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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

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Halogenated agonists for the aryl hydrocarbon receptor (AHR), such as 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), cause developmental toxicity in fish. AHR dependence of these effects is known for TCDD but only presumed for PCB126, and the AHR-regulated genes involved are known only in part. We defined the role of AHR in regulation of four cytochrome P450 1 (*CYP1*) genes and the effect of PCB126 on cell cycle genes (i.e., *PCNA* and *cyclin E*) in zebra fish (*Danio rerio*) embryos. Basal and PCB126-induced expression of *CYP1A, CYP1B1, CYP1C1*, and *CYP1C2* was examined over time as well as in relation to cell cycle gene expression and morphological effects of PCB126 in developing zebra fish. The four *CYP1* genes differed in the time for maximal basal and induced expression, i.e., *CYP1B1* peaked within 2 days postfertilization (dpf), the *CYP1C*s around hatching (3 dpf), and *CYP1A* after hatching (14–21 dpf). These results indicate developmental periods when the CYP1s may play physiological roles. PCB126 (0.3–100nM) caused concentration-dependent *CYP1* gene induction (EC_{50}: 1.4–2.7nM, Lowest observed effect concentration [LOEC]: 0.3–1nM) and pericardial edema (EC_{50}: 4.4nM, LOEC: 3nM) in 3-dpf embryos. Blockage of *AHR2* translation significantly inhibited these effects of PCB126 and TCDD. *PCNA* gene expression was reduced by PCB126 in a concentration-dependent manner, suggesting that PCB126 could suppress cell proliferation. Our results indicate that the four *CYP1* genes examined are regulated by AHR2 and that the effect of PCB126 on morphology in zebra fish embryos is AHR2 dependent. Moreover, the developmental patterns of expression and induction suggest that CYP1 enzymes could function in normal development and in developmental toxicity of PCB126 in fish embryos.

Key Words: **cytochrome P450 1 (CYP1); 3,3′,4,4′,5-pentachlorobiphenyl (PCB126); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); embryotoxicity; aryl hydrocarbon receptor (AHR); PCNA.**

Developmental effects of the potent aryl hydrocarbon receptor (AHR) agonist 2,3,7,8-tetrachlorodibenz-*p*-dioxin (TCDD) are similar among vertebrate groups (mammals, birds, and fish) and resemble some birth defects in humans. Prominent among these effects of TCDD are edemas, heart and circulatory defects, and craniofacial abnormalities (Goldstone and Stegeman, 2006; Heideman *et al*., 2005). Fish generally are more sensitive to these effects than are mammals (Hahn, 2001), and studies in zebra fish (*Danio rerio*), an important vertebrate model for developmental processes, have shown that the AHR is required for developmental effects of TCDD in fish (Prasch *et al*., 2003) as it is in mammals (Mimura *et al*., 1997). However, the identity of AHR-regulated genes involved in toxicity is not yet clear.

Recent studies have examined gene expression profiles in relation to morphological or other phenotypic indices of developmental toxicity in zebra fish to identify genes potentially involved in TCDD effects (Carney *et al*., 2006; Handley-Goldstone *et al*., 2005). As in other systems, cytochrome P450 1A (CYP1A) is the gene most strongly induced in zebra fish embryos exposed to TCDD (Carney *et al*., 2006; Handley-Goldstone *et al*., 2005). It has been appealing to consider that CYP1A may be involved in TCDD effects, possibly via its role in generating oxidative stress. TCDD causes oxidative stress, and antioxidants can protect against the toxicity of TCDD in fish embryos (Cantrell *et al*., 1996; Dong *et al*., 2002). Halogenated AHR agonists stimulate the release of reactive oxygen by uncoupling the catalytic cycle of CYP1A (Schlezinger *et al*., 2006). Induction of CYP1 enzymes also may be involved in TCDD toxicity by altering metabolism of endogenous substrates (Nebert and Dalton, 2006).

Conflicting results from studies in which CYP1A translation was blocked (Carney *et al*., 2004; Teraoka *et al*., 2003) draw into question the possible involvement of CYP1A in developmental toxicity of TCDD. This implies that other genes participate in developmental toxicity in zebra fish, possibly including other CYP1 genes. We recently described a novel CYP1 subfamily, the CYP1Cs (Godard *et al*., 2005), and established that the paralogous genes, *CYP1C1* and *CYP1C2*, are

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expressed in zebra fish adults and embryos (Jönsson *et al*., 2007). The AHR agonist 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) induced both *CYP1Cs* as well as *CYP1B1* in embryos (Jönsson *et al*., 2007), suggesting regulation by the AHR.

Other targets potentially involved in effects of TCDD in the developing embryo are genes that participate in DNA synthesis and the cell cycle. In cell cultures, AHR agonists can both inhibit and enhance cell proliferation, and AHR regulates genes involved in growth and differentiation, including growth factors, oncogenes, cytokines, and proteins involved in apoptosis (reviewed by Kawajiri and Fujii-Kuriyama, 2007). In the developing heart, TCDD has been shown to downregulate expression of *cyclin A2* and *cyclin E1* in mice (Thackaberry *et al*., 2005a) and *cyclin A2*, *cyclin B1* and the gene for proliferating cell nuclear antigen (PCNA) in zebra fish (Carney *et al*., 2006). These effects preceded a decrease in heart growth, consistent with negative effects of TCDD on proliferation of heart cells (Antkiewicz *et al*., 2005; Thackaberry *et al*., 2005b). Mitchell *et al*. (2006) also found that CDK2 activation in the G1-phase was decreased in regenerating liver by TCDD; CDK2 activation is dependent on cyclin E.

In this study we defined the developmental expression of four *CYP1* genes, including the novel *CYP1C* genes, in relation to morphological effects produced in developing zebra fish exposed to AHR agonists. We similarly examined expression of *PCNA* and *cyclin E*. Initially, we focused on PCB126 for these studies. PCB126, which is one of the most potent AHR agonists (Henry *et al*., 2001), is common in the environment and has been shown to strongly uncouple CYP1A catalysis (Schlezinger *et al*., 2006). The PCB126 dose-response relationships for effects on the various genes were compared to one another and to the dose-response relationships for developmental defects. We also employed morpholino antisense technology (Nasevicius and Ekker, 2000) to determine whether AHR2 mediates developmental effects and the induction of zebra fish *CYP1B* and the *CYP1Cs* by PCB126, as compared to the effects of TCDD. Zebra fish possess three AHR genes (AHR2, AHR1a, and AHR1b), but only AHR2 and AHR1b are activated by TCDD and planar PCBs (Andreasen *et al*., 2002; Karchner *et al*., 2005; Tanguay *et al*., 1999). AHR2 has been shown to be required for induction of CYP1A and toxicity in TCDD-exposed zebra fish embryos (Dong *et al*., 2004; Prasch *et al*., 2003), but its role in the regulation of other CYP1 forms and in the effects of PCB126 on CYP1A expression and embryotoxicity has not been determined.

MATERIALS AND METHODS

Fish Husbandry

The zebra fish used here were TLs (Tup/long fin mutations) and were maintained as previously described (Jönsson *et al*., 2007). Embryos were generated through group breedings of 30 female fish obtained from the laboratory of Mark Fishman bred with 15 male fish raised from eggs obtained from the Zebra fish International Resource Center at the University of Oregon (ZIRC, Eugene, OR) and through the reciprocal cross of ZIRC females and Fishman lab males. Embryos and larvae were maintained in Danieau's solution until day eight postfertilization, after which the juvenile fish were kept in system water (for composition of system water, refer to Jönsson *et al*., 2007). Between days 6 and 15, postfertilization larvae/juveniles were fed twice daily with marine rotifers (obtained from the zebra fish facility at the Marine Biology Laboratory, Woods Hole, MA) and twice daily with a 50:50 mix of spirulina algae (Zeigler Bros Inc., Garners, PA) and Larval AP100 (<100 um; Algae-Fest, CA; 50:50%). On day 10, first instar brine shrimp (*Artemia salina*) and larger AP100 (100–150 μm) were introduced in the feeding regimen. The developing zebra fish were held in glass Petri dishes ($d = 16$ cm) for three weeks and then were transferred to an Aquatic Habitat recirculating flowing water system. Procedures used in the experiments were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution.

Exposure to PCB126

Embryos, larvae, or juvenile fish were exposed to PCB126 or the carrier (acetone) in three sets of experiments, i.e., a 5-day time course, a 2-month time course, and a concentration-response determination. Dead embryos (normally fewer than 7%) were removed at 32 h postfertilization (hpf). No difference in mortality was observed between controls and PCB-exposed groups, and generally, no mortality was observed subsequent to 32 hpf. The embryos were held at 28°C with a 14-h light/10-h dark cycle, and water was changed daily.

Five-day time course. Groups of fertilized zebra fish TL embryos (33 ± 5) were placed in glass petri dishes ($d = 10$ cm) containing 30 ml of 0.3× Danieau's solution. At 8 hpf, a stock solution of PCB126 in acetone or acetone only was added to the Danieau's solution, yielding 100nM PCB126 and 100 ppm acetone (nominal concentrations). Embryos were held in these conditions for 24 h, after which the dosing solutions were replaced with fresh 0.3× Danieau's solution, which was changed every 24 h. Groups of PCB126- and carrier-exposed embryos/larvae ($n = 5–8$) were sampled at 32, 56, 80, 104, and 128 hpf. At each sampling time, all embryos/larvae in a dish were pooled, frozen in liquid nitrogen, and stored at − 80°C.

Two-month time course. Groups of embryos, larvae, or juvenile fish were exposed for 24 h to 100nM PCB126 or 100 ppm acetone only, as above, but with exposure beginning at different times during development, i.e., at 1, 5, 12, 19, 26, and 55 days postfertilization (dpf). Exposure was in Danieau's solution (1- and 5-dpf groups) or system water (12-, 19-, 26-, and 55-dpf groups). Exposure was performed in petri dishes for the 1-, 5-, and 12-dpf groups and in beakers with static aerated water for the 26- and 55-dpf groups. After 24 h of exposure, the dosing solutions were replaced with fresh Danieau's solution or system water, as appropriate, and after an additional 24 h, embryos, larvae, or fish were sampled. At 3, 7, 14, 21, 28, and 57 dpf, triplicate or quadruplicate groups, composed of 32, 32, 25, 10, 1, and 1 individuals per group, respectively, were frozen in liquid nitrogen and stored at − 80°C.

PCB126 concentration response. Fertilized zebra fish TL eggs (33 ± 5) were placed in glass petri dishes containing 30 ml of 0.3× Danieau's solution (28°C). At 8 hpf, stock solutions of PCB126 (in acetone) or acetone only were added to the dishes yielding PCB126 at nominal concentrations ranging from 0.3 to 100nM in 100 ppm acetone and 100 ppm acetone alone. After 24 h, the dosing solutions were replaced with fresh 0.3× Danieau's solution and dead embryos removed as described above. The embryos were held for an additional 48 h with replacement of the Danieau's solution after 24 h. At 3 dpf, the percentage of embryos having pericardial edema was determined in each dish. Subsequently (at 80 hpf), the embryos were sampled and frozen (as above) for real-time PCR analysis ($n = 5–6$ dishes, each with approximately 30 embryos, per exposure). Furthermore, at 76 or 100 hpf, embryos exposed to the range of PCB126 concentrations (0.3–100nM) or 100 ppm acetone were embedded in 3% carboxymethyl cellulose in 0.3× Danieau's solution and photographed ($n = 5–6$ embryos per exposure and time point).

In addition, three replicate samples of 25 unexposed embryos/larvae (kept in 0.3× Danieau's solution as described) were sampled every day for 7 days

(8–176 hpf). Also, unexposed juvenile fish were sampled at 28 and 57 dpf. All samples were frozen in liquid nitrogen and stored at − 80°C.

AHR2 Gene Knockdown with Morpholino Antisense Oligonucleotides and Subsequent Exposure

The zebra fish AHR2 morpholino 5′-TGTACCGATACCCGCCGACATGGTT-3′ (AHR2-MO) and the standard control morpholino 5′-CCTCTTACCTCAGTTACAATTTATA-3′ (control-MO) were obtained from Gene Tools (Philomath, OR). As in previous studies (Carney *et al*., 2004; Prasch *et al*., 2003), the effectiveness of the AHR2-MO was confirmed by its ability to block the synthesis of AHR2 protein in an *in vitro* transcription and translation system (Jenny and Hahn, in preparation). Both morpholinos were fluorescein tagged for screening purposes to guarantee that only successfully injected embryos were used for the subsequent experiments. Morpholinos were diluted to 0.18mM in 0.3× Danieau's solution. A Narishige IM-300 microinjector was used to inject 2.1 nl of morpholino into the yolk of two to four cell stage embryos, resulting in approximately 3.3 ng of morpholino per embryo. Injection volumes were calibrated by injecting solutions into mineral oil and measuring the diameter of the sphere with a stage micrometer (volume $= 4/3\pi r^3$; 160 μm diameter is equivalent to 2.1 nl). At 3 hpf, embryos were sorted to remove damaged or unfertilized eggs, and the remaining embryos were screened for fluorescence to determine injection success (>93%).

At 8 hpf, noninjected embryos or embryos injected with the AHR2-MO or control-MO were exposed to 30nM PCB126 (including 100 ppm acetone) or 100 ppm acetone in 0.3× Danieau's solution as described above. After 24 h, the dosing solutions were replaced with fresh 0.3× Danieau's solution. In another set of experiments, groups of noninjected, control-MO-injected, or AHR2-MO–injected embryos were placed in glass petri dishes with no more than three embryos per milliliter of 0.3× Danieau's and then exposed to 0.1% dimethyl sulfoxide (DMSO) or 2nM TCDD (including 0.1% DMSO) for 1 h, starting 6 hpf. After TCDD exposure, the embryos were washed three times in fresh 0.3× Danieau's, then placed in Petri dishes with 25 ml fresh 0.3× Danieau's and held as above. Danieau's solution was renewed at 24 hpf. At 48 hpf, three or four replicates of 20 pooled embryos were collected from each group, frozen in liquid nitrogen, and stored at − 80°C until RNA isolation.

Quantification of CYP1 and Cell Cycle Gene Transcripts

RNA was isolated using RNA stat-60 (Tel-Test Inc., Friendswood, TX) and the isolates were DNase treated (TURBO DNA-free kit, Ambion). The RNA quantity and quality were determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE). cDNA was synthesized using the Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA), random hexamer primers (Operon Biotechnologies Inc.), and the RNasin RNase inhibitor (Promega).

Gene-specific primers for zebra fish *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, *ARNT2* (designed to amplify a sequence common to *ARNT2a, b*, and *c*), and *β-actin* cDNAs (Jönsson *et al*., 2007) as well as those for *Cyclin E* (3′-ACGATGGATGGTTCCTTTCG-5′and 3′-CTTTCCCAGCCATTCCAAGT-5′) and *PCNA* (3′-CAATCTGAGCAGCATGTCG-5′ and 3′-CATCTCATAGTCGGACACTTTCTC-5′) were synthesized by Operon Biotechnologies Inc. Real-time PCR was performed using the iQ SYBR Green Supermix (according to the manufacturer's instructions) and an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The PCR reaction mixtures consisted of iQ SYBR Green Supermix, primer (5 pmol each of forward and reverse primer) and cDNA (derived from 0.1 μg RNA). In each sample, the genes were analyzed in duplicate with the following protocol: 95°C for 3 min and 40 cycles of 95°C for 15 s and 62°C for 1 min. To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run.

Calculations and Statistics

Relative mRNA expression of the *CYP1* genes was calculated for each reaction according to the $E^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). PCR efficiency (E) was determined by the LinRegPCR program (Ramakers *et al*., 2003). *ARNT2* was used as a reference gene for all data except for those of the TCDD-AHR2-morpholino experiment, for which *β-actin* was used as a reference gene. Expression was calculated for carrier- and PCB126-exposed developing zebra fish (0–57 dpf) using the mean values in 3-dpf carrier-controls to normalize the data (i.e., $E^{\Delta Ct\ 0\text{–}57dpf}/E^{\Delta Ct\ 3dpf\ ctrl}$). The expression in the unexposed developing fish was normalized to the mean level of expression in the 3-dpf unexposed group. The carrier (acetone) had no effect on *CYP1* gene expression. To increase the replicate number, normalized data from unexposed and carrier-exposed groups at a given time point were combined. Outliers were excluded based on the Grubbs test (Grubbs, 1969). Intervals of increases or decreases in *CYP1* expression over time were examined statistically by linear regression analysis. Linear regression was also used to determine concentration-dependent changes in *PCNA* expression after exposure to a range of PCB126 concentrations (log concentration). Significant trends are indicated by r and p values within brackets. LOEC values for induction of the *CYP1* genes or edema caused by PCB126 were determined by one-way ANOVA followed by Dunnett's test. Prism 4 by GraphPad Software Inc. (San Diego, CA) was used for the statistical examinations. EC_{50}, i.e., the PCB126 concentration causing half maximal effect was calculated by the curve-fitting routine of Prism 4 for nonlinear regression using sigmoidal dose response with variable slope.

RESULTS

Basal CYP1 Expression in Developing Zebra Fish

Basal expression of *CYP1* genes was studied in developing and growing zebra fish from 8 hpf to 57 dpf. Because there was no difference in *CYP1* gene expression between the unexposed and carrier (100 ppm acetone) -exposed groups, we consider *CYP1* expression in both unexposed and carrier-exposed embryos as representing the basal level. To compare the levels of expression at various times, all data for a given gene were normalized to the mean values for that gene at 3 days post-fertilization (refer to the section "Calculations and statistics").

Using linear regression, we observed temporal changes in basal expression for all four *CYP1* genes during the period examined in developing zebra fish (Figs. 1A–D). Expression of *CYP1A* increased continuously during the first 3 weeks ($r = 0.80$, $p < 0.001$), peaking around day 21 at approximately 10 times the expression level at 3 dpf, and decreasing thereafter ($r = 0.47$, $p < 0.05$; Fig. 1A). As shown in Figure 1B the basal expression of *CYP1B1* peaked within the first 2 days and then decreased during the following 4 days ($r = -0.58$, $p < 0.001$). The expression of *CYP1C1* and *CYP1C2* increased from 8 hpf to 4 dpf ($r = 0.75$, $p < 0.001$) and from 8 hpf to 3 dpf ($r = 0.68$, $p < 0.001$), respectively (Figs. 1C and 1D). As seen with *CYP1B1*, a decrease to less than half the 3-dpf level occurred from day 4 to 6 ($r = -0.65$, $p < 0.01$) with *CYP1C1* and from day 3 to 6 ($r = -0.49$, $p < 0.01$) with *CYP1C2* (Fig. 1C and 1D). All four *CYP1* genes tended to show increased expression (not statistically significant) between 6 and 7 dpf, i.e., at the time for depletion of yolk and onset of feeding. After day 7, *CYP1B1* and *CYP1C2* expression remained at or slightly below the 3-dpf level for the rest of the experimental period, whereas *CYP1C1* expression was at the 3-dpf level during the second and third week and then declined ($r = -0.40$, $p < 0.01$).

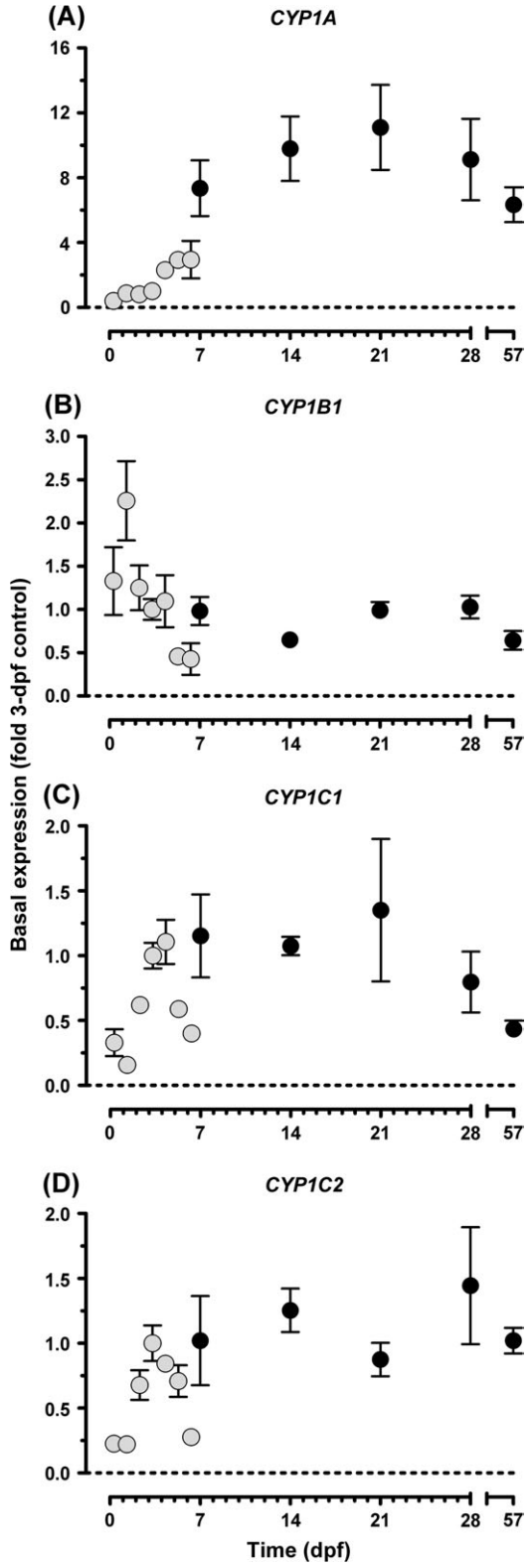

FIG. 1. Temporal patterns for basal expression of *CYP1A* (A), *CYP1B1* (B), *CYP1C1* (C), and *CYP1C2* (D) in developing zebra fish sampled at various times between 8 hpf and 57 dpf. 'Basal expression' represents data from both untreated and acetone-treated groups (i.e., we observed no effect of 100 ppm of acetone on *CYP1* gene expression). All data were normalized to the level of expression at 3 dpf using mean values for untreated or acetone-treated groups, as appropriate. Larvae and juvenile fish were fed from day 7. Data from 0–6 dpf (unfed groups, $n = 3$–9) and 7–57 dpf (fed groups, $n = 3$–4) are shown by gray and black symbols (means ± SEM), respectively. Note the difference in scale of the y-axis for the different *CYP1* forms.

PCB126-Induced CYP1 Expression in Developing Zebra fish: 1- to 5-Day Time Course

As with basal expression, temporal trends in gene expression in PCB126-exposed fish were determined by linear regression. The expression level for each gene was calculated by normalization to the corresponding mean value in the carrier-exposed embryos at 3 dpf. PCB126-induced expression was examined in developing zebra fish with two sets of time course experiments. Initially, induced *CYP1* gene expression was studied at various time (1–5 dpf) after exposure to 100nM PCB126 for a 24-h period starting at 8 hpf (gray symbols in Fig. 2). A significant induction was observed for all four genes at all time points within the period (i.e., from 32 hpf and later). The level of induced *CYP1A* expression increased during the 5-day experimental period ($r = 0.49$, $p < 0.01$), showing a 300-fold increase relative to the 3-dpf control on day 5 (Fig. 2A). The induced expression of *CYP1B1* increased during the embryo period ($r = 0.62$, $p < 0.01$), peaking on day 3 at about 20-fold the 3-dpf control, and thereafter a decreasing tendency (not significant) was observed (Fig. 2B). Similarly, the induced expression of *CYP1C1* and *CYP1C2* initially increased ($r = 0.79$ and $r = 0.88$, both $p < 0.0001$), peaked on day 3 at about 20- to 25-fold and 35- to 40-fold the 3-dpf control, respectively, and then decreased ($r = 0.58$, $p < 0.05$) and tended to decrease during day 4 and 5 (Figs. 2C and 2D).

As both the basal and the induced levels of expression vary over time, we wanted to look at the fold change at each time point separately. Table 1 shows expression after exposure to PCB126 when data for a given gene were normalized to the corresponding data in the carrier-exposed group at each sampling day. The largest fold change between basal and induced expression was observed on day 3 for *CYP1A* (236 ± 34 fold the control) and on day 5 for *CYP1B1* (43 ± 7 fold the control; Table 1). The relative induction of C*YP1C1* was about 20-fold the control over the whole 1–5 dpf-period and that of *CYP1C2* was similar from day 2 to 5 (30- to-40 fold the control; Table 1).

PCB126-Induced CYP1 Expression during a 3- to 57-Day Time Course

Earlier we observed that in adult zebra fish, *CYP1C2* is only slightly induced by PCB126, while *CYP1A* is strongly induced (Jönsson *et al*., 2007). To examine the possibility that the four *CYP1* genes vary in their responsiveness to AHR agonists over the course of development, gene expression was measured 24 h following 24-h exposures to PCB126 (100nM), when those

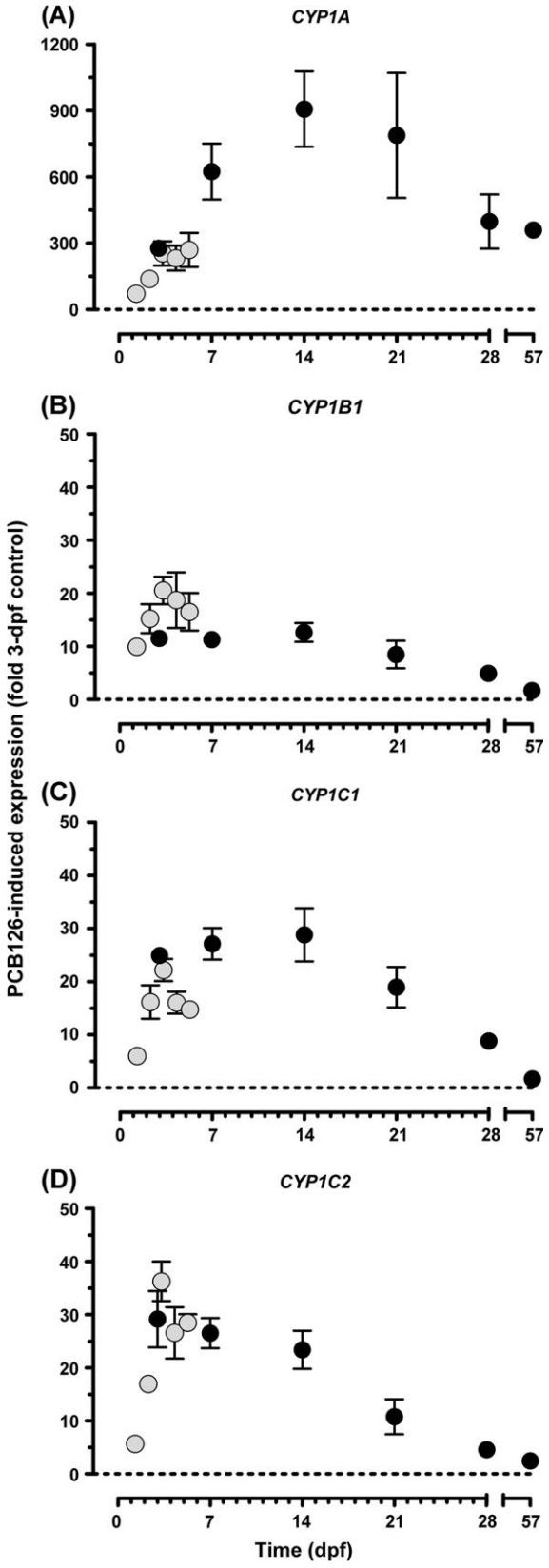

FIG. 2. Temporal patterns for PCB126-induced expression of *CYP1A* (A), *CYP1B1* (B), *CYP1C1* (C), and *CYP1C2* (D) in developing zebra fish 1–57 dpf.

exposures were initiated on day 1, 5, 12, 19, 26, or 55 postfertilization. All four *CYP1* genes were induced significantly by PCB126 compared with the control at all time points examined ($p < 0.05$; one-way ANOVA and Bonferroni's multiple comparisons test). However, the magnitude of induced expression varied greatly over the 57 days, and the temporal pattern differed among the four genes. To compare *CYP1* expression between sampling days, we normalized data in the PCB126-exposed groups to the levels in the 3-dpf carrier-controls (Fig. 2). The induced expression of *CYP1A* increased during the first 2 weeks ($r = 0.81$, $p < 0.01$) to peak at 900-fold the 3-dpf control level and then tended to decrease between days 14 and 28 (Fig. 2A). *CYP1B1* was induced similarly (i.e., slightly more than 10-fold relative to the 3-dpf basal level) between days 3 and 14, and thereafter the inducibility decreased ($r = -0.79$, $p < 0.001$; Fig. 2B). The inducibility of *CYP1C1* was similar between days 3 and 14 (about 25- to 30-fold the 3-dpf level), after which a dramatic decrease was observed ($r = -0.82$, $p < 0.001$; Fig. 2C). The inducibility of *CYP1C2* was about 30-fold at 3 dpf and then decreased between days 3 and 57 ($r = -0.81$, $p < 0.0001$; Fig. 2D). For *CYP1A*, the magnitude of induced expression did not change for treatments initiated between days 28 and 57. For the other three genes, the levels of expression that were induced by treatment during this period decreased, approaching basal levels of expression. Thus, 8 weeks after fertilization a 24-h exposure to 100nM PCB126 induced *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* by 70 ± 6, 2.4 ± 0.4, 3.9 ± 0.9, and 2.6 ± 0.3-fold (mean ± SEM), respectively, relative to the 57-dpf acetone control (based on whole-body samples of individual fish; Table 1).

Relative to the acetone control at each sampling day, *CYP1A* and *CYP1C2* showed a maximal fold induction at 3 dpf, whereas the largest increase of *CYP1B1* expression relative to the control occurred at 14 dpf (Table 1). The fold induction of *CYP1C1* expression peaked both at 3 and 14 dpf.

Dose-Response Relationship for Induction of CYP1 Genes by PCB126

All four *CYP1* genes displayed concentration-dependent increases in expression following exposure to PCB126

Embryos were exposed to 100nM PCB126 (in 0.3× Danieau's solution) for 24 h starting at 8 hpf and then held in 0.3× Danieau's solution for 0–4 days before sampling (i.e., at 32, 56, 80, 104, and 128 hpf, gray symbols). In another experiment, embryos, larvae, and small fish were treated for 24 h starting at 1, 5, 12, 19, 26, or 55 dpf and subsequently held in clean 0.3× Danieau's solution or system water for 24 h, before sampling (i.e., at 3, 7, 14, 21, and 28 dpf, black symbols). Expression in embryos exposed to the carrier (100 ppm acetone) at 3 dpf was used as a calibrator. Data are shown as means ± SEM and $n = 3$–8 groups of pooled embryos. For all four genes, a significant induction compared with the control was observed at all times examined (determined by one-way ANOVA followed by Bonferroni's multiple comparisons test). Note the difference in scale of the y-axis for *CYP1A* as compared to the other *CYP1* forms.

TABLE 1
***CYP1* Gene Induction in Developing Zebra Fish Shown as a Fold Change Relative to the Carrier-Control at Each Sampling Day (mean ± SEM)**

		Relative induction (fold control)			
Start of exposure	Time for sampling	*CYP1A*	*CYP1B1*	*CYP1C1*	*CYP1C2*
8 hpf	1 dpf	86 ± 24 a	3.9 ± 0.9 a	23 ± 2 a	21 ± 5 a
8 hpf	2 dpf	111 ± 18 a	13 ± 3 a	19 ± 3 a	34 ± 2 ab
8 hpf	3 dpf	236 ± 34 b	23 ± 4 a	23 ± 2 a	40 ± 4 b
8 hpf	4 dpf	42 ± 4 a	24 ± 7 ab	17 ± 2 a	28 ± 5 ab
8 hpf	5 dpf	82 ± 21 a	43 ± 7 b	25 ± 3 a	43 ± 4 b
1 dpf	3 dpf	277 ± 34 B	12 ± 1 BC	25 ± 2 C	29 ± 5 C
5 dpf	7 dpf	43 ± 8 A	10 ± 1 B	10 ± 1 AB	11 ± 1 AB
12 dpf	14 dpf	93 ± 17 A	19 ± 3 C	27 ± 5 C	19 ± 3 BC
19 dpf	21 dpf	71 ± 25 A	8.5 ± 2.6 AB	20 ± 4 BC	12 ± 4 AB
26 dpf	28 dpf	28 ± 9 A	6.0 ± 1.3 AB	7.2 ± 1.1 A	4.1 ± 0.3 A
55 dpf	57 dpf	70 ± 6 A	2.4 ± 0.4 A	3.9 ± 0.9 A	2.6 ± 0.3 A

Note. Embryos, larvae, or juvenile fish were exposed to 100nM PCB126 or the carrier (100 ppm acetone) for a 24-h period starting at various times and then held in 0.3× Danieaus's solution or system water until sampling. For each gene, statistically significant differences in fold induction between days were determined by one-way ANOVA followed by Tukey's multiple comparisons test and are indicated in the table by different lower case (1–5 dpf) and upper case (3–57 dpf) letters ($p < 0.05$).

(Figs. 3A–D). The EC_{50} values for induction of *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* by PCB126 in 80-hpf embryos were 2.3 ± 1.3, 2.7 ± 1.3, 1.4 ± 1.3, and 1.8 ± 1.2nM (mean ± SEM), respectively. These EC_{50} values for the four genes were not statistically different from one another. The LOEC of PCB126 was 0.3nM for *CYP1A* and *CYP1C1* and 1nM for *CYP1B1* and *CYP1C2*.

Cell Cycle Gene Expression in Response to PCB126

We examined the effect of PCB126 on two genes involved in cell proliferation, *PCNA* and *cyclin E*, as potential markers for arrest in the G1 phase of the cell cycle. *PCNA* is expressed during the S phase and *cyclin E* peaks in the G1/S phase boundary (Lew *et al*., 1991). There was an apparent dose-dependent effect of PCB126 on *PCNA* expression, whereas no significant effect was observed for *cyclin E*. Linear regression showed a gradual decrease in *PCNA* expression at the PCB126 concentrations examined, i.e., 0.3–100nM ($r = -0.65$, $p < 0.01$ for *PCNA* expression as a function of log [PCB126]). However, a statistically significant difference compared with the control was only observed with 100nM PCB126 ($p < 0.05$ by *t*-test; LOEC), at which *PCNA* expression was reduced to 40 ± 10% (mean ± SEM) of that in controls (Fig. 4).

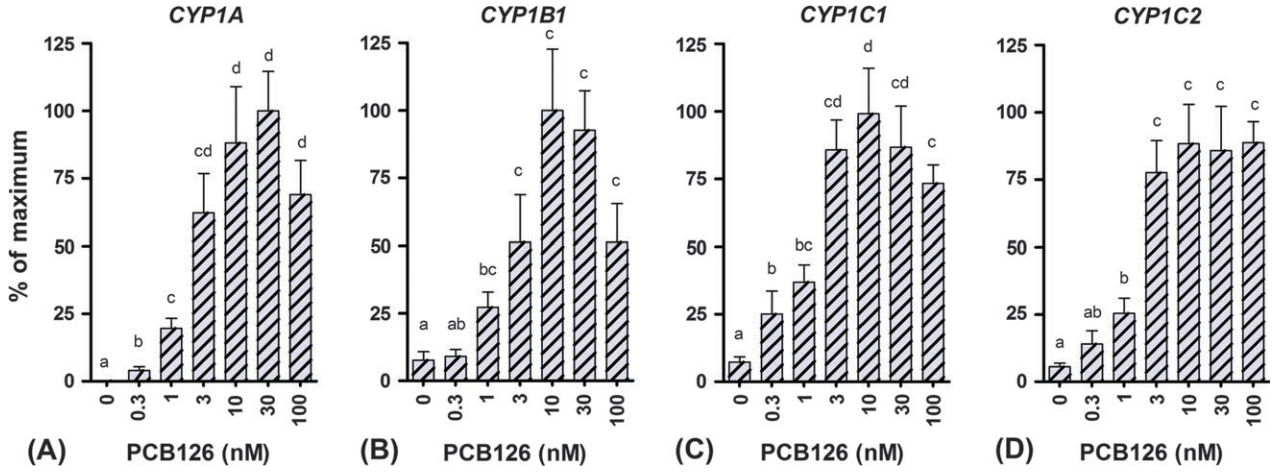

FIG. 3. Concentration response relationships for relative expression of *CYP1A* (A), *CYP1B1* (B), *CYP1C1* (C), and *CYP1C2* (D) in zebra fish embryos exposed to PCB126 (0.3–100nM), or the carrier (100 ppm acetone). The exposure lasted for 24 h, starting 8 hpf, and then the embryos were held for 48 h in clean 0.3× Danieau's solution before sampling (at 80 hpf). Statistical differences were examined by one-way ANOVA followed by Tukey's multiple comparisons test; a statistical difference is denoted by different letters. Data are shown as % of maximum (means ± SEM) and $n = 4$–6 groups of pooled embryos.

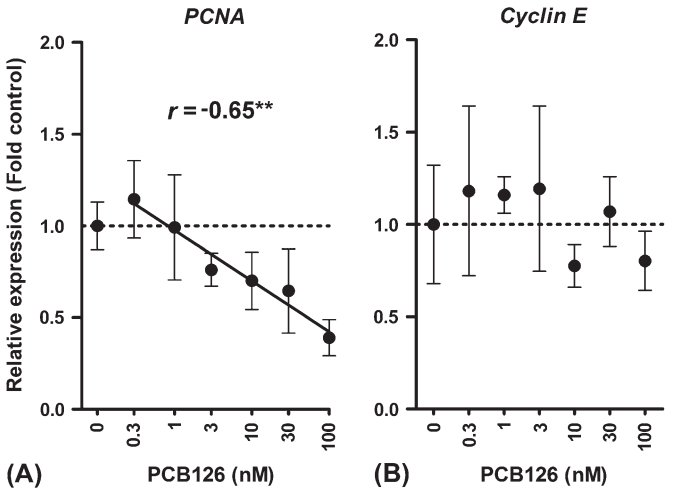

FIG. 4. Concentration-response relationships for cell cycle gene expression, i.e., *PCNA* (A) and *cyclin E* (B), in zebra fish embryos exposed to PCB126 (0.3–100nM) or the carrier (100 ppm acetone). The exposure lasted for 24 h, starting 8 hpf, and then the embryos were held for 48 h in clean 0.3× Danieau's solution before sampling (at 80 hpf). Statistical trends were examined by linear regression analysis using log-transformed data for PCB126 concentration. A statistically significant negative trend was observed for the effect of PCB126 on *PCNA* ($p < 0.01$) and is denoted by **. Data are shown as means ± SEM ($n = 3$ groups of pooled embryos).

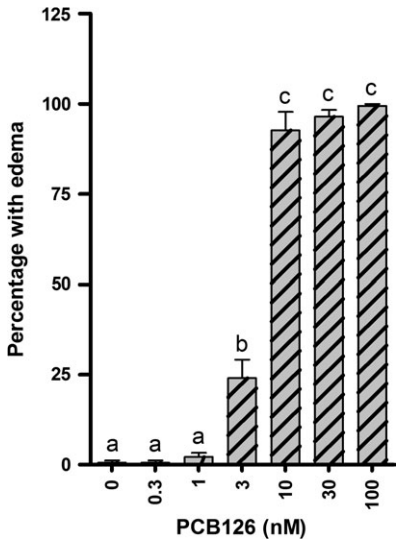

FIG. 5. Concentration-response relationship for pericardial edema in zebra fish embryos exposed to PCB126 (0.3–100nM) or the carrier (100 ppm acetone). The exposure lasted for 24 h, starting 8 hpf, and then the embryos were held for 48 h in clean 0.3× Danieau's solution before being monitored for edema at 80 hpf. Statistical differences were examined by one-way ANOVA followed by Tukey's multiple comparisons test; a statistical difference is denoted by different letters. Data are shown as a percentage of the total number of embryos in a replicate group ($n = 5$–6).

Morphological Effects of PCB126

PCB126-exposed zebra fish embryos exhibited pericardial edema as early as 2 dpf. At 3 dpf, approximately 25% of the embryos exposed to 3nM PCB126 exhibited pericardial edema, and at higher concentrations the incidence of edema was 95–100% (Figs. 5 and 6). The LOEC of PCB126 for edema was 3nM and the EC_{50} was 4.4nM, i.e., somewhat higher than the values for *CYP1* induction. In addition, at 3 dpf the groups exposed to 10–100nM PCB126 exhibited craniofacial malformations, heart malformations (hypotrophy and reduced looping), slower and weaker heartbeats, impaired circulation, and immobility (Fig. 6). At 4 dpf, these effects had become more severe and included yolk sac edema. Furthermore, a number of embryos exposed to the lower PCB126 concentrations exhibited reduced swim bladder inflation at 4 dpf; i.e., the swim bladder had not even started to inflate in 1/6, 3/6, and 3/6 embryos after exposure to 0.3, 1, and 3nM PCB126, respectively. In the control group, swim bladder inflation was observed in all embryos except one that exhibited edema. In the embryos exposed to 10–100nM PCB126, swim bladder inflation did not occur (Fig. 6). This effect of PCB126 on the swim bladder appears to be a sensitive ($EC_{50} = 1.4$nM) and consistent endpoint of PCB126 exposure (see also Fig. 9).

Effect of AHR2 Knockdown on CYP1 Gene Expression and Induction

In order to test our previous hypothesis that the *CYP1B* and *CYP1C* genes are regulated through an AHR-dependent mechanism (Jönsson *et al*., 2007) and to explore the role of AHR2 in the developmental toxicity of PCB126, embryos were injected with a standard control MO or a MO capable of blocking *AHR2* translation (AHR2-MO). Knockdown of AHR2 tended to slightly repress *CYP1* gene expression in most DMSO controls, although a significant reduction was observed only for *CYP1A* and *CYP1C2* (Table 2). Both the noninjected and the control MO-injected embryos exposed to PCB126 or to TCDD responded with a strong induction of *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2,* whereas the AHR2-MO–injected embryos exhibited a significantly lower induction of all these genes (Table 2; Fig. 7). The AHR2-MO blocked the induction of *CYP1* genes by PCB126 and TCDD by approximately 80–90% and 90–95%, respectively, when compared to induction seen in the control MO-injected embryos.

Effect of AHR2 Knockdown on Morphology and Mortality in Response to PCB126 or TCDD

Both PCB126 and TCDD caused pericardial edema by 3 dpf in 100% of the noninjected and the control MO-injected zebra fish embryos, while embryos injected with the AHR2 morpholino and subsequently exposed to PCB126 or TCDD did not begin to display pericardial edema before 5 dpf (Fig. 8A). Thus on day 5, a few of the AHR2-MO–injected and PCB126- or TCDD-exposed embryos showed pericardial and yolk sac edema, i.e., a phenotype similar to the noninjected and control-MO–injected embryos that were exposed to PCB126 or TCDD. The incidence of pericardial edema in the AHR2-MO–treated

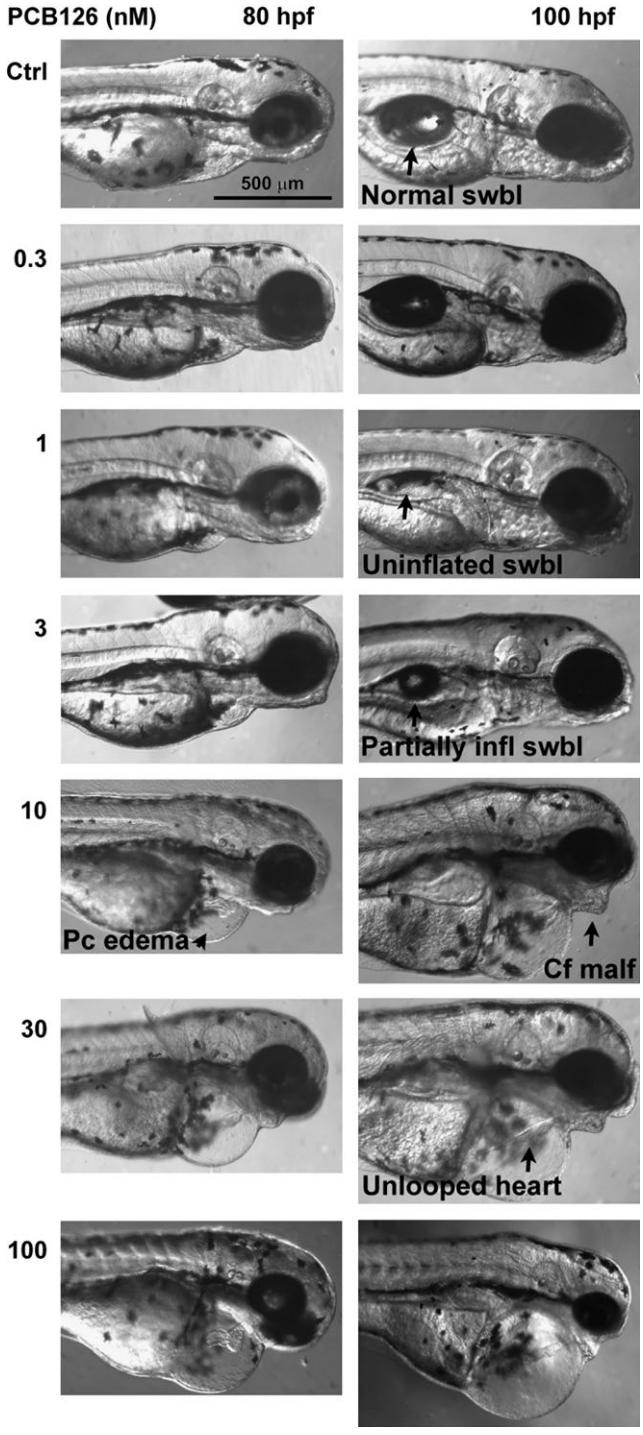

FIG. 6. Morphology of zebra fish embryos exposed to PCB126 (0.3–100nM), or the carrier (100 ppm acetone, denoted 'Ctr'), for 24 h, starting 8 hpf. After exposure, the embryos were held in clean 0.3× Danieau's solution. At 80 and 100 hpf 5 or 6 randomly sampled embryos were photographed in each exposure group (swbl, swim bladder; Pc, pericardial; infl, inflated; Cf, craniofacial; and malf, malformations).

and PCB126- or TCDD-exposed groups increased over time, whereas generally no embryo with edema was observed in groups exposed to carrier only (acetone or DMSO), regardless of morpholino pretreatment (Fig. 8A).

Between days 6 and 7, a drastic increase in mortality occurred in the noninjected and control-MO–injected groups exposed to PCB126 or TCDD. By comparison, mortality was greatly decreased in the AHR2-MO–injected PCB126-exposed group, as well as in the AHR2-MO–injected TCDD-exposed group (Fig. 8B).

As normal inflation of the swim bladder appeared to be the most sensitive morphological endpoint associated with PCB126 exposure, we examined if this effect could be prevented by blocking AHR2 translation. In this experiment, swim bladder inflation was observed at 4 dpf in individual embryos of the carrier-controls (noninjected or MO-injected embryos), and the number of embryos with inflated swim bladder increased with age (data not shown). By 6 dpf, the majority of larvae from all of the carrier-control groups had fully inflated swim bladders (Fig. 9). However, noninjected or control-MO–injected embryos exposed to PCB126 or TCDD were incapable of swim bladder inflation, and the AHR2-MO only provided minimal protection for this morphological endpoint (Fig. 9).

Overall, treatment with the AHR2-MO delayed but did not prevent the onset of several developmental phenotypes associated with PCB126 or TCDD toxicity.

DISCUSSION

In this study, we examined patterns of expression for *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* in developing zebra fish and the induction of these genes following exposure to PCB126 or carrier (acetone). The results indicate that the basal expression of *CYP1* genes is maximal at differing times, suggesting that the four genes may play physiological roles at different times during development. Furthermore, we show that *CYP1* gene expression was induced and the incidence of morphological defects in 80-hpf embryos was increased by PCB126 and that these effects occurred with similar PCB126 concentration-response relationships. Blockage of *AHR2* translation via morpholino oligonucleotides significantly inhibited *CYP1* gene induction and the occurrence of malformations by PCB126, as well as by TCDD, showing that these effects of both compounds are largely AHR2-dependent. *PCNA* gene expression decreased gradually with increasing PCB126 concentration, suggesting that toxic doses of PCB126 suppress cell proliferation.

Basal CYP1 Gene Expression in Developing Zebra Fish

Each of the four *CYP1* genes showed a characteristic time course of basal expression during zebra fish development. The first gene whose expression peaked was *CYP1B1*, which was maximally expressed within the first 2 days. In mammals,

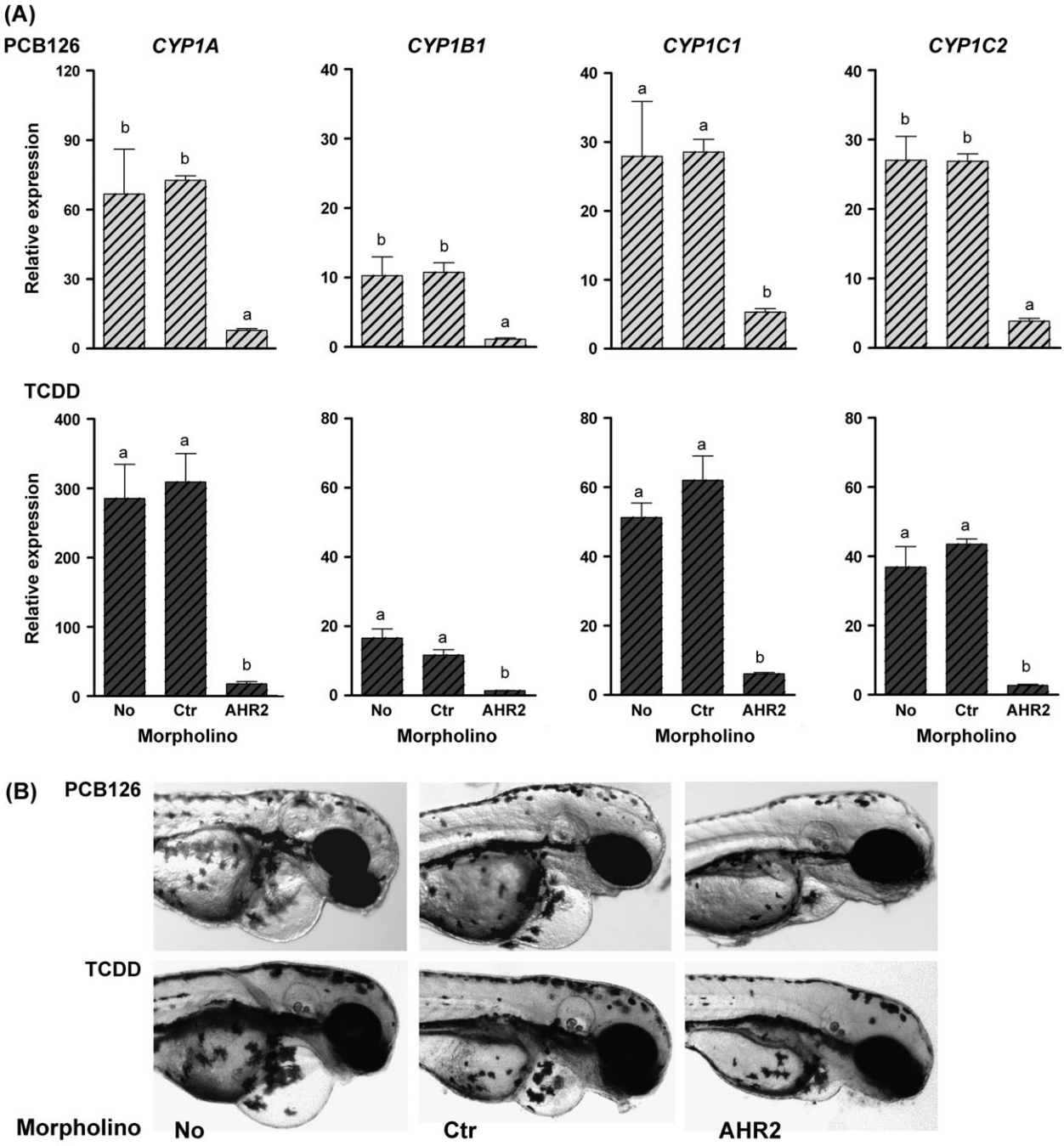

FIG. 7. Effect of AHR2 knockdown and subsequent exposure to TCDD or PCB126 on *CYP1* gene expression (A) and morphology (B) in zebra fish embryos. AHR2 translation was blocked by an AHR2 morpholino injected at the two to four cell stage. Groups of embryos injected with a control (Ctr) morpholino or no morpholino served as controls. Embryos of all three groups were exposed to 2nM TCDD or 0.1% DMSO for 1 h (starting 6 hpf) or to 30nM PCB126 or 100 ppm acetone for 24 h (starting 8 hpf). After exposure, the embryos were held in clean 0.3× Danieau's before being sampled at 48 hpf. Embryos were photographed at 76 hpf, i.e., when morphological effects were clearly observed. Statistical differences were examined by one-way ANOVA followed by Tukey's multiple comparisons test; a statistical difference is denoted by different letters. Expression in the PCB126- or TCDD-exposed groups was normalized to the level in the corresponding carrier-treated ctrl-MO group and is shown as means ± SEM ($n = 3$–4).

TABLE 2
***CYP1* Gene Expression at 48 hpf in Zebra Fish Embryos Treated with a Morpholino Blocking AHR2 Translation (AHR2), a Control Morpholino (Ctrl), or No Morpholino (No) and Subsequently Exposed to 30nM PCB126, 2nM TCDD, or the carriers (100 ppm Acetone or 0.1% DMSO)**

Exposure	Morpholino	*CYP1A*	*CYP1B1*	*CYP1C1*	*CYP1C2*
Acetone	No	0.9 ± 0.2	1.7 ± 0.4	1.2 ± 0.2	1.0 ± 0.1
	Ctrl	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
	AHR2	1.2 ± 0.6	1.1 ± 0.2	0.7 ± 0.2	0.8 ± 0.1
PCB126	No	67 ± 19	10 ± 3	28 ± 8	27 ± 3
	Ctrl	73 ± 2	11 ± 1	29 ± 2	27 ± 1
	AHR2	7.7 ± 0.8**	1.1 ± 0.2**	5.3 ± 1.5**	3.9 ± 0.4**
DMSO	No	0.7 ± 0.2	0.5 ± 0.1	1.1 ± 0.1	1.3 ± 0.3
	Ctrl	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.0
	AHR2	0.2 ± 0.1*	0.8 ± 0.3	0.6 ± 0.1	0.4 ± 0.1*
TCDD	No	183 ± 32	7.7 ± 1.2	57 ± 5	46 ± 7
	Ctrl	261 ± 35	11 ± 2	60 ± 7	43 ± 1
	AHR2	15 ± 3**	1.3 ± 0.1**	5.8 ± 0.4**	2.7 ± 0.3**

Significant decreases relative to the similarly exposed group treated with the control morpholino were determined by one-way ANOVA followed by Dunnett's multiple comparisons test and are shown by $*p < 0.05$ or $**p < 0.01$.

CYP1B1 has been shown to be essential for normal eye development (Choudhary *et al*., 2006; Libby *et al*., 2003) and is expressed in corneal and ciliary epithelia and in retina (Choudhary *et al*., 2006). The localization pattern of *CYP1B1* has not been studied in zebra fish embryos, although the eye (together with brain and heart) is among the organs having the highest basal expression of *CYP1B1* in adult zebra fish (Jönsson *et al*., 2007). The high basal expression of *CYP1B1* early in the developing zebra fish embryo could be related to formation of the eye, as retina starts to develop at about 30 hpf and vision begins around hatch in zebra fish (Easter and Nicola, 1996). It could also be related to development of brain, heart, and other structures. In the developing chicken embryo, *CYP1B1* is expressed in several structures including the eye, brain, and notochord, and the *CYP1B1* expression pattern is temporally and spatially dynamic (Chambers *et al*., 2007). Chambers *et al.* (2007) showed that CYP1B1 may contribute to retinoic acid synthesis during embryonic patterning, and hence may play a regulatory function during embryogenesis.

In a recent study, we showed that the organ distribution patterns for basal *CYP1C1* and *CYP1C2* expression in adult zebra fish are similar to one another and that they resemble that of *CYP1B1* (Jönsson *et al*., 2007). Both *CYP1B1* and the *CYP1C* genes show a high basal expression in the adult zebra fish eye and heart (Jönsson *et al*., 2007). This could mean that the functions of *CYP1B1* and the *CYP1C* genes are related. However, the time courses for basal expression of the *CYP1C*s differed from that of *CYP1B1* in developing zebra fish. *CYP1C1* and *CYP1C2* transcript levels increased until around hatch (3–4 dpf) and then remained at this level for several weeks. Similarly, the basal expression of *CYP1C1* in killifish (*Fundulus heteroclitus*) embryos increased rapidly before hatch and then the increase slowed down (Wang *et al*., 2006). Together these results suggest *CYP1C1* and *1C2* may play some physiological roles in newly hatched fish, perhaps related to transition from embryonic to free-swimming life.

The basal expression of zebra fish *CYP1A* increased several fold at hatching and then continued to increase during the first weeks of development, peaking around day 21 at about 30-fold the 8-hpf level. The increase at hatching is similar to that first reported by Binder and Stegeman (1984) who showed that the basal level of aryl hydrocarbon hydroxylase activity (a CYP1A function) increased sharply during the first 24 h after hatching in killifish. More recently, Wang *et al*. (2006) showed that the basal level of *CYP1A* mRNA also increased after hatching in killifish. These findings suggest CYP1A may play different roles before and after hatching.

Expression of all four zebra fish *CYP1* genes tended to increase between days 6 and 7 in control fish. This coincided with initiation of feeding, which could mean that AHR agonists were present in the diet, and hence caused a slight induction of the *CYP1* genes.

Morphological Effects and CYP1 Gene Expression in Developing Zebra Fish Exposed to PCB126

The morphological effects observed in zebra fish embryos exposed to PCB126 doses from 3 to 100nM appeared identical to those in embryos exposed to TCDD (Henry *et al*., 1997; Teraoka *et al*., 2003). Reduced swim bladder inflation was detected in some embryos at even lower PCB126 concentrations (0.3–1nM), suggesting that this process is particularly sensitive to AHR agonists. Interestingly, the PCB126 EC_{50} values for induction of the four *CYP1* genes at 3 dpf were in the range of those for reduction of swim bladder inflation and increase of pericardial edema. However, while expression of

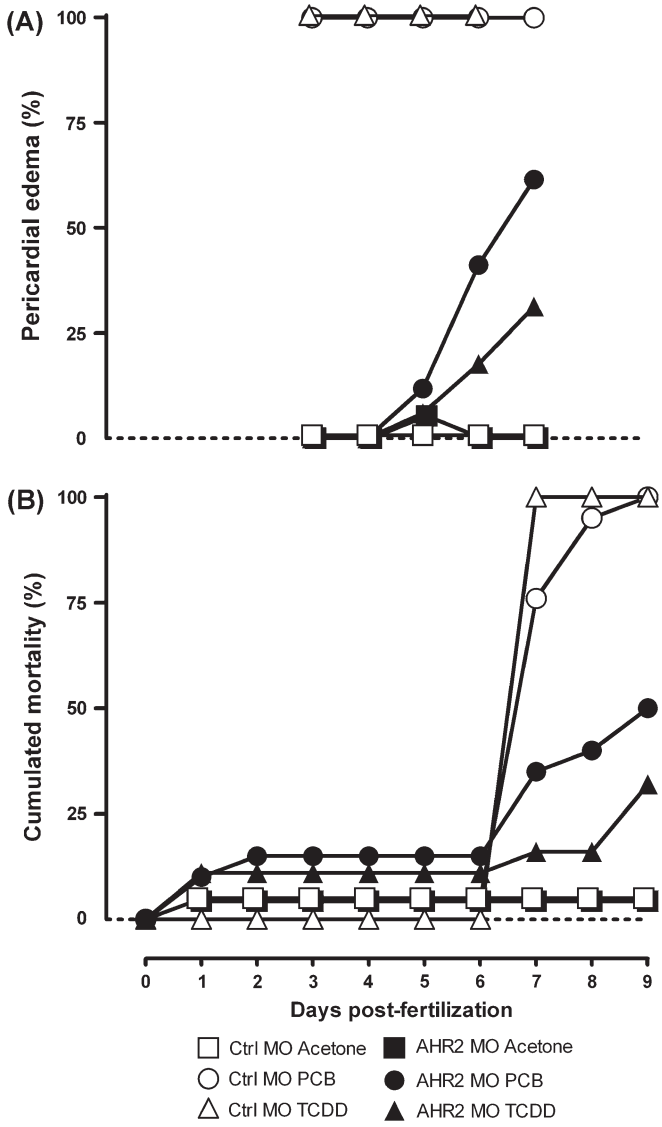

FIG. 8. Pericardial edema (A) and embryo mortality (B) in zebra fish embryos following blockage of AHR2 translation and subsequent exposure to PCB126 or TCDD. Zebra fish embryos were treated with a control morpholino (open symbols) or a morpholino blocking AHR2 synthesis (filled symbols) at the two to four cell stage. The embryos were subsequently exposed to 30nM PCB126 (circles), 2nM TCDD (triangles), 100 ppm of acetone (PCB126 carrier-control; squares) or 0.1% DMSO (not shown). MO, morpholino.

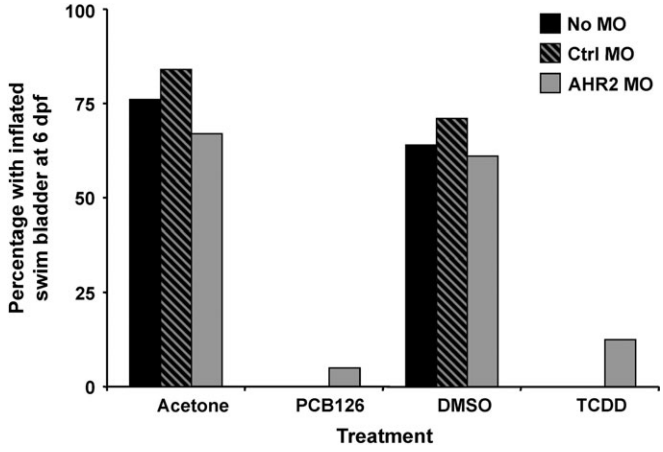

FIG. 9. Percentage of zebra fish larvae (at 6 days postfertilization) showing swim bladder inflation following injection with a morpholino (MO) blocking AHR2 translation and subsequent exposure to 30nM PCB126 or 2nM TCDD or the carriers (100 ppm acetone and 0.1% DMSO, respectively). Average sample size for each treatment was 43 individuals (range of $n = 38–50$).

the *CYP1* genes was induced by 32 hpf, edema was first observed at 48 hpf. Since the swim bladder inflates after hatching, the effect of PCB126 on this endpoint could be examined first at 4 dpf. The time course for induced expression of each *CYP1* gene roughly followed that for basal expression of the same gene; *CYP1B1*, *CYP1C1*, and *CYP1C2* reached the maximal level of induction around hatching (2–4 dpf), whereas *CYP1A* was maximally induced only after 14 days. Hence, the induced expression of *CYP1B1*, *CYP1C1*, and *CYP1C2* was maximal at the time when developmental defects of PCB126 became obvious. This could mean that *CYP1B1, CYP1C1*, and/or *CYP1C2* somehow are involved in the toxicity of PCB126 in fish embryos. Notably, at the same time *CYP1A* showed the maximal fold induction (Table 1). Conceivably, all four CYP1 forms could contribute to embryotoxicity.

At 3 dpf, we also observed a PCB126 dose-dependent inhibition of *PCNA* expression. The effect on *PCNA* expression was significant only at the highest PCB126 dose, but using regression analysis a gradual negative trend was observed within the whole range of PCB126 concentrations. Specific target cells or organs for this effect of PCB126 are unknown since we analyzed *PCNA* gene expression in pools of whole embryos. It is possible the effect is 'diluted' in the whole-embryo homogenate, i.e., some cell types could be more sensitive than others. Recently, Carney *et al.* (2006) observed that TCDD downregulated expression of *PCNA* and other genes involved in proliferation in the zebra fish embryo heart to a considerably higher degree than in a homogenate of the rest of the body. Although these results suggest that PCB126 and TCDD may suppress cell proliferation, the underlying mechanism is not known. *PCNA* gene expression is regulated by the E2F transcription factor, which in turn is regulated by the retinoblastoma protein pRB. AHR has been shown to interact with pRB and affect E2F function (Ge and Elferink, 1998; Puga *et al.*, 2000) and E2F expression is repressed by Aroclor 1254 in human hepatocytes (Reymann and Borlak, 2006). A link to the cell cycle is further suggested by the finding of a putative binding element for E2F upstream of the transcriptional start site in several of the *CYP1* genes (Jönsson *et al.*, 2007). It is therefore possible that AHR is involved in the effect on proliferation but if or how CYP1 enzymes could be implicated in this effect is unclear.

CYP1A was strikingly more inducible than the other three *CYP1* genes at all times examined, out to 57 days. It is possible that this characteristic of *CYP1A* is associated with a

physiological function of the enzyme. This could be to rapidly eliminate endogenous and exogenous AHR agonists (Nebert and Dalton, 2006; Nebert *et al*., 2000). The dynamics in magnitude of *CYP1A* induction suggest that the capability to initiate a strong and rapid increase of CYP1A enzyme may be physiologically beneficial at particular periods of development.

In 1-month-old zebra fish, the induced levels of *CYP1B1*, *1C1*, and *1C2* expression were less than 10-fold the 3-dpf control level, whereas *CYP1A* expression was almost 400-fold the 3-dpf control level. We have recently demonstrated a generally low *CYP1C2* induction response in adult zebra fish, the liver being the only organ (of those we studied) showing a significant induction by PCB126 (ca fivefold) (Jönsson *et al*., 2007). In 2-month-old zebra fish, the induction of *CYP1C2* by PCB126 (100nM) was only two- to threefold. Since the analyses in the present study were based on pooled embryos/whole-body homogenate of juvenile fish, a strong expression in specific organs/cell types could be masked. However, it is possible that *CYP1C2* is regulated differently in embryos as compared to juveniles and adults (Jönsson *et al*., 2007). The different time courses of the four genes and the variation over time of a gene suggest that the genes may be regulated individually, possibly via other transcription factors and/or epigenetic mechanisms.

Regulation of CYP1 Gene Expression via AHR2

The PCB126- and TCDD-induced expression of the four *CYP1* genes was strongly inhibited by the presence of the AHR2-MO. Previously, the induction of CYP1A by TCDD was shown to be AHR2 dependent (Prasch *et al*., 2003; Teraoka *et al*., 2003). Here, we have demonstrated that the induction of *CYP1B1*, *CYP1C1*, and *CYP1C2* in zebra fish embryos also appears to be regulated primarily by AHR2. Furthermore, we found that the control of *CYP1* induction by AHR2 extends to other AHR ligands, in this case PCB126. In addition to inhibiting *CYP1* induction, the AHR2-MO also protected against pericardial edema and embryo mortality in PCB126- or TCDD-exposed zebra fish. The AHR2-MO did not provide complete protection but rather delayed the onset of toxicity and reduced its severity.

There are at least two possible explanations for the lack of complete inhibition of CYP1 induction and embryotoxicity by the AHR2-MO. First, as noted earlier (Nasevicius and Ekker, 2000; Prasch *et al*., 2003), morpholino knockdown is transient and does not completely eliminate protein expression. Thus, a residual amount of AHR2 protein in the AHR2-MO–treated embryos could be responsible for the slight degree of CYP induction that remained when these embryos were exposed to inducer. Similarly, the late onset of edema and mortality in AHR-MO–treated, PCB126- or TCDD-exposed embryos could be due to reduced effectiveness of the AHR2-MO over time, leading to resynthesis of AHR2 protein at the later times. Since PCB126 and TCDD are slowly metabolized and eliminated, they may well remain in the tissues of the growing larvae at amounts high enough to activate the AHR when the morpholino effect declines. Similar to Prasch *et al*. (2003), we observed that the AHR2-MO did not protect against inhibition of swim bladder inflation. In carrier-controls the swim bladder inflated first at 4–6 dpf, and by then the AHR2-MO had begun to lose its protective effect against edema, which indicates AHR2 protein was being resynthesized (Fig. 8A). In addition, inhibition of swim bladder inflation by PCB126 showed a lower EC_{50} value than edema. Thus, if the effect on swim bladder inflation is AHR2-dependent, a smaller amount of activated AHR2 would be required to block swim bladder inflation, than to cause pericardial edema.

A second explanation for the lack of complete inhibition of the effects by PCB126 and TCDD by the AHR2-MO, which is not mutually exclusive with the first, is that other zebra fish AHRs play a role in regulation and/or toxicity. Zebra fish AHR1A (Andreasen *et al*., 2002) was found to be nonfunctional in *in vitro* studies. More recently, however, Incardona *et al*. (2006) suggested a role for AHR1A in the toxicity of certain PAHs, such as pyrene. Zebra fish also possess a second AHR1 form, AHR1B, which is functional *in vitro* (Karchner *et al*., 2005). Either of these AHRs could be involved in *CYP1* regulation, perhaps in a tissue- or stage-specific manner. Similarly, each of these AHRs could contribute to the embryotoxicity of PCB126 or TCDD, especially for effects not measured here.

Despite the possible roles of other AHRs, our results demonstrate clearly the important role of AHR2 in *CYP1* induction and embryotoxicity caused by exposure of embryos to PCB126 or TCDD. Interestingly, AHR2 is not an ortholog of the human AHR, which is an AHR1 and thus more closely related to the zebra fish AHR1 forms (Karchner *et al*., 2005). Examination and phylogenetic analysis of AHRs in a variety of species suggest that AHR2 has been lost in at least some mammals, including humans, while it has been retained in some other vertebrate groups (Hahn *et al*., 2006). Our results and those of previous studies (Prasch *et al*., 2003; Teraoka *et al*., 2003) suggest that in zebra fish, AHR2 is the predominant AHR form involved in gene regulation and toxicity by halogenated aromatic hydrocarbons during development, and thus it is functionally equivalent to the mammalian AHR in this regard (Mimura *et al*., 1997). Further studies will be needed to define the roles of zebra fish AHR1 forms in gene regulation and toxicity of various compounds and at different life stages. Similarly, further studies are required to establish the roles of the various CYP1s in physiology and/or toxicity of xenobiotics.

SUPPLEMENTARY DATA

Color version of Figures 6 and 7 are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Andreasen, E. A., Hahn, M. E., Heideman, W., Peterson, R. E., and Tanguay, R. L. (2002). The zebrafish (Danio rerio) aryl hydrocarbon receptor type 1 is a novel vertebrate receptor. *Mol. Pharmacol.* **62,** 234–249.

Antkiewicz, D. S., Burns, C. G., Carney, S. A., Peterson, R. E., and Heideman, W. (2005). Heart malformation is an early response to TCDD in embryonic zebrafish. *Toxicol. Sci.* **84,** 368–377.

Binder, R. L., and Stegeman, J. J. (1984). Microsomal electron transport and xenobiotic monooxygenase activities during the embryonic period of development in the killifish, Fundulus heteroclitus. *Toxicol. Appl. Pharmacol.* **73,** 432–443.

Cantrell, S. M., Lutz, L. H., Tillitt, D. E., and Hannink, M. (1996). Embryotoxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): The embryonic vasculature is a physiological target for TCDD-induced DNA damage and apoptotic cell death in Medaka (Orizias latipes). *Toxicol. Appl. Pharmacol.* **141,** 23–34.

Carney, S. A., Chen, J., Burns, C. G., Xiong, K. M., Peterson, R. E., and Heideman, W. (2006). Aryl hydrocarbon receptor activation produces heart-specific transcriptional and toxic responses in developing zebrafish. *Mol. Pharmacol.* **70,** 549–561.

Carney, S. A., Peterson, R. E., and Heideman, W. (2004). 2,3,7,8-Tetrachlorodibenzo-p-dioxin activation of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator pathway causes developmental toxicity through a CYP1A-independent mechanism in zebrafish. *Mol. Pharmacol.* **66,** 512–521.

Chambers, D., Wilson, L., Maden, M., and Lumsden, A. (2007). RALDH-independent generation of retinoic acid during vertebrate embryogenesis by CYP1B1. *Development* **134,** 1369–1383.

Choudhary, D., Jansson, I., Sarfarazi, M., and Schenkman, J. B. (2006). Physiological significance and expression of P450s in the developing eye. *Drug Metab. Rev.* **38,** 337–352.

Dong, W., Teraoka, H., Tsujimoto, Y., Stegeman, J. J., and Hiraga, T. (2004). Role of aryl hydrocarbon receptor in mesencephalic circulation failure and apoptosis in zebrafish embryos exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol. Sci.* **77,** 109–116.

Dong, W., Teraoka, H., Yamazaki, K., Tsukiyama, S., Imani, S., Imagawa, T., Stegeman, J. J., Peterson, R. E., and Hiraga, T. (2002). 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: Local circulation failure in the dorsal midbrain is associated with increased apoptosis. *Toxicol. Sci.* **69,** 191–201.

Easter, S. S., Jr, and Nicola, G. N. (1996). The development of vision in the zebrafish (Danio rerio). *Dev. Biol.* **180,** 646–663.

Ge, N. L., and Elferink, C. J. (1998). A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein. Linking dioxin signaling to the cell cycle. *J. Biol. Chem.* **273,** 22708–22713.

Godard, C. A., Goldstone, J. V., Said, M. R., Dickerson, R. L., Woodin, B. R., and Stegeman, J. J. (2005). The new vertebrate CYP1C family: Cloning of new subfamily members and phylogenetic analysis. *Biochem. Biophys. Res. Commun.* **331,** 1016–1024.

Goldstone, H. M., and Stegeman, J. J. (2006). Molecular mechanisms of 2,3,7,8-tetrachlorodibenzo-p-dioxin cardiovascular embryotoxicity. *Drug Metab. Rev.* **38,** 261–289.

Grubbs, F. E. (1969). Procedures for detecting outlying observations in samples. *Technometrics* **11,** 1–21.

Hahn, M. E. (2001). Dioxin toxicology and the aryl hydrocarbon receptor: Insights from fish and other non-traditional models. *Mar. Biotechnol.* **3,** S224–S238.

Hahn, M. E., Karchner, S. I., Evans, B. R., Franks, D. G., Merson, R. R., and Lapseritis, J. M. (2006). Unexpected diversity of aryl hydrocarbon receptors in non-mammalian vertebrates: Insights from comparative genomics. *J. Exp. Zool.* **305A,** 693–706.

Handley-Goldstone, H. M., Grow, M. W., and Stegeman, J. J. (2005). Cardiovascular gene expression profiles of dioxin exposure in zebrafish embryos. *Toxicol. Sci.* **85,** 683–693.

Heideman, W., Antkiewicz, D. S., Carney, S. A., and Peterson, R. E. (2005). Zebrafish and cardiac toxicology. *Cardiovasc. Toxicol.* **5,** 203–214.

Henry, T. R., Nesbit, D. J., Heideman, W., and Peterson, R. E. (2001). Relative potencies of polychlorinated dibenzo-p-dioxin, dibenzofuran, and biphenyl congeners to induce cytochrome P4501A mRNA in a zebrafish liver cell line. *Environ. Toxicol. Chem.* **20,** 1053–1058.

Henry, T. R., Spitsbergen, J. M., Hornung, M. W., Abnet, C. C., and Peterson, R. E. (1997). Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish (Danio rerio). *Toxicol. Appl. Pharmacol.* **142,** 56–68.

Incardona, J. P., Day, H. L., Collier, T. K., and Scholz, N. L. (2006). Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. *Toxicol. Appl. Pharmacol.* **217,** 308–321.

Jönsson, M. E., Orrego, R., Woodin, B. R., Goldstone, J. V., and Stegeman, J. J. (2007). Basal and 3,3,'4,4′,5-Pentachlorobiphenyl-induced expression of Cytochrome P450 1A, 1B and 1C Genes in Zebrafish. *Toxicol. Appl. Pharmacol.* **221,** 29–41.

Karchner, S. I., Franks, D. G., and Hahn, M. E. (2005). AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: Tandem arrangement of ahr1b and ahr2 genes. *Biochem. J.* **392,** 153–161.

Kawajiri, K., and Fujii-Kuriyama, Y. (2007). Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. *Arch. Biochem. Biophys.* **464,** 207–212.

Lew, D. J., Dulic, V., and Reed, S. I. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell* **66,** 1197–1206.

Libby, R. T., Smith, R. S., Savinova, O. V., Zabaleta, A., Martin, J. E., Gonzalez, F. J., and John, S. W. (2003). Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science* **299,** 1578–1581.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25,** 402–408.

Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T. N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., *et al.* (1997). Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. *Genes Cells* **2,** 645–654.

Mitchell, K. A., Lockhart, C. A., Huang, G., and Elferink, C. J. (2006). Sustained aryl hydrocarbon receptor activity attenuates liver regeneration. *Mol. Pharmacol.* **70,** 163–170.

Nasevicius, A., and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26,** 216–220.

Nebert, D. W., and Dalton, T. P. (2006). The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. *Nat. Rev. Cancer* **6,** 947–960.

Nebert, D. W., Roe, A. L., Dieter, M. Z., Solis, W. A., Yang, Y., and Dalton, T. P. (2000). Role of the aromatic hydrocarbon receptor and (Ah) gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem. Pharmacol.* **59,** 65–85.

Prasch, A. L., Teraoka, H., Carney, S. A., Dong, W., Hiraga, T., Stegeman, J. J., Heideman, W., and Peterson, R. E. (2003). Aryl hydrocarbon receptor 2 mediates 2,3,7,8-tetrachlorodibenzo-p-dioxin developmental toxicity in zebrafish. *Toxicol. Sci.* **76,** 138–150.

Puga, A., Barnes, S. J., Dalton, T. P., Chang, C., Knudsen, E. S., and Maier, M. A. (2000). Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest. *J. Biol. Chem.* **275,** 2943–2950.

Ramakers, C., Ruijter, J. M., Deprez, R. H., and Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339,** 62–66.

Reymann, S., and Borlak, J. (2006). Transcriptome profiling of human hepatocytes treated with Aroclor 1254 reveals transcription factor regulatory networks and clusters of regulated genes. *BMC Genomics* **7,** 217.

Schlezinger, J. J., Struntz, W. D., Goldstone, J. V., and Stegeman, J. J. (2006). Uncoupling of cytochrome P450 1A and stimulation of reactive oxygen species production by co-planar polychlorinated biphenyl congeners. *Aquat. Toxicol.* **77,** 422–432.

Tanguay, R. L., Abnet, C. C., Heideman, W., and Peterson, R. E. (1999). Cloning and characterization of the zebrafish (Danio rerio) aryl hydrocarbon receptor. *Biochim. Biophys. Acta* **1444,** 35–48.

Teraoka, H., Dong, W., Tsujimoto, Y., Iwasa, H., Endoh, D., Ueno, N., Stegeman, J. J., Peterson, R. E., and Hiraga, T. (2003). Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish. *Biochem. Biophys. Res. Commun.* **304,** 223–228.

Thackaberry, E. A., Jiang, Z., Johnson, C. D., Ramos, K. S., and Walker, M. K. (2005a). Toxicogenomic profile of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the murine fetal heart: Modulation of cell cycle and extracellular matrix genes. *Toxicol. Sci.* **88,** 231–241.

Thackaberry, E. A., Nunez, B. A., Ivnitski-Steele, I. D., Friggins, M., and Walker, M. K. (2005b). Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on murine heart development: Alteration in fetal and postnatal cardiac growth, and postnatal cardiac chronotropy. *Toxicol. Sci.* **88,** 242–249.

Wang, L., Scheffler, B. E., and Willett, K. L. (2006). CYP1C1 Messenger RNA Expression is Inducible by Benzo[a]pyrene in Fundulus heteroclitus Embryos and Adults. *Toxicol. Sci.* **93,** 331–340.