



Published in final edited form as:

Aquat Toxicol. 2007 December 30; 85(4): 241–250.

Synergistic induction of AHR regulated genes in developmental toxicity from co-exposure to two model PAHs in zebrafish

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are pollutants created by the incomplete combustion of carbon, and are increasing in the environment largely due to the burning of fossil fuels. PAHs occur as complex mixtures, and some combinations have been shown to cause synergistic developmental toxicity in fish embryos, characterized by pericardial edema and craniofacial malformations. Previous studies have indicated that in the zebrafish model, this toxicity is mediated by the aryl hydrocarbon receptor 2 (AHR2), and enhanced by inhibition of CYP1A activity. In this study, we further examined this interaction of the model PAH and AHR agonist β -naphthoflavone (BNF) with and without the AHR partial agonist/antagonist and CYP1A inhibitor α -naphthoflavone (ANF) to determine 1) whether ANF was acting as an AHR antagonist, 2) what alterations BNF and ANF both alone and in combination had on mRNA expression of the AHR regulated genes cytochrome P450 (*cyp*) *1a*, *1b1*, and *1c1*, and the AHR repressor (*ahrr2*) prior to vs. during deformity onset, and 3) compare CYP1A enzyme activity with mRNA induction. Zebrafish embryos were exposed from 24–48 or 24–96 hpf to BNF, 1–100 $\mu\text{g/L}$, ANF, 1–150 $\mu\text{g/L}$, a BNF+ANF co-exposure (1 $\mu\text{g/L}$ + 100 $\mu\text{g/L}$), or a DMSO solvent control. RNA was extracted and examined by quantitative real time PCR. Both BNF and ANF each individually resulted in a dose dependent increase *CYP1A*, *CYP1B1*, *CYP1C1*, and *AHRR2* mRNA, confirming their activities as AHR agonists. In the BNF+ANF co-exposures prior to deformity onset, expression of these genes was synergistic, and expression levels of the AHR regulated genes resembled the higher doses of BNF alone. Gene induction during deformities was also significantly increased in the co-exposure, but to a lesser magnitude than prior to deformity onset. EROD measurements of CYP1A activity showed ANF inhibited activity induction by BNF in the co-exposure group; this finding is not predicted by mRNA expression, which is synergistically induced in this treatment. This suggests that inhibition of CYP1A activity may alter metabolism and/or increase the half-life of the AHR agonist(s), allowing for increased AHR activation. This study furthers a mechanistic understanding of interactions underlying PAH synergistic toxicity.

Keywords

quantitative real-time PCR; CYP1A; CYP1B1; CYP1C1; AHRR; zebrafish; polycyclic aromatic hydrocarbons; aryl hydrocarbon receptor

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Nomenclature note: Gene names are indicated with lowercase italics; gene products are indicated with uppercase lettering, with mRNA products noted in italics to differentiate from protein names.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants of both natural and anthropogenic sources, derived from the incomplete combustion of carbon. Unlike many persistent organic pollutants, levels of PAHs are increasing in the environment, largely due to the burning of fossil fuels (Van Metre and Mahler, 2005). In aquatic systems, atmospheric deposition and urban runoff pose significant threats to early life stages of fishes, which are sensitive to toxicity of PAHs (Brinkworth et al., 2003; Incardona et al., 2004; Wassenberg and Di Giulio, 2004; Incardona et al., 2006). Resulting deformities include pericardial edema and craniofacial malformations. Other environmental contaminants, such as chlorinated dioxins and polychlorinated biphenyls (PCBs), cause similar toxicity in developing fish, and this toxicity has been shown to be mediated by the aryl hydrocarbon receptor (Billiard et al., 2006; Carney et al., 2004).

The aryl hydrocarbon receptor is a ligand activated member of the basic helix-loop helix Per-ARNT-Sim family of nuclear receptors. The cytosolic AHR can bind a wide variety of ligands including natural indoles from cruciferous vegetables and tryptophan metabolites (Denison and Heath-Pagliuso, 1998), as well as environmentally relevant pollutants including some chlorinated dioxins, PCBs, and PAHs. The AHR pathway is activated upon ligand binding to the AHR, which then translocates to the nucleus and forms a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT) protein, binds to xenobiotic response elements (XRE) in promoter regions and initiates transcription of numerous genes. The so-called “AHR battery” of genes largely includes Phase I metabolic enzymes and Phase II conjugating enzymes which then in turn metabolize a variety of organic substrates (Nebert et al., 1993).

For the most part, PAHs are excellent substrates for metabolism by the Phase I cytochrome P450 enzymes (Hawkins et al., 2002; Shimada, 2006). However, within the PAH class, there are varying structures based on the number of rings (2–6), bonding patterns of those rings, and heteroatom substitutions. This variety makes it difficult to ascribe a general biologic mechanism to these contaminants; several PAHs are AHR agonists, while some PAHs can also inhibit the enzymatic activity of the Phase I enzyme cytochrome P450 1A (CYP1A), the fish homolog of the mammalian CYP1A1 and 1A2. Recent studies in our laboratory have shown that co-exposure of several 4- and 5-ring PAH-type AHR agonists with a CYP1A inhibitor results in synergistic toxicity in developing killifish (*Fundulus heteroclitus*) and zebrafish embryos (Billiard et al., 2006; Wassenberg and Di Giulio, 2004).

To further understand the interactions underlying this AHR mediated synergistic toxicity, in this study we used two well characterized synthetic flavonoids commonly used as surrogate model PAHs, β -naphthoflavone (BNF) as an AHR agonist, and α -naphthoflavone (ANF) which acts as a reversible competitive CYP1A inhibitor that can bind to either the active site or the ferric heme (Goujon et al., 1972; Testa and Jenner, 1981; Miranda et al., 1998). Also, there is evidence that metabolites of ANF may be even more potent in this action than the parent compound (Nesnow and Bergman, 1981; Nesnow et al., 1982). However, ANF has also been described as a partial AHR agonist/antagonist able to displace TCDD from the receptor, resulting in a reduction in *CYP1A1* mRNA expression (Merchant et al., 1990; Merchant et al., 1992; Merchant et al., 1993). To complicate matters, other studies have reported that ANF was able to induce CYP1A in its own right, albeit to a lesser extent than the more potent agonist BNF (Aluru et al., 2005).

In our previous studies, we measured CYP1A enzymatic activity using an *in vivo* ethoxyresorufin-O-deethylase activity assay (EROD) and showed that ANF was able to inhibit activity induction caused by exposure to BNF; this inhibition coincides with a synergistic increase in deformities (Billiard et al., 2006; Wassenberg and Di Giulio, 2004). While our

studies suggested ANF was inhibiting CYP1A protein activity, they did not rule out the possibility that the observed EROD inhibition was the result of ANF acting as an AHR antagonist.

In the current study, we examined mRNA expression of *ahr2* and four genes known to be upregulated by the AHR pathway, *cyp1a*, *cyp1b1*, *cyp1c1*, and *ahrr2* (Hahn, 2001; Andreasen et al., 2002; Evans et al., 2005; Wang et al., 2006; Willett et al., 2006). Zebrafish have three AHR receptors, AHR1A, AHR1B, and AHR2; AHR2 has been shown to be necessary for dioxin and BNF toxicity, and was thus chosen for analysis (Prasch et al., 2003; Dong et al., 2004; Billiard et al., 2006). Our objectives were to determine whether ANF was functioning as an AHR antagonist in the synergistic toxicity, what alterations BNF and ANF both alone and in combination had on mRNA expression of *ahr2* and AHR regulated genes, how induction of these genes compare prior to versus during deformity onset, and how mRNA expression of *cyp1a* compares to the protein's enzymatic activity.

2. Materials and Methods

2.1 Chemicals

The chemicals α -naphthoflavone (ANF), and β -naphthoflavone (BNF), and 7-ethoxyresorufin were obtained from Sigma Aldrich (Saint Louis, MO), and dimethyl sulfoxide (DMSO) was purchased from Mallinckrodt Baker (Phillipsburg, NJ). All chemicals were dissolved in DMSO and kept protected from light at -20°C and vortexed prior to administration.

2.2 Animals

Zebrafish embryos were collected following mating of AB* strain adults maintained at 28°C on a 14 hr light, 10 hr dark light cycle. Embryos were maintained in 1x Danieau water (Nasevicius and Ekker, 2000) under the same temperature and photoperiod conditions for the duration of experiments.

2.3 Exposure

Chemicals and dosing concentrations were selected based on our earlier observations of synergistic PAH interactions in killifish and zebrafish (Wassenberg and Di Giulio, 2004; Billiard et al., 2006). Model PAHs used in this study were BNF (AHR agonist) and ANF (CYP1A activity inhibitor and potential AHR agonist/antagonist). At 24 hours post fertilization (hpf), embryos were dosed in replicate pools of 5 embryos, with 3 replicates per treatment, in 20 mL glass vials. DMSO concentration was kept at 0.03% in each vial and total volume set at 7.5 mL 1x Danieau water. The dose response for BNF included doses of 0, 1, 10, 50, and 100 $\mu\text{g/L}$; the dose of 50 $\mu\text{g/L}$ causes a slight increase in pericardial edema, while 100 $\mu\text{g/L}$ embryos appear similar to those in the 1 $\mu\text{g/L}$ BNF+ 100 $\mu\text{g/L}$ ANF co-exposures at 96 hpf (Billiard et al., 2006); no deformities are present at 48 hpf. The dose response for ANF included 0, 1, 10, 50, 100, and 150 $\mu\text{g/L}$, doses which are not embryotoxic by themselves (Billiard et al., 2006). Dose response experiments were repeated at least twice, and each data point represents an *n* of at least 6 pools of 5 embryos. Co-exposure experiments consisted of exposures to DMSO, 1 $\mu\text{g/L}$ BNF alone, 100 $\mu\text{g/L}$ ANF alone, and 1 $\mu\text{g/L}$ BNF + 100 $\mu\text{g/L}$ ANF in combination. Co-exposure experiments were repeated at least four times, and each data point represents an *n* of 12 pools of 5 embryos. For experiments examining *in vivo* EROD, the 1x Danieau water contained 21 $\mu\text{g/L}$ 7-ethoxyresorufin. Embryos were left in original dosing solutions for the duration of the experiments. At 48 hpf, embryos were rinsed with 1x Danieau water, manually dechorionated, fixed in RNA *later* (Ambion, Austin, TX) and stored at -80°C . At 96 hpf, larvae were washed and either fixed in RNA *later*, or imaged and scored blind for measurement of *in vivo* EROD activity.

Although deformity analysis was not a focus of this paper, our previous work on this matter demonstrated that no deformities are observed with DMSO, or single compound exposures of 1 or 10 µg/L BNF or 1–150 µg/L ANF at 96 hpf; pericardial edema and craniofacial malformations are only observed with the BNF+ANF co-exposure at the two highest doses of BNF tested, 50 and 100 µg/L (Billiard et al., 2006). These deformities are not yet manifested at 48 hpf.

2.4 *In vivo* EROD activity

We used a slightly modified *in vivo* ethoxyresorufin-O-deethylase (EROD) activity assay (Billiard et al., 2006) to measure the CYP1A activity in hatched zebrafish embryos (i.e. larvae) at 96 hpf. At 24 hpf, embryos were dosed in 1x Danieau water containing 21 µg/L 7-ethoxyresorufin (7-ER, in DMSO) as described above until 96 hpf when embryos were rinsed, anesthetized in MS-222, and mounted in a left lateral orientation in 3% methylcellulose. The accumulated fluorescent product of CYP1A metabolism of 7-ER, resorufin, was visualized in the gastro-intestinal tract and imaged using a rhodamine red fluorescent filter set under 50x magnification (Zeiss Axioskop, Thornwood, NY.). Images were then scored blind for EROD activity measured as fluorescence intensity minus background, quantified digitally by IP Lab software (Scanalytics Inc., Fairfax, VA). *In vivo* EROD values were expressed as a percentage of fluorescent intensity in control embryos.

2.5 Total RNA extraction & reverse transcription

A hand-held homogenizer was sterilized with 70% ethanol, sprayed with RNaseZap (Ambion), and washed three times with DEPC water and sterile dH₂O before each 30 second sample homogenization. RNA extractions were carried out according to the RNeasy Micro Kit protocol (Qiagen, Inc; Valencia, CA). RNA quantity and quality were analyzed spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). The Omniscript cDNA synthesis kit for Reverse Transcription (Qiagen) was used according to the manufacturer's instructions using 500 ng of RNA, random hexamers, and RNase inhibitor and carried out in a thermocycler for 1 hour at 37°C. cDNA was diluted to a working concentration of 1.88 ng/µl.

2.6 Quantitative real-time PCR

β-actin primers were designed using Light Cycler Probe Design software (Roche, Indianapolis, IN). *CYP1A*, *IBI*, and *ICI* primers were designed using PrimerQuest software (Integrated DNA Technologies, Inc, www.idtdna.com). *AHR2* and *AHRR2* primers were published previously (Andreasen et al., 2002; Evans et al., 2005). Primer efficiencies were also tested to ensure the housekeeping and target genes amplified at the same rate. Primer sequences and source sequence accession numbers are provided in Table 1.

A 25 µl reaction contained 200 nm of each primer, 12.5 µl 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 9.5 µl dH₂O, and 3.75 ng cDNA template (2 µl of 1.88 ng/µl). QRT-PCR was carried out on an ABI PRISM 7300 Real Time PCR System under the following thermal cycle conditions: 10 minutes at 95°C, followed by 40 replicates of 15 seconds at 95°C, then 1 minute at 60°C, followed by a dissociation curve calculation step. All samples were run in duplicate.

QRT-PCR data were analyzed using the ABI PRISM 7000 Sequence Detection System Software, Version 1.1 (Applied Biosystems). The comparative C_T (threshold cycle) method was used to determine average fold induction of mRNA by comparing the C_T of the target gene to that of the reference gene (*β-ACTIN*) according to the ABI PRISM users manual (Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, Applied Biosystems, https://www2.appliedbiosystems.com/support/apptech/#rt_pcr2).

Reference gene expression was not altered by treatment. To find fold induction, the C_T of each technical replicate was averaged and the following formulas were used:

$$\begin{aligned} C_T(\text{target gene}) - C_T(\beta - ACTIN) &= dC_T \\ dC_T(\text{treatment}) - dC_T(\text{control}) &= ddC_T \\ 2^{(-ddCT)} &= \text{fold change} \end{aligned}$$

Lastly, the fold change obtained for each biological replicate pool of 5 embryos was averaged for treatments; 6–15 replicates were used to obtain final induction averages and standard error per treatment per time point. As this study was designed to examine gene induction and not to compare basal gene expression levels, each gene was analyzed individually and induction determined based on control expression of that gene for each time point.

2.7 Statistical analysis

Data were analyzed with Statview for Windows (version 5.0.1; SAS Institute, Cary, NC). EROD values and mRNA fold changes were analyzed by factorial analysis of variance (ANOVA). When ANOVA yielded significance ($p \leq 0.05$), Fisher's protected least-significant differences (PLSD) was used as a *post hoc* test. We used significance of the interaction term between factors of BNF and ANF to test our null hypothesis that mixtures tested would yield additive responses. Data are presented as mean \pm S.E., and n defined as number of pools of 5 embryos or larvae.

3. Results

3.1 Dose dependent mRNA induction in single compound exposures

The BNF dose response curve resulted in a dose dependent increase in mRNA expression of *ahr2*, as well as the AHR regulated genes *cyp1a*, *cyp1b1*, *cyp1c1*, and *ahrr2* (Figure 1). *AHR2* was only slightly upregulated by BNF (2-fold). *CYP1A* exhibited the most dramatic increase in expression by BNF, 250-fold at the 100 $\mu\text{g/L}$ dose, while *CYP1B1* and *CYP1C1* both had the same level of induction, with a maximum mean induction of 40-fold. *AHRR2* showed the lowest increase in expression of the AHR2 regulated genes examined, with a maximal mean induction of 18-fold.

The ANF dose response curve produced similar mRNA induction patterns as the BNF dose response curve, although at a lower magnitude, consistent with its characterization as a weaker AHR agonist. *AHR2* was induced two fold, but only in the 100 $\mu\text{g/L}$ dose, and the induction was not statistically significant. Similar to the BNF gene induction, *CYP1A* had the highest upregulation, 30-fold compared to BNF's 250-fold at the same dose of 100 $\mu\text{g/L}$. *CYP1B1* had a maximum induction of nearly 3-fold, while *CYP1C1* had a maximum induction of nearly 6-fold, both at the 100 $\mu\text{g/L}$ dose. *AHRR2* was induced nearly 5-fold at the same dose. Overall, the AHR2 regulated genes were induced roughly ten times more by BNF than ANF.

3.2 Additive vs. synergistic mRNA induction in co-exposure groups

In embryos co-exposed to BNF+ANF, *AHR2* was induced 3-fold ($p = 0.03$) at 48 hpf, but by 96 hpf, this induction had ceased and values returned to control levels (Figure 2). The response observed at 48 hpf was consistent with an additive model of induction (Table 2).

In these same embryos, at 48 hpf a statistically significant effect of BNF, ANF, and a synergistic interaction between the two ($p < 0.0001$) was observed in all AHR regulated genes. With the exception of *AHRR2*, 1 $\mu\text{g/L}$ BNF and 100 $\mu\text{g/L}$ ANF single compound exposures significantly induced expression of all four genes (Figure 3). *CYP1A* exhibited the highest induction, 16.1-fold by BNF ($p = 0.0326$) and 30.5-fold by ANF ($p < 0.0001$). *CYP1B1* was also significantly

induced by BNF and ANF, although to a much lower extent than *CYP1A* (3.3 and 2.9-fold respectively, $p = 0.0002$, $p = 0.0027$). *CYP1C1* induced slightly more than *CYP1B1*, 3.5-fold by BNF and 5.7-fold by ANF ($p < 0.0001$ for both). *AHRR2* induction by BNF alone was not significantly different from DMSO, while ANF caused a 4.5-fold increase ($p = 0.0031$). In the co-exposures *CYP1A* showed the highest induction level of 190.5-fold, *CYP1C1* showed the second highest induction at 60.5-fold; followed by *CYP1B1* at 27.3-fold; and *AHRR2* at 22-fold (all $p < 0.0001$) (Figure 3). These observed inductions were at least three times greater than what would be predicted from an additive model (Table 2).

At 96 hpf, gene induction in the BNF or ANF single compound treatments either persisted at similar levels or in some cases increased above levels at 48 hpf (Figure 3). BNF and ANF single treatments showed significant effects (ANOVA $p \leq 0.0006$). BNF induced *CYP1A* 36.3-fold ($p = 0.0134$), more than double the expression level observed at the earlier time-point. ANF caused a 34-fold change ($p = 0.0215$) which was similar to the expression level at the 48 hpf time-point. *CYP1B1* induction levels by BNF were similar to those at 48 hpf, with a 4-fold induction ($p = 0.0172$) compared to 3.3-fold. ANF was also somewhat higher at 96 hpf at 5.5-fold ($p = 0.0018$) compared to 2.9-fold at 48 hpf. *CYP1C1* expression showed a significant effect of BNF and ANF ($p \leq 0.0002$), and a significant interaction between the two factors ($p = 0.0441$). Expression levels in response to BNF were higher at 96 hpf than at 48 hpf, 5.1-fold ($p = 0.0009$) compared to 3.5-fold, and remained similar in the ANF treatment, 6.2-fold, ($p = 0.0001$), compared to 5.7-fold. *AHRR2* expression showed a significant effect of BNF ($p = 0.0172$), ANF ($p = 0.0035$), and a significant interaction term ($p = 0.0481$). Induction in the BNF treatment was not different from DMSO control, while induction by ANF was 2.7-fold ($p = 0.0016$), down from 4.5-fold at 48 hpf.

In the BNF+ANF co-exposure group at 96 hpf, expression of all AHR- regulated genes was about half of the induction observed at 48 hpf, and only *CYP1C1* and *AHRR2* retained significant interaction terms ($p = 0.0441$, $p = 0.0481$). *CYP1A* was induced 80.4-fold ($p < 0.0001$) less than half of the 190.5-fold induction at the earlier time-point. *CYP1B1* induction levels fell from 27.3-fold at 48 hpf to 15-fold at 96 hpf ($p < 0.0001$). *CYP1C1* induction levels showed the most dramatic decrease, 17.8-fold ($p < 0.0001$), less than a third of the earlier 60.5-fold. *AHRR2* expression was 9.2-fold ($p = 0.0374$), less than half of the 22-fold observed at 48 hpf.

The expression of all four AHR- responsive genes in the co-exposure group at 96 hpf was much less than levels observed in this treatment at 48 hpf, while single compound exposures either remained steady or increased in expression (Figure 3). This difference could be due to a number of factors such as alternate metabolic clearance mechanisms. Still, the level of AHR- regulated gene induction at 96 hpf is quite substantial in spite of deformities, highlighting the robustness of the AHR pathway response.

3.3 Gene induction vs. protein activity

Our previous studies have shown that *CYP1A* enzymatic activity as measured by *in vivo* ethoxyresorufin-O-deethylase (EROD) activity was inhibited by ANF in the BNF+ANF co-exposures (Wassenberg and Di Giulio, 2004; Billiard et al., 2006). This study repeated those experiments and further show that this enzyme activity inhibition is in contrast to the mRNA expression of *CYP1A*, which is synergistically increased by this same exposure (Figure 4). In addition, the increase in EROD activity in the BNF but not the ANF single exposures is also not predicted by their similar levels of mRNA induction (Figure 4). These results highlight a disparity between *CYP1A* mRNA induction and enzyme activity.

4. Discussion

The data presented in this study help clarify the role of ANF in this synergistic toxicity. We observe that ANF acts as a comparatively weak AHR agonist by itself, and in BNF+ ANF co-exposures, it is not acting as an AHR antagonist at the described doses. We would expect that if ANF was acting as an AHR antagonist in the co-exposures, we would have observed a lack of mRNA induction of genes regulated by the AHR. On the contrary, we observed a synergistic induction of mRNA expression of genes regulated by the AHR at 48 hpf, and continued upregulation after deformity onset through 96 hpf. In most instances, the induction observed in the co-exposures at 48 hpf was comparable to induction levels observed at the two highest concentrations of the BNF dose response curve. These two highest doses of BNF also begin to exhibit signs of toxicity by 96 hpf, in great similarity to the co-exposure groups (Billiard et al., 2006). This result coupled with the observed inhibition of CYP1A EROD activity suggests that through ANF's ability to inhibit CYP1A's enzymatic activity, metabolism is altered such that the half-life of the AHR agonist(s) is being extended. This would allow for continual activation of the AHR and correlates with the appearance of deformities.

These deformities are similar to those observed by others following zebrafish embryo exposure to 2,3,7,8,-tetrachlorodibenzo-*p*- dioxin (TCDD) (Teraoka et al., 2003; Carney et al., 2004). It is thus hypothesized that the mechanism of toxicity behind the synergistic developmental toxicity of these model polycyclic aromatic hydrocarbons may, as with TCDD, be due to the continual activation or misregulation of the AHR during critical periods of development. Alternatively, upregulation of additional CYP1s could cause a shift in metabolism and increased production of reactive metabolites.

Our observation of ANF's interaction with BNF as a weak AHR agonist agrees with a study by Aluru *et al.* (2005), in which rainbow trout hepatocytes treated with BNF and ANF single treatments both increased mRNA abundance in a dose dependent manner, but that ANF's maximal induction of AHR and CYP1A protein was about half that of BNF's. The authors confirmed ANF's activity as a partial AHR agonist, and not an AHR antagonist. BNF+ANF co-exposures did not decrease BNF's induction of CYP1A mRNA or protein; instead, similar to our observation of increased mRNA expression in BNF+ANF co-exposures, the authors observed increased hepatocyte CYP1A protein (Aluru et al., 2005).

We propose that it is ANF's activity as a CYP1A inhibitor that is critical to the synergistic embryo toxicity of BNF+ ANF, not ANF's activity as a weak AHR agonist. This is supported by previous work in the killifish model (*Fundulus heteroclitus*) where we observed that treatment with numerous CYP1A inhibitors including ANF, fluoranthene, 2-aminoanthracene, and dibenzothiophene also increased toxicity of BNF and resulted in synergistic embryotoxicity (Wassenberg and Di Giulio, 2004; Wassenberg et al., 2005). We have also observed in our previous zebrafish work, that morpholino knockdown of CYP1A resulted in exacerbation of a nontoxic combination of BNF+ANF, (1 µg/L BNF + 50 µg/L ANF), and also synergized toxicity of a single nontoxic dose of BNF (10 µg/L), such that deformities of both experiments were of similar severity to those observed in the BNF+ANF (1 µg/L BNF + 100 µg/L ANF) synergistic toxicity (Billiard et al, 2006). These studies clearly indicate the importance of CYP1A metabolic activity in protecting from toxicity.

In addition to inhibition of CYP1A activity, it is possible that this toxicity may also be due to the subsequent increased half-life of the AHR agonist(s). We observed a synergistic upregulation of genes regulated by the AHR during co-exposure of BNF and ANF at mRNA expression levels that were similar to those observed with higher doses of BNF alone. While our studies did not include metabolite analysis, our interpretation of these data is supported by microcosm studies examining metabolism of the PAH benzo (a) pyrene (BaP) with co-exposure

to the CYP- inhibitor piperonyl butoxide in mosquito fish (*Gambusia affinis*) which resulted in reduced metabolism of BaP and increased accumulation of the parent compound by 21% in a 3-day aquatic microcosm study and by 12% in a 33-day terrestrial-aquatic study (Lu et al., 1977).

As CYP1A enzyme inhibition appears to result in increased ligand and activation of the AHR, it then follows that we must further examine the importance of this receptor. Not only does the AHR play an important role in toxicant detoxification as an adaptive response, but it also serves critical physiologic functions during normal development; both overactivation as well as inhibition can have severe consequences for normal development, as reviewed in Carney et al (2006a) and Hahn (2003). Our study demonstrated a transient 3-fold upregulation of *AHR2* at 48 hpf (24 h post dose administration) in the BNF+ ANF co-exposure group. This is within the range of the 2-4- fold upregulation of *AHR2* mRNA observed in TCDD exposed zebrafish embryos (10–100 nM concentrations administered 1 hpf and analyzed 24 hpf; analyzed by Northern blot) (Tanguay et al., 1999). While only small fold changes were observed with *AHR2* mRNA, the genes regulated by the AHR exhibit tremendous responsiveness to the ligand dependent induction by AHR2. It remains unclear whether the upregulation of genes by AHR2 is due to increased abundance of the receptor, increased ligand concentration, or perhaps both, which contribute to the synergistic upregulation of genes by AHR in our experiments.

Three days after treatment administration (96 hpf), BNF and ANF single treatment groups (1 and 100 µg/L) still had elevated induction of *CYP1* mRNA. Also notable is that this low level continual activation of the AHR pathway did not lead to deformities, while the higher activation level observed in the co-exposure groups did. One explanation for the lowered mRNA induction observed at the 96 hpf time point compared to the 48 hpf time point in the co-exposure treatment group is that perhaps metabolism by CYP1B1 and CYP1C1 is occurring, and thereby lowering the concentration of AHR ligand.

We examined mRNA expression of four genes upregulated by the AHR pathway: *CYP1A*, *CYP1B1*, *CYP1C1*, and *AHR2*. *CYP1A* has been widely studied in fish. Upregulation of this highly responsive AHR regulated gene by various AHR agonists has been shown in numerous RNA and protein expression studies (Andreasen et al., 2002; Meyer et al., 2003; Teraoka et al., 2003; Chung-Davidson et al., 2004; Volz et al., 2005), and it is often used as a biomarker for exposure to environmental contaminants. *CYP1B1* on the other hand, is less well studied in fish. In channel catfish, BaP treatment induced *CYP1B1* mRNA in blood, liver, gonad, in cultured gill cells (Willett et al., 2006). In zebrafish, *CYP1B1* mRNA has been found to be inducible by BNF in adult gill, and by PCB-126 in heart, liver, kidney, gut, gill, eye, and brain, and by PCB-126 in zebrafish embryos (Jonsson et al., 2007). *CYP1B1* has also been shown to be inducible in zebrafish by TCDD through microarray analysis (Handley-Goldstone et al., 2005; Carney et al., 2006).

This study also provides new information about the newly identified CYP1C1. This enzyme was recently identified along with CYP1C2 in scup and zebrafish (Godard et al., 2005) and has also been found in carp and killifish (Itakura et al., 2005; Wang et al., 2006). The zebrafish CYP1C2 cDNA sequence was not available at the start of this study. Homologues to CYP1C1 or the CYP1C2 genes have not been identified in mammals, suggesting this may be a fish specific gene. While only very few studies of CYP1C1 have yet been published, XREs were identified in both *cyp1c* promoters (Jonsson et al., 2007). Both zebrafish *CYP1C1* and *CYP1C2* were inducible in embryos exposed to PCB-126, and *CYP1C1* was inducible by PCB126 in adult eye, heart, gill, liver, and kidney (Jonsson et al., 2007). *CYP1C1* has also been shown to be inducible in the killifish model by the PAH benzo(a)pyrene (Wang et al., 2006) and in zebrafish embryos by TCDD (Carney et al., 2006). Our study contributes a mRNA dose

response of *CYP1C1* by both BNF and ANF that was quite similar compared to the *CYP1B1* dose response; neither was as responsive as *CYP1A*.

We also examined *AHRR2*, one of two zebrafish co-orthologs of the mammalian *AHRR*. While the function of this protein in toxicity is not yet understood, *AHRR* has been shown to repress the transactivation ability of the *AHR/ARNT* heterodimer *in vitro* (Mimura et al., 1999). Additionally, both *AHRR1* and *AHRR2* mRNA have been shown to be upregulated by TCDD exposure in developing zebrafish embryos (Evans et al., 2005). Here we show that *AHRR2* is also responsive to induction by BNF and ANF.

While we have previously shown that *AHR2* mediates the synergistic toxicity of BNF+ANF in zebrafish (Billiard et al., 2006), it remains unclear as to whether it is the overactivation of the *AHR2* and its downstream interactions with other signaling pathways, or the upregulation of *CYP1* metabolic activity altering xenobiotic and endogenous metabolism, that is responsible for toxicity.

The important role of *CYP1* metabolic activity in this synergistic interaction is also demonstrated by the work of Hodson et al (2007). In this recent paper, 100 µg/L ANF (the same dose used in our co-exposure experiments) enhanced toxicity of the alkyl-substituted PAH and *AHR* agonist retene (1-methyl-7-isopropyl phenanthrene) to rainbow trout (*Oncorhynchus mykiss*) juveniles. Similar to our observations with BNF, higher levels of retene have also been shown to cause blue sac disease and induction of *CYP1A* (Brinkworth et al., 2003). Metabolite analysis during this retene + ANF (100 µg/L) co-exposure showed decreased concentrations of polar dihydroxylated metabolites, and increased concentrations of less polar monohydroxylated metabolites, suggesting that ANF's inhibition of *CYP1A* is causing a shift in metabolism, perhaps to other *CYP1* enzymes. Concurrently, protein concentrations of *CYP1A* increased, indicating increased activation of the *AHR* pathway. Similar to our previous study in which morpholino knockdown of *AHR2* prevented toxicity of BNF+ANF co-exposure, rainbow trout blue sac disease symptoms were substantially lessened when concentrations of ANF (320 µg/L) were high enough to inhibit retene metabolism via *AHR* antagonism as demonstrated by a decrease in *CYP1A* protein, increased parent compound concentration and very few retene metabolites (Hodson et al., 2007). Other studies further substantiate the observation for dual modes of action of ANF, and that these modes will differ depending on the dose (Gasiewicz and Rucci, 1991; Santostefano et al., 1993; Aluru et al., 2005).

There is also strong evidence that misregulation of the *AHR* could be contributing to this toxicity. The *AHR* has been shown to have a critical endogenous role in normal cardiovascular development in vertebrates (Hahn, 2003). Continual activation of this receptor, or misregulation, is the primary mechanism by which TCDD is thought to cause toxicity, although downstream effects remain unclear. The *AHR* has been shown to also interact with numerous pathways which could disrupt development including p53, NFκB, steroid receptors, and retinoblastoma protein, and has been the topic of several reviews (Huang and Elferink, 2005; Marlowe and Puga, 2005; Puga et al., 2005). Further studies are necessary to distinguish whether altered metabolism, overactivation of the *AHR* with subsequent cell signaling disruptions, or both, are involved in developmental toxicity of some PAHs.

5. Conclusions

This study provides additional insight into the mechanisms underlying synergistic developmental toxicity of PAHs. We have confirmed that ANF is not antagonizing the *AHR2* in these co-exposures, but is instead acting as a weak *AHR* agonist. Furthermore, mRNA induction of the *AHR* regulated genes *CYP1A*, *CYP1B1*, *CYP1C1*, *AHRR2* was synergistically

upregulated in BNF+ANF co-exposures groups prior to the onset of deformities to levels that resembled the highest doses of BNF alone in the dose-response curve experiments. Induction of *CYP1A* mRNA was synergistically induced to 190-fold, which starkly contrasts to the *CYP1A* enzyme activity, which was inhibited in the BNF+ANF co-exposures. These data suggest that through inhibition of the metabolic clearance provided by *CYP1A*, the half-life of the AHR agonist(s) is being extended, thus allowing for prolonged activation of the AHR. This hypothesis, however, remains to be confirmed.

Acknowledgements

Di Giulio Laboratory, Deena Wassenberg, David Volz, Elwood Linney, Superfund Basic Research Program (P42 ES10356), Integrated Toxicology & Environmental Health Program (T32 ES07031), EPA STAR (to A. T.-L.).

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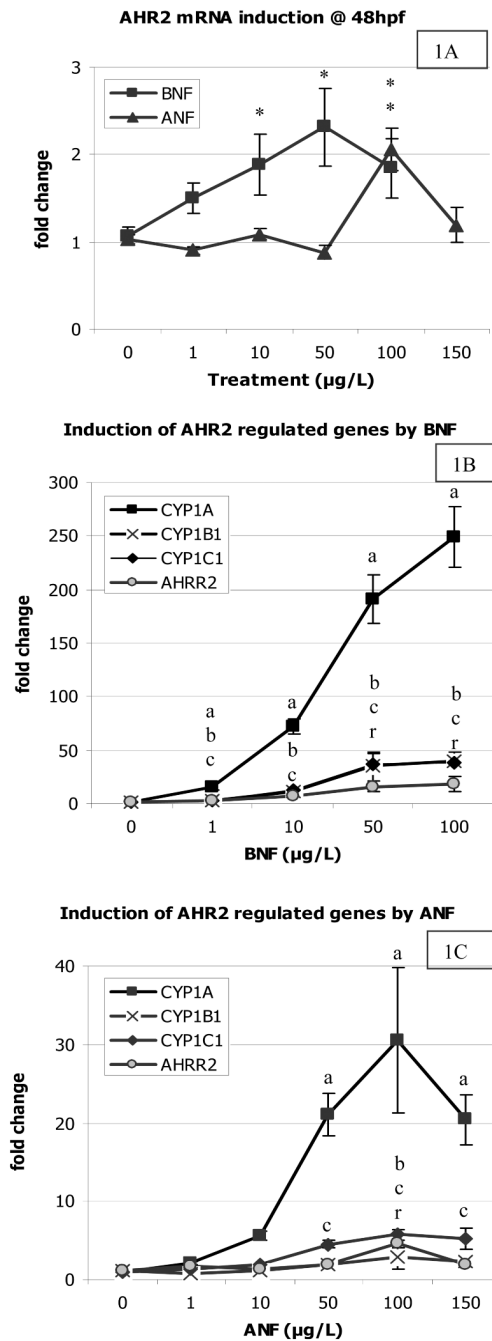


Figure 1. Dose response of BNF and ANF on mRNA induction of *AHR2* and *AHR2* regulated genes at 48 hpf after exposure for 24 hours. A) Induction of *AHR2* by BNF and ANF dose response curves. A “*” indicates a significant difference from control levels. B) BNF induction of *CYP1A*, *1B1*, *1C1*, and *AHRR2*. To differentiate significance between overlapping data points, the letter “a” indicates a significant difference from control levels for *CYP1A* induction, letter “b” for *CYP1B1*, letter “c” for *CYP1C1*, and the letter “r” for *AHRR2* expression. C) ANF induction of *CYP1A*, *1B1*, and *1C1*, and *AHRR2*. Significance is indicated by the letters described above. Data presented as mean ± S.E.; $n \geq 6$ replicates of pooled embryos; significance defined as $p \leq 0.05$.

AHR2 mRNA induction over time

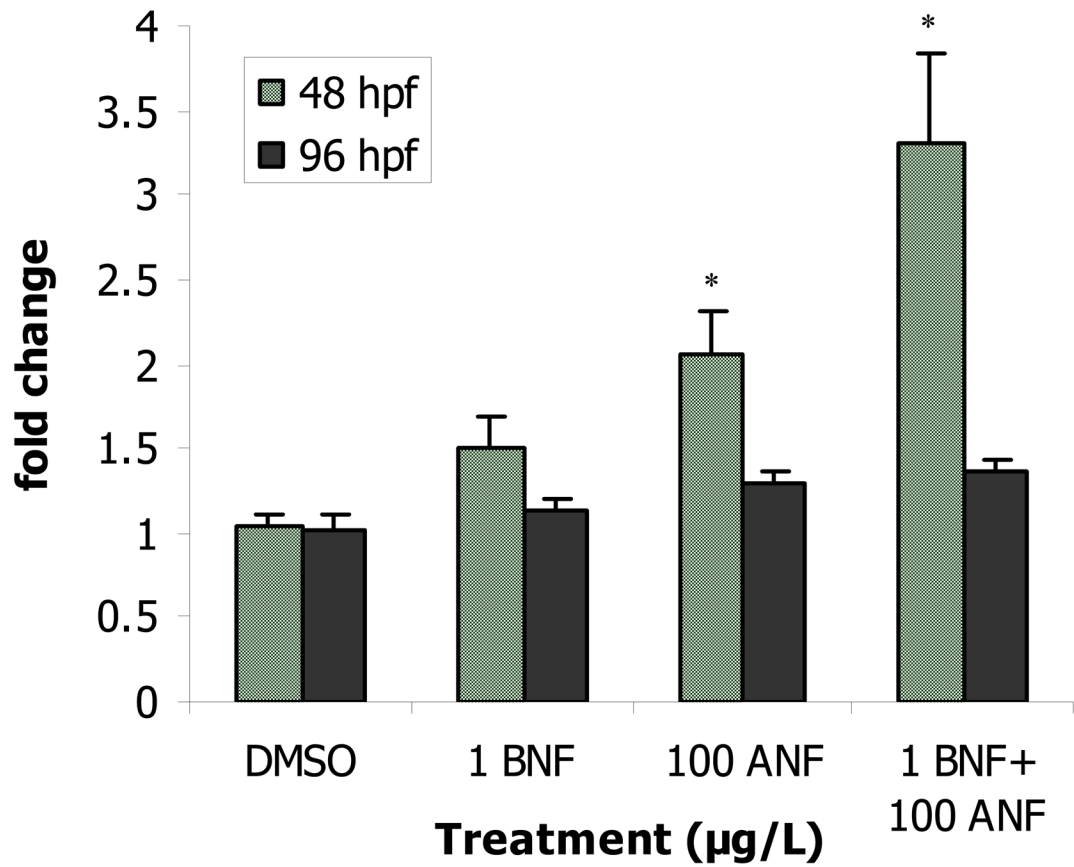


Figure 2.

Induction of *AHR2* mRNA at 48 hpf and at 96 hpf. A "*" indicates a significant difference from time-matched controls ($p \leq 0.05$). Data presented as mean \pm S.E.; 48 hpf time-point data $n \geq 15$ replicates of pooled embryos; 96 hpf time-point data $n = 6$.

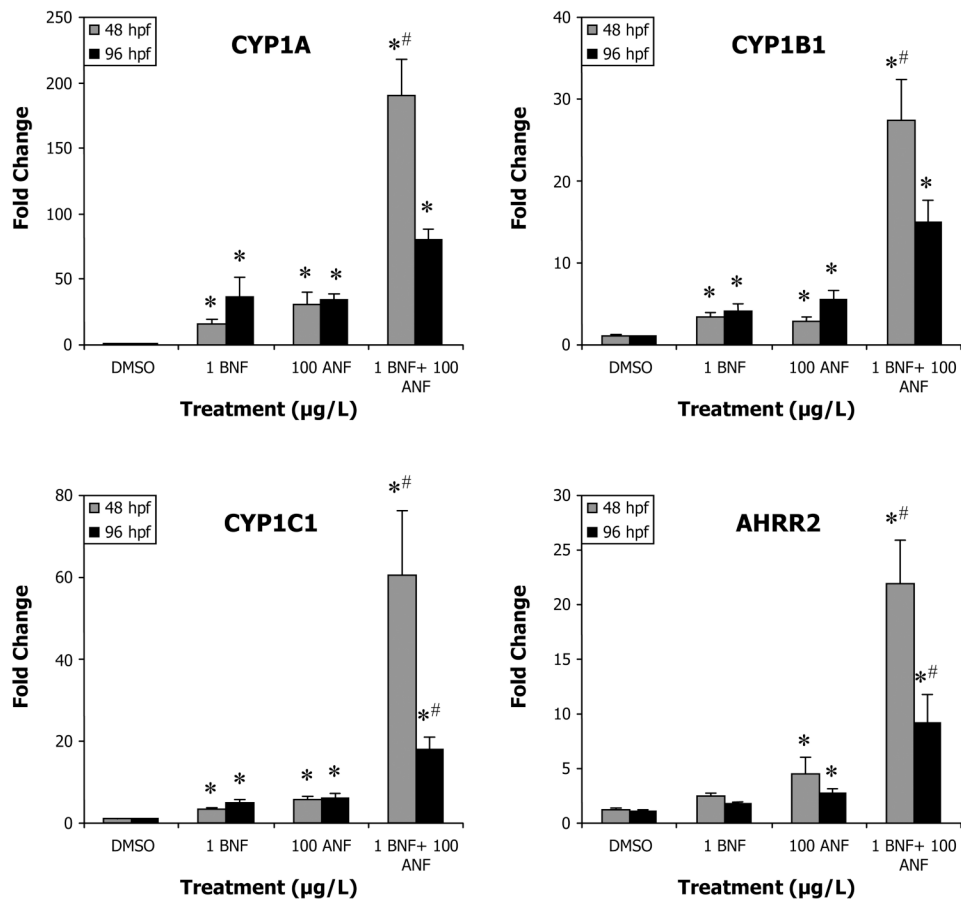


Figure 3. Induction of AHR2 regulated genes in the BNF+ANF co-exposure experiments at 48 and 96 hpf. A “#” indicates a synergistic interaction (ANOVA interaction term $p \leq 0.05$). A “*” indicates a significant difference from time-matched control ($p \leq 0.05$). Data presented as mean \pm S.E.; for 48 hpf data $n \geq 12$ replicates of pooled embryos; for 96 hpf data $n \geq 6$.

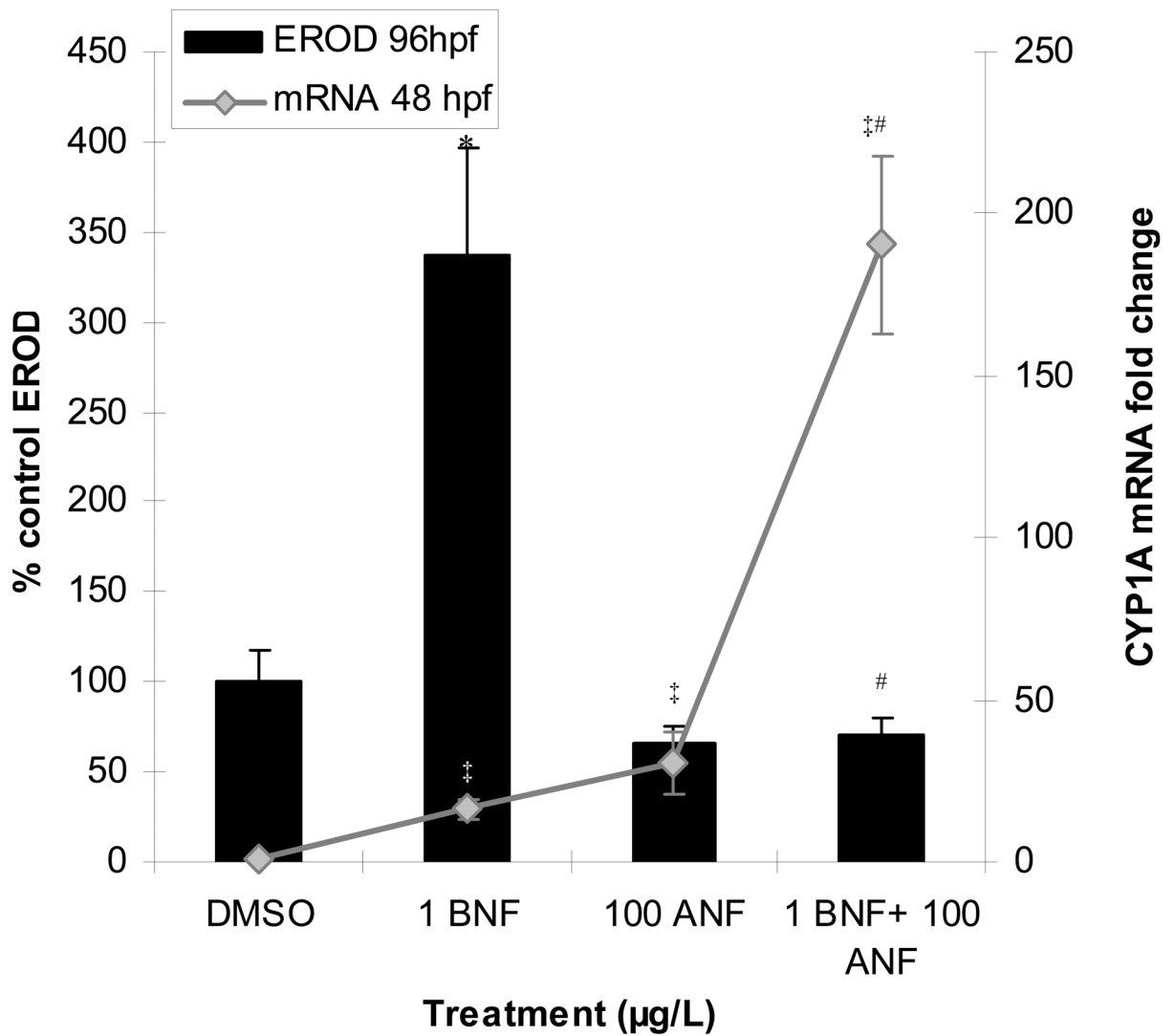


Figure 4. CYP1A EROD activity vs. CYP1A mRNA induction. BNF induction of EROD activity (96 hpf) is inhibited by ANF while mRNA induction (48 hpf) is synergistically enhanced. A “#” indicates a synergistic interaction (ANOVA interaction term $p \leq 0.05$). A “*” indicates a significant difference from control among EROD data, and “†” indicates a significant difference from controls among mRNA data ($p \leq 0.05$). Data are presented as mean \pm S.E.; EROD $n = 3$ replicates of pooled embryos and is representative of 75 larvae. mRNA $n \geq 12$ replicates of pooled embryos.

Table 1

cDNA target genes, GenBank identification, and primers used for QRT-PCR.

Gene	GenBank ID	Forward primer (5'-3') Reverse primer (5'-3')
<i>β-ACTIN</i>	<u>AF057040</u>	ACATCCGTAAGGACCTG GGTCGTTCGTTGAATCTC
<i>CYP1A</i>	<u>NM_131879</u>	AGGACAACATCAGACACATCACCG GATAGACAACCGCCAGGACAGAG
<i>CYP1B1</i>	<u>NM_001013267</u>	CCACCCGAACTCTGAAACTC AAACACACCATCAGCGACAG
<i>CYP1C1</i>	<u>NM_001020610</u>	TGGAGGCTGAGTTTGGACTGAAGA GAGGAAGAAGAGGATGACGAAGGATG
<i>AHRR2</i>	<u>NM_001020610</u>	AAACAGAAGCTCTGGCCGAGTGA CTGTATGCCCATGAAGCGTCCTGAG
<i>AHR2</i>	<u>AF063446</u>	CTGTATGCCCATGAAGCGTCCTGAG CTGTATGCCCATGAAGCGTCCTGAG

Table 2

Predicted vs. observed gene mRNA induction at 48 hpf. Expression levels of the AHR2 display additive induction while the AHR2 regulated genes in the BNF+ANF co-exposure groups exhibit mRNA induction levels at least three times greater than those predicted by simple additive induction of single compound exposures.

Gene	BNF (1 µg/L)	ANF (100 µg/L)	BNF+ANF Predicted Additive Induction	BNF+ANF Observed Induction
<i>AHR2</i>	1.5	2.1	3.6	3.3
<i>CYP1A</i>	16.1	31.6	47.6	190.4
<i>CYP1B1</i>	3.3	2.9	6.2	27.3
<i>CYP1C1</i>	3.5	5.7	9.2	60.6
<i>AHRR2</i>	2.4	4.5	6.9	22.0