been shown to upregulate *CYP1A1* expression seemingly without activating the AHR in mice, which means EROD and CYP1A mRNA expression potentially are unspecific biomarkers for AHR activation (Hu et al., 2007). To confirm AHR activation it can therefore be useful to also determine the response of other AHR-regulated genes in addition to *CYP1A*, *e.g.*, other *CYP1* family genes.

The CYP1 family typically consists of five genes in fish: *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, and *CYP1D1* (Godard et al., 2005, Goldstone et al., 2009, Goldstone and Stegeman, 2008, Leaver and George, 2000). However, in rainbow trout (*Oncorhynchus mykiss*) at least six *CYP1* genes are present, *i.e.*, two *CYP1A* forms (*CYP1A1* and *CYP1A3*), *CYP1B1*, and three *CYP1C* forms (*CYP1C1*, *CYP1C2*, and *CYP1C3*) (Jönsson et al., 2010). *CYP1A* mRNA and protein are strongly induced in animals exposed to various AHR agonists (Denison and Nagy, 2003, Denison et al., 2002). *CYP1B1* and the *CYP1C*s are inducible by AHR agonists in both adult and embryonic stages of several fish species (Jönsson et al., 2010, Jönsson et al., 2007a, 2007b, Wang et al., 2006, Zanette et al., 2009). The recently discovered *CYP1D1*, which has been studied in zebrafish (*Danio rerio*), killifish (*Fundulus heteroclitus*), clawed frog (*Xenopus tropicalis*), and rhesus macaque (*Macaca fascicularis*) does not appear to be transcriptionally induced by typical AHR agonists (*i.e.*, TCDD, PCB126, and FICZ), suggesting that *CYP1D1* is not AHR-regulated (Goldstone et al., 2009, Jönsson et al., 2011, Zanette et al., 2009). Given their various degree of responsiveness to pollutants, expression patterns of the *CYP1A*, *CYP1B*, and *CYP1C* genes in fish could become useful biomarkers in environmental monitoring (Jönsson et al., 2010).

It is well established that CYP1A and CYP1B1 catalyze bioactivation and detoxification of PAHs such as benzo(a)pyrene (BaP) and dimethylbenz(a)anthracene in mammals and fish

(Conney, 1982, Granberg et al., 2003, Scornaienchi et al., 2010, Shimada and Fujii-Kuriyama, 2004). In addition, binding of persistent PCBs to CYP1A and CYP1B1 may result in uncoupling of the enzyme with a subsequent release of reactive oxygen species (ROS) and oxidative stress as shown *in vitro* in fish and human (Green et al., 2008, Schlezinger and Stegeman, 2001). A recent study examining catalytic activity of the five zebrafish CYP1s reported that all five enzymes including the CYP1Cs and CYP1D1 could metabolize BaP, although CYP1D1 had a different regioselectivity for the molecule than the other four enzymes (Scornaienchi et al., 2010). However, physiological and toxicological functions of proteins in the CYP1C and CYP1D subfamilies remain poorly understood.

CYP1A catalytic activity is classically measured with an EROD assay, and EROD induction is commonly used to assess exposure to AHR agonists in aquatic and terrestrial vertebrates (Bucheli and Fent, 1995, Whyte et al., 2000). As indicated in a previous study in rainbow trout, EROD activity correlates more with *CYP1A1* and *CYP1A3* mRNA expression than with expression of the other *CYP1* forms in this species (Jönsson et al., 2010). EROD activity and *CYP1A* expression have been applied in several biomonitoring studies with three-spined stickleback (Sanchez et al., 2008, Wartman et al., 2009). However, information on mRNA expression of other *CYP1* isoforms than *CYP1A* is lacking in stickleback. The objective of the present study was therefore to identify all *CYP1* genes expressed in three-spined stickleback, and determine their degree of inducibility by PCB 126 in four different organs (liver, brain, gill, and kidney). Another aim was to determine temporal mRNA expression patterns of the full suite of *CYP1* genes in gills and liver of stickleback exposed to either a transient or a persistent *CYP1* inducer (indigo and PCB 126, respectively) for 24 h and then transferred to clean water.

2. Material and Methods

2.1. Animals

Three-spined stickleback caught along the coast near Skanör in south-western Sweden in December 2008, were held in the aquarium facility at the Department of Environmental Toxicology, Uppsala University, Sweden. Fish were maintained in tanks in clean Uppsala tap water (i.e., freshwater; pH 7.5) for more than 5 months before they were used. The fish were fed a mixture of bloodworms (*Chironomus*; Ruto B.V., Zevenhuizen, the Netherlands) and brine shrimp (*Artemia franciscana*, Kordon LLC, Hayward, CA, USA) every other day. To keep the fish in a non-reproductive state, they were held at a low temperature (10° C) and a photoperiod of 8 h light/16 h dark (three-spined stickleback breed in spring-summer). The experiments of the current study were approved by the Local Ethics Committee for Research on Animals in Uppsala.

2.2. Cloning, gene mapping, and sequence analysis

Ensembl 58 (stickleback genome assembly v1, Broad Institute) was used as the genome resource. Five *CYP1* genes and their predicted transcripts were identified, i.e., *CYP1A*, *CYP1B1*, *CYP1C1*, *4CYP1C2*, and *CYP1D1*. Ensembl predictions were either accepted or refined using Genewise. For each putative transcript, four primers were designed: one pair targeting the untranslated regions of the transcript, and one pair targeting part of the coding region. In order to guarantee a high *CYP1* mRNA yield, total RNA was isolated from six organs of stickleback exposed to β -naphthoflavone (1 μ M, 24 h). Total RNA was isolated from liver, brain, gill, heart, eye, and kidney using the Aurum™ total RNA fatty and fibrous Tissue kit (Bio-Rad Laboratories Inc., Hercules CA, USA) and mRNA was isolated from the pooled RNA preparations using the MicroPoly(A)Pure kit (Ambion, Austin, TX, USA). Subsequently cDNA was synthesized by the iScript cDNA Synthesis kit (Bio-Rad). *CYP1* cDNA was amplified by PCR using the gene specific primers with Advantage® cDNA PCR

Kit & Polymerase Mix (Clontech Laboratories Inc., Mountain View, CA, USA with cDNA. The PCR products were separated on a 1% agarose gel and purified using the Mini-Elute gel extraction kit from Qiagen (Valencia, CA, USA). The nucleotide sequences of the PCR products were determined by Uppsala Genome Center (Uppsala University). The cloned nucleotide sequences were aligned using ClustalW resulting in four full length *CYP1* gene coding sequences and the deduced amino acid sequences of these *CYP1*s were compared with homologous sequences in zebrafish by sequence identity analysis in BioEdit (Hall, 1999). Substrate recognition regions (SRS) of the CYP1 proteins were located based on the predictions by Lewis et al. (2003).

A search for the consensus core DRE sequence KNGCGTG in the upstream *cis* regulatory regions of all of the CYP1 genes was performed using the EMBOSS program 'fuzznuc'. Other putative regulatory regions were identified using a matrix similarity search with MatInspector (Genomatix Software GmbH, Munich, Germany).

2.3. Exposures

Fish used in the experiments were randomly collected from the holding tank without selection for sex. In wild stickleback EROD activity has shown to vary between sexes during spring and summer but not in the autumn (Sanchez et al., 2008). To minimize the effect of sex we used fish in non-reproductive state and all experiments were made in autumn. Fish were exposed to indigo (Sigma–Aldrich, St. Louis, MO, USA), PCB 126 (Larodan, Malmö, Sweden), or the carriers (acetone or DMSO) in three sets of experiments, i.e., an organ distribution study, a concentration-response relationship study, and a 10-day time course study. All concentrations given in the text are nominal. No adverse effect of the treatment was observed in any fish.

2.3.1. Organ distribution study—Exposure was performed using glass beakers containing plastic bags (food grade) filled with 2 L continuously aerated Uppsala tap water. In total four beakers were prepared and kept in a trough with running tap water to maintain the water temperature at 10°C. Groups of six randomly selected fish were placed in each beaker. Acetone was added to two of the beakers (solvent controls) and PCB 126 dissolved in acetone was added to the remaining two beakers (PCB 126 exposure groups), yielding 100 ppm of acetone and 100 nM PCB 126 (including 100 ppm of acetone), respectively. After 24 h of exposure, the water was replaced with clean tap water. After being kept in clean water for another 24 h, the fish were decapitated, and liver, brain, gill, and kidney were dissected. For each tissue, six replicates were prepared per exposure group (control and PCB 126). Every replicate was composed of pooled tissue from two fish, i.e., one fish from each of the two beakers belonging to the same exposure group. The fish body weight was 1.2 ± 0.3 g (mean $\pm$ standard deviation of the mean; SD). The samples were stored in RNAlater® (Ambion, Austin, TX, USA) for 24 h (4 °C) before qPCR analysis.

2.3.2. Indigo and PCB 126 concentration response—Groups of six fish were exposed in plastic bags containing 2 L of aerated Uppsala tap water (10°C) as described above (Subsection 2.3.1). Stock solutions for exposure to PCB 126 at various concentrations were prepared in acetone. Each PCB 126 solution was added to the water of one beaker yielding nominal concentrations of 1, 3, 10, 30, and 100 nM of PCB 126 (including 100 ppm of acetone). One beaker containing 100 ppm of acetone served as a PCB 126 solvent control. Indigo exposure solutions were prepared similarly but by using DMSO as a solvent (indigo is more soluble in DMSO than in acetone). In an initial experiment fish were exposed to indigo at nominal concentrations of 1 nM, 10 nM, 100 nM, 1 μ M (all including 50 of DMSO), or 10 μ M (including 500 ppm DMSO), or to solvent controls (50 and 500 ppm DMSO). However all indigo concentrations in this interval induced maximal gill EROD

activity (no statistically significant difference was observed among the groups). Therefore, a second experiment was performed where fish were exposed to a range of lower indigo concentrations (1 pM, 10 pM, 100 pM, and 1 nM, all including 50 ppm of DMSO) or to 50 ppm DMSO (control). Only data from this experiment are shown in the results. Previous studies have shown that indigo is a potent AHR agonist which gives rise to a transient CYP1 induction (Adachi et al., 2001, Guengerich et al., 2004, Jönsson et al., 2006). Therefore we exposed fish to indigo for a short period (6 h) whereas the PCB 126 exposure lasted for 48 h (i.e., 24 h exposure + 24 h clean water). The concentration range and exposure time for PCB 126 were chosen based on data from Jönsson et al. (2006). After exposure, the fish were decapitated (body weight: 1.4 ± 0.3 g) One gill arch was dissected and put into ice-cold HEPES-Cortland (HC) buffer (Jönsson et al., 2002) in order to analyze EROD activity.

2.3.3. Ten-day time course study—The exposure regimen of this experiment is shown in Figure 1. Exposure was carried out in large transparent polyethylene bags (85×60 cm; VWR International, LLC, West Chester, PA, USA). Exposure in such bags has no effect on EROD activity in rainbow trout (Jönsson et al., 2002). The bags were placed in three boxes ($35 \times 20 \times 19$ cm) and filled with 20 L of continuously aerated tap water. Groups of 42 fish were placed in the water in each bag (body weight: 1.3 ± 0.2 g; biomass: 2.3 g/L). Indigo, PCB 126 (both dissolved in DMSO), or DMSO was added to the water in the bags, yielding nominal concentrations of 1 nM indigo, 10 nM PCB 126 (both including 50 ppm DMSO), or 50 ppm DMSO only. The indigo and PCB 126 concentrations used were selected based on results from the concentration-response study, where these concentrations induced maximal gill filament EROD activity. After 3, 6, 9, and 24 h of exposure six fish were randomly sampled from each of the three bags. The fish were decapitated. From each fish one gill arch was dissected and placed in ice-cold HC buffer to be used for determination of EROD activity in gill filaments. The filaments of the rest of the gill arches (cartilage removed) and the liver were frozen in liquid N₂ for later use in real time PCR. The 18 fish remaining at 24 h were transferred to new bags with clean tap water to be kept for another 2 or 9 days, at which six fish from each bag were sampled as described above. The water in each bag was replaced with fresh water after 4 days in clean water. Six and four fish per exposure group were used in the gill EROD assay and real PCR analyses, respectively. The temperature was kept stable (10 ± 0.5 °C) over the whole period of the experiment.

2.4. Gill filament EROD assay

Gill filament EROD activity was analyzed immediately after fish sampling. The method used was the one described by Andersson et al. (2007). In brief, the cartilage was removed and the middle part of the gill arch was cut out resulting in a piece having approximately 20 primary filaments. These pieces were placed in the wells of a 12-well tissue culture plate containing HC buffer. The buffer was replaced by 0.5 mL reaction buffer (consisting of 1 μ M 7-ethoxyresorufin and 10 μ M dicumarol in HC buffer). After 20 min of pre-incubation in room temperature, the buffer was replaced with 0.7 mL fresh reaction buffer. After incubation for 10 and 30 min (exposed fish), or 40 and 60 min (control fish), aliquots of 0.2 mL were transferred from each well to a Fluoronunc 96-well plate. The longer incubation time used for controls was intended to allow the resorufin fluorescence to attain a linear increase during the two samplings (Jönsson et al., 2002). The fluorescence was determined in a multi-well plate reader (VICTOR3, Wallac Oy, Turku, Finland) at 590 nm (emission) and at 544 nm (excitation). EROD activity was calculated using data for the difference in resorufin concentration and time between the two samplings and expressed as picomoles of resorufin formed per filament per minute (the number of filaments assumed being 20).

2.5. Quantitative real time PCR

Total RNA was isolated and DNase-treated using the Aurum™ Total RNA Fatty and Fibrous Tissue kit (Bio-Rad) according to Bio-Rad's instructions. The purity and quantity of RNA were determined spectrophotometrically (260/280 and 260/230 nm ratios were generally 2 or above; NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis kit (Bio-Rad).

Gene-specific quantitative real-time PCR primers (amplicon length: 75-150 bp) for three-spined stickleback *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, and β -actin were synthesized by Sigma-Aldrich (St. Louis, MO, USA) (Table 1). Quantitative PCR was conducted by using Bio-Rad's iQ SYBR Green Supermix and an Rotor Gene 6000 Real-Time PCR Machine (Qiagen, Hilden, Germany). The samples were analyzed in duplicate with the following protocol: 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s and 62°C for 45 s. At the end of each PCR run a melt curve analysis was performed in the range from 55°C to 95°C.

2.6. Calculations and Statistics

Relative mRNA expression of target genes was calculated by $E^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). For all target genes the $E^{-\Delta CT}$ value ($E^{CT_{reference}-CT_{target}}$) of each sample was divided by the mean $E^{-\Delta CT}$ value of the controls, i.e., $E^{-\Delta CT_{[sample]}}/\text{mean } E^{-\Delta CT_{[control]}}$. Mean values of PCR efficiency (E) for within-experiment amplicon groups were determined by the LinRegPCR program (using data within 10% of group median) (Ramakers et al., 2003, Ruijter et al., 2009). The E values obtained ranged from 1.80 to 1.92. Three potential reference genes (β -actin, *ARNT*, and *L13*) were analyzed in the four organs (gill, liver brain, and kidney). Of these, β -actin was selected as the most appropriate because it showed a low individual variation in mRNA expression levels within an organ, and its expression was not significantly affected by the different exposures. Basal levels of mRNA expression in the different organs were studied using data from carrier controls. Because all reference genes showed a considerable variation in expression among organs basal levels were calculated without normalization to an internal control, i.e., by E^{-CT} (Schmittgen and Livak, 2008). Outliers were excluded based on Grubbs test (1969). Statistical analysis was performed using Prism 4 by GraphPad Software Inc. (San Diego, CA, USA) with log-transformed data. The statistical methods used were student's t test and one-way ANOVA followed by Tukey's or Dunnett's post hoc tests. EC_{50} for induction by indigo or PCB 126, i.e., the concentrations causing half maximal response, was calculated by the curve-fitting routine of Prism 4 for nonlinear regression using sigmoidal dose response with variable slope. Specific methods for calculation and statistics and other details are given in the figure captions.

3. Results

3.1. Cloning of CYP1 transcripts and localization of DREs

Using tissues from adult fish, we were able to clone the transcripts of three-spined stickleback *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2*. A complete prediction of the *CYP1D1* gene was previously found in the stickleback genome (Goldstone and Stegeman, 2008). Seeking a transcript of this gene, we used primers targeting predicted untranslated and coding regions. No *CYP1D1* transcript was detected. However, primers designed to amplify part of the predicted *CYP1D1* coding region generated a product covering part of exon 3 and the intron between exons 3 and 4. This was presumably a PCR product of genomic DNA (amplified due to minor contamination of the extracted total RNA).

The deduced amino acid sequences of the cloned stickleback *CYP1* cDNAs were aligned with the corresponding zebrafish sequences, revealing a high identity between orthologs in the two species (*CYP1A* 74%, *CYP1B1* 65%, *CYP1C1* 76%, and *CYP1C2* 75%; Fig. 2). Examination of the *CYP1* gene structures revealed a relative arrangement on the various chromosomes highly similar to the exon-intron structure of the *CYP1*s in zebrafish. The *CYP1* genes in stickleback are compressed on the chromosome (Fig. 3) relative to the zebrafish *CYP1*s (Goldstone et al., 2009, Goldstone and Stegeman, 2008, Jönsson et al., 2007b), possibly reflecting the smaller genome size in stickleback (714 Mb) relative to zebrafish (1.7 Gb) (Ishikawa, 2000).

Putative DREs were located within the 15 kb region upstream from the start codons of the *CYP1* genes using the consensus DRE sequence KNGCGTG (Fig. 3 and Table 2). A varying number of putative DREs were identified in the 15 kb-upstream region of the *CYP1*s, the *CYP1A* gene having the largest number (39, 12, 19, and 17 DREs in the *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* genes, respectively; Fig. 3 and Table 2). Sixteen DREs were located within the 0-3 kb upstream region of the *CYP1A* gene, while *CYP1B1*, *CYP1C1*, and *CYP1C2* had one, three, and two DREs, respectively, in this region (Fig. 3 and Table 2). Within 1 kb upstream from the start codon six DREs were found in the *CYP1A* gene, one DRE was found in the *CYP1B1* and *CYP1C2* genes, while no DRE was found within 1 kb upstream of *CYP1C1*. Note that *CYP1C1* and *CYP1C2* are syntenic, separated on linkage group XV by only about 1 kb (Fig. 3). Goldstone and Stegeman (2008) found four DREs in the 10 kb upstream region of *CYP1D1*.

3.2. Basal expression of CYP1

Stickleback *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2* transcripts were all detected in liver, brain, gill, and kidney from control fish (Fig. 4). For *CYP1A* the highest and lowest levels of expression were found in liver and brain, respectively (liver > kidney > gill > brain; Fig. 4A). On the contrary, *CYP1B1* expression was highest in brain and lowest in liver (brain > gill = kidney > liver; Fig. 4B). The *CYP1C1* transcript was most highly expressed in brain and showed no difference in expression level in the other three organs (brain > liver = gill = kidney; Fig. 4C). Expression of *CYP1C2* was dominant in kidney and very low in gill (kidney > brain = liver > gill; Fig. 4D).

The basal levels of the four *CYP1* genes were also compared within a tissue (Fig. 4E-H). Data were calculated as a percentage of the expression of the most abundant transcript and are shown below within parenthesis in the following order: *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2*. Liver showed a considerably higher expression of *CYP1A* than of the other *CYP1* genes (100%, 0.06%, 0.4%, and 2%; Fig. 4E), while the difference among the genes was smaller in the brain, *CYP1B1* and *CYP1C2* showing the highest expression (23%, 89%, 18%, and 100%; Fig. 4F). In the gill *CYP1A* was most highly expressed and *CYP1C2* was most weakly expressed (100%, 14%, 6% and 2%; Fig. 4G), and in the kidney *CYP1A* and *CYP1C2* were much more highly expressed than *CYP1B1* and *CYP1C1* (83%, 1%, 1%, and 100%; Fig. 4H).

3.3. PCB 126-induced CYP1 expression

No treatment-related adverse effect in response to PCB 126 was observed among the fish after 48 h of exposure. The results of PCB 126-induced *CYP1* expression are illustrated in Figure 5. *CYP1A* and *CYP1C1* showed a statistically significant induction over the control in all four tissues examined (Fig. 5A and 5C). For *CYP1A* the highest induction was observed in brain and kidney (115- and 94-fold over the control; Fig. 5A). *CYP1B1* showed a relatively low induction in liver, gill, and kidney (7-, 10-, and 10-fold over the control) and no induction in the brain (Fig. 5B). *CYP1C1* and *CYP1C2* showed similar expression

patterns, although for *CYP1C2* no induction in brain and gills and a very weak induction in kidney were recorded (Figs. 5C and 5D). The liver showed a stronger induction of *CYP1C1* and *CYP1C2* than of *CYP1A* (103- and 68-fold over the control versus 52-fold over the control; Fig. 5E), whereas *CYP1A* exhibited a much stronger induction than the other three *CYP1* transcripts in brain, gill, and kidney (Figs. 5F-H). *CYP1A* and *CYP1C1* were significantly induced in brain (115- and 3-fold over the control), whereas *CYP1B1* and *CYP1C2* did not show any induction in the brain (Fig. 5F). The level of *CYP1A* induction was lower in gills than in kidney, but otherwise the induction pattern of the four genes was similar in these two tissues (Fig. 5G and 5H).

3.4. Concentration response to indigo and PCB 126 of gill EROD activity

Both indigo and PCB 126 induced EROD activity in gill filaments in a concentration dependent manner (Fig 6). All concentrations examined, including the lowest (1 pM indigo and 1 nM PCB 126), yielded a significant induction (Fig. 6A). For indigo maximal induction was observed at 1 nM (Fig. 6A), and higher concentrations (up to 10 μ M) caused no further effect (data not shown). In PCB 126-treated fish, the maximal gill EROD activity was achieved at 100 nM nominal concentration (Fig. 6B). The nominal EC₅₀ values for EROD induction in gills by indigo and PCB 126 were approximately 0.09 nM and 6 nM, respectively, although the actual EC₅₀ values may be lower due to sorptive loss particularly of PCB 126.

3.5. Time courses for EROD activity and CYP1 expression after exposure to indigo and PCB 126

Groups of three-spined stickleback were exposed to indigo (1 nM), PCB 126 (10 nM), or solvent (50 ppm DMSO) for 3, 6, 9, or 24 h, while other groups were exposed for 24 h and subsequently held in clean water for 2 or 9 days (for experimental design see Fig. 1).

3.5.1 Gill EROD activity—Figure 7 shows the time course of gill EROD induction. The EROD induction level peaked after 9 h and 24 h of exposure to indigo and PCB 126, respectively. In fish exposed to indigo and subsequently transferred to clean water, gill EROD activity was markedly reduced after 2 days, and had returned to the control level after 9 days (Fig. 7A). The level of EROD induction in PCB 126-exposed fish tended to decrease after transfer to clean water, although no statistically significant change was observed (Fig. 7A). DMSO-treated fish showed no significant change in EROD activity over time (Fig. 7A).

When compared to the control at each sampling time point a statistically significant induction by indigo was observed as soon as after 3 h of exposure (3-fold over the control), whereas an EROD induction by PCB 126 was not observed until after 6 h (12-fold over the control; Fig. 7B). The highest recorded levels of EROD induction by indigo (9 h) and PCB 126 (24 h) were 100- and 110-fold over the controls, respectively. All indigo-exposed fish, except for those exposed for 1124 h and then held in clean water for 9 days showed a significant EROD induction compared to the controls (Fig. 7B). EROD induction by PCB 126 was observed at all time points except for the first (3 h; Fig. 7B). Hence, EROD induction by indigo was transient whereas induction by PCB 126 persisted for the duration of the experiment.

3.5.2. CYP1 expression in gills and liver—Figure 8 shows the time courses for expression of the four *CYP1* transcripts in DMSO-, indigo- or PCB 126-exposed fish, and Figure 9 shows the effect of indigo and PCB 126 versus the DMSO controls at each sampling time point.

Gills: Both indigo and PCB 126 induced *CYP1A* expression in the gills after only 3 h of exposure, and *CYP1A* induction peaked after 6 h and 6-9 h of exposure to indigo and PCB 126, respectively. After 24 h of exposure *CYP1A* expression was back to the control level in indigo-exposed fish while it had declined to about 50% of maximum in PCB126-exposed fish. Following 9 days in clean water the *CYP1A* transcript was no longer induced in indigo-exposed fish, while it remained induced at 30% of maximum in PCB 126-exposed fish. The *CYP1B1* and *CYP1C1* transcripts displayed time trends resembling that of *CYP1A* in indigo-exposed fish although the induction levels were generally lower. *CYP1B1* expression in gills of PCB 126-exposed fish remained induced over the course of the experiment varying between 4- and 8-fold over the 3-h control. PCB 126 induced a lower level of *CYP1C1* expression in gills than indigo, but unlike indigo PCB 126 tended to give rise to persist induction from 6 h and during the rest of the experiment. Neither indigo nor PCB 126 had any clear effect on *CYP1C2* expression over time. In gills of indigo-exposed fish the maximal levels of induction of *CYP1A*, *CYP1B1*, and *CYP1C1*, were 200-, 14-, and 8-fold over the control, respectively (all after 6 h of exposure), and in gills of PCB 126-exposed fish the maximal levels of induction of these transcripts were 130-, 8-, and 3-fold over the control, respectively (after 6 or 9 h of exposure).

Liver: In the liver, *CYP1A* induction reached the maximal level after 6 and 24 h of exposure to indigo and PCB 126, respectively. After holding indigo-exposed fish in clean water for 2 days *CYP1A* expression was reduced to 20% of maximum and after 9 days *CYP1A* expression was back to the control level. A statistically significant induction of *CYP1B1*, *CYP1C1*, and *CYP1C2* by indigo was only observed in the 6-h exposure group. In PCB 126-exposed fish *CYP1A* expression was strongly induced at all time points including after 9 days in clean water. *CYP1B1* expression in PCB 126-exposed fish showed a weak induction after 9 h of exposure, tended to remain induced after 2 days in clean water, and was back to the control level after 9 days in clean water. *CYP1A* and *CYP1C2* expression tended to increase over time within the 24-h period of PCB 126 exposure, and stayed induced after transfer to clean water. *CYP1C1* expression showed trends similar to that of *CYP1C2* in both indigo- and PCB 126-exposed fish, but the levels of induction were lower. The maximal levels of *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2* induction by indigo were 24-, 6-, 2-, and 3-fold over the control, respectively, and the maximal levels of induction by PCB 126 of the same transcripts were 100-, 3-, 2-, and 16-fold over the control, respectively.

The four *CYP1* transcripts showed some degree of variability in the DMSO treated group. We have previously observed that EROD activity is slightly affected by DMSO (Jönsson et al., 2006). The reason for this is not known.

4. Discussion

4.1. Three-spined stickleback CYP1 genes in environmental monitoring

Cytochrome P450 family 1 (CYP1) enzymes play important roles in the metabolism of certain combustion products, pharmaceuticals and other compounds. Generally, this metabolism leads to detoxification, but may in certain cases result in bioactivation, mutagenesis, cancer induction and other types of toxicity (Conney, 1982, Gelboin, 1980). CYP1 induction is commonly used as a biomarker for pollution with chemicals activating the AHR.

In the current study we developed methods for evaluation of the full suite of pollutant-inducible CYP1 mRNAs (*CYP1A*, *1B*, *1C1* and *1C2*) in stickleback. Based on the results obtained, we conclude that stickleback is a useful model species for monitoring exposure to AHR agonists in polluted waters, including both marine and fresh water. The advantage of stickleback for this purpose is that other biomarker endpoints can also be recorded

simultaneously in the same fish, such as androgenic, antiandrogenic, and estrogenic responses (Andersson et al., 2007). In addition to its proposed usefulness in field studies, the small size of stickleback is attractive when characterizing water samples, individual pharmaceuticals, and other chemicals in the laboratory. We have shown that three-spined stickleback can be used also to evaluate inhibition of EROD activity by pharmaceuticals (Beijer et al., 2010), and studies are underway to characterize wastewater treatment plant effluent samples both with regard to CYP1 induction and inhibition in stickleback. The different temporal and spatial induction patterns of the four AHR-regulated CYP1 genes observed here could provide a basis for using the induced CYP1 patterns to characterize AHR ligand potency and persistence in environmental samples.

4.2. CYP1 gene structure

The number of predicted coding exons in the stickleback *CYP1A* and *CYP1B1* genes were 6 and 2 (Fig. 3), the same as in the zebrafish and medaka orthologs. Both *CYP1C1* and *CYP1C2* were found to be single exon genes in stickleback as they are in zebrafish. The two *CYP1C* genes occur immediately adjacent to each other in the fish genomes, presumably as result of a tandem duplication event. In the stickleback genome, we found that the *CYP1C1* gene was located about 1 kb upstream of the *CYP1C2* gene. This is not consistent with the *CYP1C* gene structure in zebrafish where *CYP1C1* is located about 4 kb downstream from *CYP1C2* (Jönsson et al., 2007b). Molecular phylogenetic analysis indicates that the stickleback CYP1 proteins are more closely related to the CYP1s in killifish and medaka than to those in zebrafish (Zanette et al., 2009). A fifth gene, *CYP1D1*, is also present in the stickleback genome (Goldstone and Stegeman, 2008), but we could not detect any mRNA for this gene, suggesting that *CYP1D1* was not expressed in any of the pooled organs examined (liver, brain, gill, heart, eye, and kidney). In zebrafish and killifish, liver and brain were among the tissues having the highest *CYP1D1* expression levels (Goldstone et al., 2009, Zanette et al., 2009) and these two tissues were included in the cDNA pool used in the current study. Furthermore, we obtained a PCR product covering a partial exon and intron of the *CYP1D1* gene indicating that at least one primer pair used was appropriate. Together these facts suggest that the likelihood of finding a *CYP1D1* transcript would have been high if it were expressed. It is presently not known whether the three-spined stickleback *CYP1D1* gene is expressed at earlier developmental stages or in organs not examined in the current study. It is also possible that *CYP1D1* is not expressed at all in this species.

Putative binding sites for the AHR/ARNT complex (*i.e.*, DREs) were searched for in the 15 kb regions upstream from the start codon in the three-spined stickleback *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2* genes, using the consensus DRE sequence, KNGCGTG (ZeRuth and Pollenz, 2007). The presence of abundant putative DREs upstream of all cloned *CYP1* transcripts and the *CYP1* induction by PCB 126 and indigo strongly suggests that the genes are AHR-regulated. Elimination of CYP1 gene induction by morpholino oligonucleotide knockdown of AHR would confirm this finding (Jönsson et al., 2007a).

4.3. Basal and PCB 126-induced CYP1 expression patterns in three-spined stickleback

The basal and induced CYP1 expression patterns determined in control and PCB 126-exposed stickleback varied in the organs examined suggesting that the four genes have tissue-specific regulation and possibly different functions. Basal levels of *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* expression were highest in liver, brain, brain, and kidney, respectively. The results for *CYP1A* and *CYP1B1* are consistent with previous findings in zebrafish, killifish, and mouse where basal expression of *CYP1A* was high in liver and low in brain while the opposite situation occurred for *CYP1B1* (Choudhary et al., 2005, Jönsson et al., 2007b, Zanette et al., 2009). The chicken embryo also expresses a high level of *CYP1B1* mRNA in the brain (Chambers et al., 2007). Endogenous functions of CYP1A and

CYP1B1 are poorly understood. CYP1A has been proposed to play a role in controlling AHR activation via a negative feed-back loop by metabolizing endogenous AHR ligands (Chiaro et al., 2007). Chambers et al. (2007) found that CYP1B1 can catalyze the formation of retinoic acid and suggested it plays a role in retinoic acid mediated patterning during embryogenesis. CYP1B1 has also proven crucial for normal development of the eye in mammals (Choudhary et al., 2007), and in line with this, *CYP1B1* is highly expressed in the zebrafish eye (Jönsson et al., 2007b). It is also notable that *CYP1B1* occurs at higher levels in various cancers (breast, colon, lung, skin, brain, testis, etc.) than in the corresponding healthy tissues in human (Murray et al., 1997).

The *CYPIC* genes were only recently described and little is known about their functions. *CYPIC*s have been identified in fish, frogs, and birds but not in mammals (Godard et al., 2005, Goldstone et al., 2007, Jönsson et al., 2011, Jönsson et al., 2010, Jönsson et al., 2007b, Zanette et al., 2009). The CYPIC protein sequences are more similar to CYP1B1 proteins than to CYP1A proteins and phylogenetic analyses indicate that CYPIC and CYP1B1 proteins are more closely related to each other than to the CYP1As and CYP1D1s (Godard et al., 2005, Jönsson et al., 2011, Jönsson et al., 2010). In line with a close relationship the expression patterns of the CYPICs and CYP1B1 showed a marked similarity when examined in eight zebrafish organs (Jönsson et al., 2007b). Stickleback *CYPIC1* and *CYPIC2* were most highly expressed in brain and kidney, respectively, and of the four organs currently examined brain and kidney had the highest expression of the CYPICs also in zebrafish and killifish (Jönsson et al., 2007b, Zanette et al., 2009). In zebrafish, a high CYPIC expression was also observed in eye and heart (Jönsson et al., 2007b). Together the results in fish suggest the CYPICs could have particular functions in brain, kidney, eye, and heart.

Although *CYP1* mRNA expression patterns yield some information on potential CYP1 enzyme functions, enzyme activities depend on a range of events downstream from transcription, such as mRNA degradation, translation efficiency, protein stability, and cofactor levels. Hence, CYP1 mRNA levels and enzyme activities are not always directly correlated. Cellular localization and catalytic activity of the CYP1 enzymes is required to improve our understanding of their function, in any species. The inducibility of the four *CYP1* genes was determined in fish exposed to 100 nM PCB 126 via the ambient water. This concentration produces malformations and edema in zebrafish embryos and is high enough to ensure an effect on inducible genes in both embryonic and adult zebrafish (Jönsson et al., 2007b). The CYP1A enzyme/gene is induced in a range of tissues in fish, including all those examined in the current study (Chung-Davidson et al., 2004, Jönsson et al., 2007b, Smolowitz et al., 1992). Accordingly, stickleback *CYP1A* was strongly induced in brain and kidney (about 100-fold over the control), as well as in liver and gills (about 50-fold over the control). Induction of *CYP1B1* by PCB 126 (up to 10-fold over the control) was observed in stickleback liver, gill and kidney, but not in brain. Similarly, zebrafish and killifish showed strong or moderate induction of *CYP1B1* in liver, gill and kidney, while brain showed a slight *CYP1B1* induction (Jönsson et al., 2007b, Zanette et al., 2009). Since brain had a high basal level of *CYP1B1* in all these species it seems possible that the expression could not increase much above this level. The two stickleback *CYPIC*s were highly inducible by PCB126 in liver, but while *CYPIC1* responded to PCB 126 with induction in the other three organs as well, *CYPIC2* showed no induction in brain and gill and only a very slight induction in kidney. In killifish and zebrafish liver *CYPIC1* is highly induced by PCB 126 whereas *CYPIC2* induction is weaker; in fact the liver was the only organ showing induction of *CYPIC2* in zebrafish (Jönsson et al., 2007b, Wang et al., 2006, Zanette et al., 2009). For a comparison, PCB 126 induced the three rainbow trout *CYPIC* genes strongly in the gills (80–90-fold over the control) but only moderately in the liver (4–17-fold over the control), *CYPIC1* being the most responsive and *CYPIC2* the least

responsive in liver (Jönsson et al., 2010). In conclusion, the current results indicate that the CYP1 genes respond to PCB 126 in a tissue specific manner, suggesting they are differently regulated in different cell types. Furthermore, the relative induction level appears to partly depend on the basal level, such as for *CYP1B1* and *CYP1C2* which had high basal expression in brain and kidney, respectively, but which showed no or only slight induction in the same organs.

4.4. Indigo and PCB 126 concentration response study

Indigo is a natural AHR agonist with a high AHR binding affinity in mammalian cells (Denison et al., 2002), and a transient effect *in vivo* (Guengerich et al., 2004, Jönsson et al., 2006). Accordingly, in this experiment the CYP1 induction potency and persistency of indigo was compared with that of PCB 126. The gill filament EROD assay was applied, because it is a rapid and sensitive method to assay AHR activation (Jönsson et al., 2002). We observed that 6 h of exposure to indigo was more potent than 48 h of exposure to PCB 126 in stickleback based on their nominal EC₅₀ induction values (approximately 0.09 nM versus 6 nM). However, since exposure was performed via the water, the EC₅₀ values observed were likely influenced by the different hydrophobicities of indigo (Log P_{ow}: 3.6) and PCB 126 (Log P_{ow}: 7.0) (Hou et al., 1991, Kong et al., 2007). Consequently, the actual PCB 126 EC₅₀ value is likely to be considerably lower than 6 nM, depending on sorptive losses in the experimental system. Nonetheless, indigo proved to be a potent AHR agonist in stickleback with a maximal gill EROD induction level observed at a nominal concentration as low as 1 nM.

4.5. Indigo and PCB 126 time course study

Indigo and PCB 126 displayed different induction patterns over time, particularly following transfer to clean water (Fig. 8). Indigo significantly induced the *CYP1* mRNA expression level and EROD activity in the gills after 3 h of exposure, while after 6 h, the induction response to both indigo and PCB 126 was strong, indicating that both compounds rapidly reached intracellular concentrations sufficiently high to activate the AHR in gill cells. Notably, the *CYP1A* mRNA induction pattern was consistent with the EROD induction pattern in gills, with the exception that mRNA level peaked after 6 h while EROD activity peaked after 9 h. This delay most probably reflects the time lag between mRNA and protein synthesis.

After replacement of the indigo exposure solution with clean water, EROD activity and *CYP1* transcript levels decreased rapidly. Furthermore, in indigo-exposed fish the liver showed a much weaker *CYP1A* induction response than the gill: hepatic *CYP1B1* and *CYP1C* induction occurred only in the 6-h exposure group. These observations conform to the notion that indigo was rapidly eliminated after uptake by the gills. The CYP1A-activated carcinogen benzo[a]pyrene undergoes first-pass metabolism in the fish gill (Andersson and Pärt, 1989, Stegeman et al., 1984); this is probably true also for indigo. In mammals, the indigo derivatives indirubin and indirubin 3'-oxime are metabolized by CYP1A1 and/or CYP1B1 (Adachi et al., 2004, Guengerich et al., 2004, Spink et al., 2003). FICZ is an indole derivative, which has proved to be an exceptionally good substrate for human CYP1 enzymes (Rannug et al., 1987, Wincent et al., 2009). Zebrafish embryos exposed to FICZ exhibit higher levels of induced *CYP1A* after 6 h of exposure than after 12 h, suggesting that FICZ is rapidly degraded also in fish (Jönsson et al., 2009). It seems likely that both indigo and FICZ are metabolized by induced CYP1 and phase II enzymes, although this has not been determined experimentally in fish.

Whereas *CYP1* induction by indigo was transient, induction by PCB 126 persisted during the whole 10-day period monitored, possibly reflecting a difference in degradability of the

two inducers. Similar results were reported for EROD induction by indigo and PCB 126 in rainbow trout (Jönsson et al., 2006). In the trout, EROD induction by PCB 126 was delayed in the liver compared to the gill, and after transfer to clean water the EROD activity decreased in the gill whereas it increased in the liver. These findings were interpreted to reflect a redistribution of PCB 126 from gills and other tissues to the liver, resulting in PCB 126 levels in the liver increasing with time. Although the current results in stickleback resemble those in rainbow trout (Jönsson et al., 2006), no increased *CYP1* induction was observed in stickleback liver following transfer of the fish to clean water. This difference may be due to kinetic reasons, e.g. different body size and rate of redistribution of inducer following absorption via the gills.

Conclusion

We cloned four *CYP1* genes in three-spined stickleback, *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2*, and demonstrated that they are constitutively expressed in several organs. The observation that the magnitude of induction of these genes varied in different organs and at different exposure times in indigo- and PCB 126-exposed fish suggest that that induced stickleback *CYP1* transcript patterns may be useful biomarkers for AHR agonistic environmental pollutants in water.

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Experimental design

Duration of exposure
 Exposure: DMSO
 Indigo
 PCB126
 Total no. fish / exposure = 42
 Sampling (No. fish / exp.
 and time point)

Methods of analyses
 Gill EROD
 qPCR in gill and liver

Figure 1.

Exposure regimen for the time course study. Groups of 42 three-spined stickleback were exposed to 50 ppm DMSO (carrier control), 1 nM indigo or 10 nM PCB 126 for 24 h. Fish were sampled after 3 h, 6 h, 9 h and 24 h. In addition, fish were sampled after 24 h of exposure followed by holding in clean water for 2 days (+2d) or 9 days (+9d). At each time point, 6 fish were randomly selected for analysis.

SB CYP1A		MVLN	MLPLVGVSV	SGSLVMTTL	CLVYLIVKFF	HTETPKGLR	RLGPKPLPL	IGNVLELSR	PYLSLTAMSK	73
ZF		A.T	I.L	I.L		IMRLR	R.K	D.A	Q	73
SB CYP1B1		MS	SNR	KVL	I.CY		LFSLILMR	RLR	ARRSSS	63
ZF		MM	SVLLA	LRD	LGLS	R	SVLLS	IMVCL	MLMFRRRQ	65
SB CYP1C1		MALDTEFGVK	GSSIIREW	G	QVQPAIVASF	VFLVCLACL	WVRNLRLEK		FAN	79
ZF		MEAEFG	K	GSSIIREW	G	QVQPAIVASF	VFLVCLACL	WVRNLRLEK		79
SB CYP1C2		MYDTEFGVK	GSSIIREW	G	QVQPAIVASF	VFLVCLACL	WVRNLRLEK		FAN	79
ZF		MAQSD	S	KPSILKEW	G	QVQPAIVASF	VFLVCLACL	WVRNLRLEK		76
SB CYP1A		RYGHIPIQIGI	GMRFVVVLSS	ND	TLAQALI	KQGDFA	RP	DLTSPILIND	GSLAPSTDQ	152
ZF		C	RV		VI					152
SB CYP1B1		K	NV		KL	A.T				140
ZF		K	DV		KL	S.N				142
SB CYP1C1		K	NV		KL	CSD				157
ZF		K	NV		KL	CSD				155
SB CYP1C2		K	NV		KL	CSD				157
ZF		K	NV		KL	CSD				154
SB CYP1A		LGTTTETSC	MLEKILKES	KYLIOQLKDV	MASDGSFDFP	RSIVSVANV	ICMGCFORRY	SHDDQELLG	VLLGCEFGD	232
ZF		VQ	KS		K					232
SB CYP1B1		GNAR	KK		AP	B	V	C.V		217
ZF		ANIQ	KQ		TP	K	VS	I	GE	219
SB CYP1C1		GNIS	KK		AP	Q	TA	A	ME	234
ZF		ANSQ	KK		TP	Q	VVG	A	MD	232
SB CYP1C2		ANSQ	KK		AP	BQ	AA	A	TE	234
ZF		ANSQ	KK		SP	K	VA	A	VD	227
SB CYP1A		AGSGNPADFI	PALRPLEN	ALMTKEDI	WAFNIFVQK	IVSEHYATYD	KDRIIDITDS	LIDGCEDRKL	DENSNVMSD	309
ZF		V								309
SB CYP1B1		V	A	SLV	VM		W	QTF	PT	295
ZF		V	A	SLV	VM		W	QTF	PT	295
SB CYP1C1		V	A	SLV	VM		W	QTF	PT	309
ZF		V	A	SLV	VM		W	QTF	PT	307
SB CYP1C2		V	A	SLV	VM		W	QTF	PT	310
ZF		V	A	SLV	VM		W	QTF	PT	303
SB CYP1A		KIVIVVNDL	PAGAFDTPT	TLSSWSVMVM	ANPETQERLI	QELKDKVOLD	RSPLLSKPFN	LPFLKAPLE	SLRSLPFLP	389
ZF										389
SB CYP1B1										375
ZF										378
SB CYP1C1										389
ZF										387
SB CYP1C2										390
ZF										383
SB CYP1A		ETP	CTTKDT	SLNGYPIPKD	TCVPIHQGV	NQDPELWQDP	STYFNPRLS	ADGTEINKL	GERVWAPNS	469
ZF										469
SB CYP1B1										454
ZF										457
SB CYP1C1										468
ZF										466
SB CYP1C2										469
ZF										462
SB CYP1A		RNEVILFLAI	IVQKLPHRAI	PGELINITE	TLITIME	KRC	HLRATMBARS	EE		521
ZF										519
SB CYP1B1		KLQLE	TYL	LAHQ	ITVD	AR	VRLDPN			525
ZF		KLQLE	TSV	LAHQ	K	E	STP			526
SB CYP1C1		KV	F							525
ZF		KV	F							523
SB CYP1C2		KV	IFM							523
ZF		KV	F	IS						514

Figure 2.

Alignment of deduced amino acid CYP1 sequences of three-spined stickleback *Gasterosteus aculeatus* L. (SB) and zebrafish *Danio rerio* (ZF). Five *CYP1*-like genes (*CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, and *CYP1D1*) were identified in the stickleback genome. Except for *CYP1D1* all transcripts could be cloned. “SRS” = substrate binding site; “Heme bind” = heme binding site. The SRS positions were searched according to Lewis et al. (2003).

Figure 3.

Gene structure and dioxin response elements (DRE) for the four *CYP1* transcripts cloned in three-spined stickleback. Exons are shown as black boxes. The numbers above the coding region start refer to the location on the chromosome (Ensembl 58). Shown also are the location of calculated DRE sequences (KNGCGTG).

Figure 4.

Relative basal expression of the cloned three-spined stickleback *CYP1* transcripts illustrated in two different ways: as comparisons of a gene among organs (A-D) and among genes within an organ (E-H). Expression was calculated by E^{-CT} using data from carrier controls (100 ppm of acetone; refer to Fig. 5). Data are presented as a percentage of the level in the tissues with highest *CYP1* transcript level (A-D), or one of the *CYP1* transcripts in each tissue with highest level (E-H). The dotted lines indicate 100%. Difference within each bar graph was determined by one-way ANOVA followed by Tukey's post hoc test after log transformation of data (mean $\pm$ SD, $p < 0.05$). Different letters indicate a statistically significant difference. The bars for *CYP1B1* expression in kidney represent data from five replicates. All other bars represent data from six replicates.

Figure 5.

Relative *CYP1* mRNA expression level in liver, brain gill, and kidney of three-spined stickleback exposed to 100 nM PCB 126 (including 100 ppm acetone) for 24 h and then kept in clean water during 24 h. The results are shown as comparisons of a gene among organs (A-D) and among genes within an organ (E-H). Statistically significant differences versus the carrier control (100 ppm acetone) were determined by unpaired *t* test with Welch's correction (mean $\pm$ SD, ***= p <0.001 and **= p <0.01; n = 5-6). The bars for *CYP1C1* expression in liver and *CYP1C2* expression in brain represent data from five replicates. All other bars represent data from six replicates. The dotted line (1-fold) stands for expression of the control groups.

Figure 6.

Concentration-response relationships of EROD activity in gill filaments of three-spined stickleback exposed to indigo (A) or PCB 126 (B). Fish were exposed to indigo (1 pM - 1 nM) plus 50 ppm DMSO (carrier) or 50 ppm DMSO only for 6 h. Other fish were exposed to PCB 126 (1 - 100 nM) plus 50 ppm acetone (carrier) or 50 ppm acetone only for 24 h and subsequently held in clean water for 24 h. Statistical differences were determined by one-way ANOVA followed by Dunnett's test (Mean $\pm$ SD, ***= p <0.001 and **= p <0.01). Bars for 10 and 30 nM PCB 126 represent data from five replicates. All other bars represent six replicates.

Figure 7.

Time course of EROD induction (A) and induction level at various exposure times (B) in gills of three-spined stickleback exposed to indigo or PCB 126. Groups of fish were exposed to the carrier (50 ppm of DMSO), indigo (1 nM), or PCB 126 (10 nM) for 3, 6, 9, and 24 h, and groups were exposed for 24 h and subsequently held in clean water for 2 days (+2 days) or 9 days (+9 days). A) For each compound (DMSO, indigo, or PCB 126) levels of EROD activity at different exposure time points were compared by one-way ANOVA followed by Tukey's test using log transformed data. Statistically significant differences among the groups are indicated by different letters ($p < 0.05$) below the graph. B) At each time point, differences between EROD activity in the exposure groups (indigo or PCB 126) and the control group (DMSO) were determined using ANOVA followed by Dunnett's test. Statistically significant differences are indicated by stars ($*** = p < 0.001$, $** = p < 0.01$ and $* = p < 0.05$). Data are shown as mean $\pm$ SD, $n = 6$. A detailed exposure regimen is given in Figure 1.

Figure 8.

Time course for relative expression of *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* in three-spined stickleback gills and liver following exposure to carrier (50 ppm of DMSO), indigo (1 nM), or PCB 126 (10 nM). For each chemical, *CYP1* expression was analyzed after 3, 6, 9, and 24 h of exposure, and after 24 h of exposure followed by holding in clean water for 2 days (+2 days) or 9 days (+9 days). Calculations were made using β -actin as reference gene and the 3-h DMSO group as a calibrator. For each compound (DMSO, indigo, or PCB 126) expression levels at different exposure time points were compared by one-way ANOVA followed by Tukey's test using log transformed data. Statistically significant differences among the groups are indicated by different letters ($p < 0.05$) below each graph. Data are shown as mean $\pm$ SD. The data point for gills, 9 h of exposure to PCB126 represents data from three replicates and all other points represent data from four replicates.

Figure 9.

Relative expression of *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* in three-spined stickleback gills and liver following exposure to carrier (50 ppm of DMSO), indigo (1 nM), or PCB 126 (10 nM). For each chemical, *CYP1* expression was analyzed after 3, 6, 9, and 24 h of exposure, and after 24 h of exposure followed by holding in clean water for 2 days (+2 days) or 9 days (+9 days). Calculations were made using β -actin as reference gene and the 3-h DMSO group as a calibrator. Statistically significant differences between the exposure groups (indigo or PCB 126) and the control group (DMSO) at each time point were examined by one-way ANOVA followed by Dunnett's test using log-transformed data. Statistically significant differences are indicated by stars (***= $p<0.001$, **= $p<0.01$ and *= $p<0.05$). Data are shown as mean $\pm$ SD. The data point for gills, 9 h of exposure to PCB126 represent data from three replicates and all other points represent data from four replicates.

Table 1

Primer sequences (5' to 3') used to determine mRNA expression levels of the cloned three-spined stickleback CYP1s.

Gene	Forwardprimer	Reverse primer	GenBank Acc. No
<i>CYP1A</i>	ACCTATGACAAGGACCACAT TCGT	ACAATTCCCACAATCTTCTC GTCT	HQ202281
<i>CYP1B1</i>	GACTACGTGACTCCCACAAT AG	GGAACCTTGACAAGCAAGAG G	HQ202282
<i>CYP1C1</i>	AGCAAACAGTGGAAGCAC A	GAATACCTGCACCAGCTCC AT	HQ202283
<i>CYP1C2</i>	GAAGAGCATGACGTTCAAC AG	TGTGGCTTCAGCAGCTATTT GG	HQ202284
<i>β-actin</i>	ACATCAGGGAGTGATGGTG G	CAGGATACCTCTCTTGCTCT G	DQ018719

Table 2

Localization and number of putative dioxin response elements (DREs) upstream from the start codon in the three-spined stickleback (*Gasterosteus aculeatus* L.) *CYP1* genes.

Gene	Number of consensus DREs ^a within						
	-1 kb	-2 kb	-3 kb	-4 kb	-5 kb	-10 kb	-15 kb
<i>CYP1A</i>	6	9	16	16	19	34	39
<i>CYP1B1</i>	1	1	1	5	5	9	12
<i>CYP1C1</i>	0	0	3	6	6	11	19
<i>CYP1C2</i>	1	1	2	3	3	10	17

^aThe consensus DRE sequence [KNGCGTG] was searched for on both strands (ZeRuth and Pollenz, 2007).