ALTERING CYTOCHROME P4501A ACTIVITY AFFECTS POLYCYCLIC AROMATIC HYDROCARBON METABOLISM AND TOXICITY IN RAINBOW TROUT (ONCORHYNCHUS MYKISS)

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Abstract—The polycyclic aromatic hydrocarbons (PAHs) phenanthrene and retene (7-isopropyl-1-methyl phenanthrene) are lethal to rainbow trout (Oncorhychus mykiss) larvae during chronic exposures. Phenanthrene is a low-toxicity, non–cytochrome P4501A (CYP1A)–inducing compound that accumulates in fish tissues during exposure to lethal concentrations in water. Retene is a higher toxicity CYP1A-inducing compound that is not detectable in tissue at lethal exposure concentrations. The metabolism, excretion, and toxicity of retene and phenanthrene were examined in juvenile and larval rainbow trout during coexposure to the model CYP1A inducer β-naphthoflavone (βNF), or to the inducer–inhibitor piperonyl butoxide to determine if modulating CYP1A activity affected PAH metabolism and toxicity. Phenanthrene metabolism, excretion rate, and toxicity increased with coexposure to βNF. Piperonyl butoxide inhibited phenanthrene metabolism and reduced the excretion of all phenanthrene metabolites. As a consequence, embryo mortality rates increased but rates of sublethal effects did not. Coexposure of trout to retene and βNF caused no change in retene metabolism and excretion, but retene toxicity increased, perhaps due to additivity. Piperonyl butoxide inhibited retene metabolism, decreased the excretion of some retene metabolites while increasing the excretion of others, and increased the toxicity of retene. These results support the role of CYP1A activity in PAH metabolism and excretion, and the role of the CYP1A-generated metabolites of PAHs in chronic toxicity to larval fish.

Keywords—Cytochrome P450, Retene, Phenanthrene, Metabolism, Trout

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants produced by the incomplete combustion of organic compounds. The main source of PAHs in aquatic ecosystems is atmospheric deposition of fossil fuel combustion by-products, and other important sources include industrial discharges, forest fires, decomposition of organic materials, and oil spills [1]. Because of their hydrophobicity, PAHs are readily taken up by aquatic organisms in proportion to their octanol–water partition coefficients (Kow) [2].

After uptake in rainbow trout (Oncorhynchus mykiss), some PAHs induce cytochrome P4501A (CYP1A) enzymes through the aryl hydrocarbon receptor (AhR) pathway [3]. Initial CYP-mediated PAH oxygenation (phase I metabolism) may produce an epoxide, which can undergo further metabolism to a diol or diol epoxide [4]. Both the diol and diol epoxide have increased polarity and reactivity, and may result in covalent interactions with nucleic acids and proteins [5]. Phase I metabolites are available for phase II conjugation with glutathione, glucuronide, or sulfate, leading to decreased reactivity and increased excretion [6]. Although primary and secondary metabolism of PAHs are required for biliary excretion, they may result in the activation of relatively nontoxic parent compounds to toxic metabolites, an example of which is the bioactivation of benzo[a]pyrene to a potent carcinogen [5]. Evidence for the role of CYP1A in PAH metabolism is supported by correlations among PAH exposure, increases in CYP1A mRNA and enzyme levels, and increased PAH depuration in rainbow trout [7] and in primary cultures of trout cells [8]. Because all PAHs do not induce CYP1A activity, differences possibly exist among PAHs in the nature and extent of metabolism under conditions of low endogenous CYP1A activity or high induced activity. Assuming that the metabolites of PAHs have differing toxicities than their parent compounds, we measured the extent of CYP1A induction, rates of accumulation and excretion, and chronic toxicities to fish of two PAHs, a CYP1A inducer and a noninducer, in the presence and absence of a model CYP1A inducer and a CYP1A inhibitor.

Retene (7-isopropyl-1-methyl phenanthrene) (Fig. 1) has been found in sediments downstream of pulp mills at concentrations of up to 3,500 μg/g dry weight [9]. Phenanthrene (Fig. 1) and alkyl-substituted phenanthrenes have been found in crude oil [10], fish exposed to crude oil [10], and industrially contaminated sediments (P. Hodson, unpublished data). The molecular weights of PAHs increase with increasing alkylation, resulting in a higher Kow and hydrophobicity. The increase in the degree of alkylation also increases the induction and activity of CYP1A protein, and the toxicity to early life stages (ELSs) of medaka [11; Y. Kiparissis, personal communication].

Retene has a Kow of 6.4 [11], so it is relatively insoluble in water and is rapidly accumulated by fish [7]. However, because it also binds to fish AhR [12], it induces CYP1A in juvenile trout [13], and is excreted as metabolites in the bile [13]. At lethal exposure concentrations, retene tissue concentrations are below 2.3 × 10⁻³ mmol/kg [14]. Waterborne retene has a chronic median lethal concentration (LC50) for ELSs of trout of 7.56 × 10⁻⁴ mmol/L [15], and its toxicity resembles dioxinlike blue-sac disease (BSD), with pericardial and yolk-sac edema, craniofacial malformations, hemorrhaging, decreased growth, and increased mortality [16].
Phenanthrene is an unsubstituted PAH with a \( K_{ow} \) of 4.46 [2], which makes it somewhat more soluble in water than retene. Phenanthrene does not have a detectable binding affinity to trout AhR proteins or in trout cell lines [12], and does not induce CYP1A activity in trout cells in vitro [8] or in vivo [11]. Consequently, phenanthrene has a 9-d half-life [17], and at death whole-body concentrations in sac fry are 7.07 mmol/kg [15], a value within the range of 2 to 8 mmol/kg for the critical body residue for narcosis [18]. Although exposure of trout ELSs to phenanthrene will elicit BSD symptoms, phenanthrene has a relatively low toxicity with a chronic LC50 of \( 5.61 \times 10^{-2} \) mmol/L [15].

Enhanced metabolism and excretion of retene suggests that retene metabolites may be the toxic forms. Reactive metabolites such as epoxides can bond to proteins and nucleic acids, causing toxicity. Alternatively, the toxicity of PAHs to ELSs of fish may be a consequence of oxidative stress, as is proposed for dioxin [15,19]. In this model, excess production of free radicals due to the continual induction and activity of oxidative enzymes causes lipid peroxidation, loss of membrane fluidity and integrity, and edema and hemorrhaging [19]. This mechanism would be consistent with the symptoms of BSD, and previous research has indicated that the potency of dioxins, furans, and polychlorinated biphenyls for BSD correlates with the potency for induction and activity of CYP1A [20].

The null hypothesis that CYP1A activity plays no role in PAH metabolism and toxicity was tested by coexposure of trout to retene and phenanthrene in the presence of a CYP1A inducer (\( \beta \)-naphthoflavone \([\betaNF]\)) or inhibitor (piperonyl butoxide \([PBO]\)). Metabolism was measured by the rate of accumulation (or loss) of PAHs from the tissues of juvenile trout, and the occurrence of metabolites in their bile during a short-term exposure. Toxicity was measured by the incidence of symptoms of BSD in embryos and larvae of trout during chronic exposure. \( \beta \)-Naphthoflavone is a model inducer of the AhR pathway, and leads to an increase in CYP1A levels and activity [21]. Piperonyl butoxide, although a CYP1A inducer, is also a quasi-irreversible inhibitor [22]. After metabolism by CYP1A enzymes, PBO forms a metabolite intermediate complex with the CYP1A catalytic site. This results in an inactive metabolite intermediate complex and disables the CYP1A protein from further oxidative metabolism [22]. Therefore coexposures of fish to PAHs and \( \betaNF \) should increase PAH metabolism and excretion in vivo, whereas coexposure to PAHs and PBO should decrease PAH metabolism and excretion.

**MATERIALS AND METHODS**

**Experimental design**

Juvenile or embryonic rainbow trout were exposed to either waterborne retene or phenanthrene, or to retene or phenanthrene in combination with a CYP1A inducer (\( \betaNF \)) or inhibitor (PBO) to determine the effect of modulated CYP1A activity on PAH pharmacokinetics and toxicity. Each experiment consisted of nine treatment groups, including methanol (MeOH; solvent control), \( \betaNF \), PBO, retene, phenanthrene, retene with \( \betaNF \), retene with PBO, phenanthrene with \( \betaNF \), and phenanthrene with PBO. Juvenile trout were exposed to nominal waterborne concentrations of \( 3.67 \times 10^{-3} \) mmol/L (10 \( \mu \)g/L) \( \betaNF \), \( 5.9 \times 10^{-3} \) mmol/L (2000 \( \mu \)g/L) PBO, \( 4.27 \times 10^{-4} \) mmol/L (100 \( \mu \)g/L) retene, or \( 5.61 \times 10^{-4} \) mmol/L (100 \( \mu \)g/L) phenanthrene. Nominal chemical concentrations in the ELS treatments were \( 3.67 \times 10^{-3} \) mmol/L (10 \( \mu \)g/L) \( \betaNF \), \( 1.0 \times 10^{-3} \) mmol/L (338 \( \mu \)g/L) PBO, \( 4.27 \times 10^{-4} \) mmol/L (100 \( \mu \)g/L) retene, and \( 2.81 \times 10^{-3} \) mmol/L (500 \( \mu \)g/L) phenanthrene.

Retene and phenanthrene exposure concentrations in the two experiments were selected based on the concentrations needed for maximal ethoxyresorufin-\( O \)-deethylase (EROD) induction in juvenile fish [15; retene] and the concentrations needed to cause a high prevalence of BSD symptoms in embryos and larvae [16]. For the ELS experiment, phenanthrene exposure concentrations were higher than in the pharmacokinetic experiment to ensure that symptoms of toxicity would be observed. The concentration of \( \betaNF \) used in both experiments was on the linear portion on an exposure response curve for EROD induction in juvenile trout [23]; above this concentration the curve reached a plateau. Exposure concentrations for PBO in the juvenile experiment were adapted from Stuthridge et al. [24] and reduced in the ELS experiment to avoid killing the test fish.

Chemical concentrations for coexposures were the same as those used in single chemical exposures for each respective chemical. Juveniles were exposed for 96 h, whereas ELSs were exposed for 22 d. Three replicates of all treatments were conducted in both experiments, and the entire pharmacokinetic
study with juvenile trout was repeated to confirm significant differences.

**Chemicals**

Retene (98% purity) was obtained from ICN Biomedicals (Costa Mesa, CA, USA), phenanthrene and PBO (98% purity) were from Aldrich Chemical (Milwaukee, WI, USA), and β-NF (98% purity) was from Acros Chemical (Fair Lawn, NJ, USA). All chemicals were carried in high-performance liquid chromatography (HPLC)-grade MeOH (Fisher Scientific, Ottawa, ON, Canada).

The water for all aspects of acclimatization and chemical exposure was Kingston (ON, Canada) municipal water from Lake Ontario that was filtered, and then dechlorinated in the laboratory by charcoal filtration. Water hardness, conductivity, and pH were 135 mg/L as CaCO₃, 300 μS/cm, and 7.8, respectively.

**Maintenance of ELSs**

Juvenile rainbow trout (~1–3 g) were acquired from Rainbow Springs Trout Hatchery (Thamesford, ON, Canada) and acclimated for at least one week at 15°C on a 16:8 h light:dark photocycle. Dissolved oxygen and temperature were monitored daily. Rainbow trout eggs (Rainbow Springs Trout Hatchery) were acquired at the eyed-egg stage, and maintained at 10 ± 1°C (mean ± standard deviation) in dechlorinated water for at least 24 h on a 24-h dark photocycle before chemical exposure.

**Juvenile rainbow trout exposure**

Juvenile trout were exposed in 10 L of water in 20-L covered black plastic buckets lined with clear, food-grade polyethylene bags (Apache Plastic, Burlington, ON, Canada). Each bucket contained five 1- to 3-g fish, for a loading density of approximately 1 g/L. Photoperiod was maintained at 16:8 h light:dark, and temperature was maintained at 15 ± 1°C. Dissolved oxygen (8.0 ± 1.5 mg/L) and pH (7.5 ± 0.5) were monitored daily before water changeover. At 96 h, fish were sacrificed and gallbladders and livers were placed in separate 1.0-ml cryovials (Fisher Scientific), quick-frozen in liquid nitrogen, and stored at −80°C. Fish carcasses were stored individually at −20°C.

**Rainbow trout egg exposure**

For each treatment replicate, 50 eyed eggs were held at 10 ± 1°C in 7 L of aerated water in glass aquaria on a 24-h dark cycle until swim-up, when the fish were sampled. Dissolved oxygen (11 ± 0.25 mg/L) and pH (8.25 ± 0.25) were monitored daily. Sac fry were scored for BSD symptoms after 12 d (hatch) and 22 d (swim-up) of chemical exposure. Mortality was recorded daily and dead eggs or sac fry were removed and discarded.

**Assay for EROD activity and protein concentration**

Tissue S-9 fractions were prepared from livers sampled for EROD assays, frozen in liquid nitrogen, and stored at −80°C [13]. The extent of CYP1A induction was estimated by a kinetic assay of EROD activity in S-9 fractions as described by Fragoso et al. [13]. The fluorescence of the product, resorufin, was measured at excitation–emission wavelengths of 490 and 580 nm, respectively, with a Spectra Max Gemini microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). The S-9 protein concentrations were measured against a standard curve of bovine serum albumin with the BIORAD colorimetric assay (BIORAD Laboratories, Hercules, CA, USA) with a Spectra Max Plus microplate spectrophotometer (Molecular Devices). Activity as fluorescence units per minute was converted to specific molar activity (pmoles resorufin/min/mg protein) by reference to a standard curve of resorufin included with each microplate, and by dividing by the protein concentration of the S-9 fraction.

**PAH extraction**

Juvenile body and liver tissue and whole sac fry were homogenized in 5 ml/g, 100 ml/g, and 30 ml/g cyclohexane: chloroform (3:1, v/v) respectively. Homogenates were sonicated for 25 min below 30°C, after which 1 ml was eluted through an Isolute Fl florisil solid phase extraction cartridge (International Sorbent Technologies, Chromatographic Specialties, Brockville, ON, Canada) at a flow rate of 0.1 ml/min. The cartridge was rinsed with an additional 3 ml of solvent. The total eluant (4 ml) comprised a sample for analysis, and the percent recovery for this method was 82 ± 9%.

**PAH metabolite extraction**

Tissue samples were placed in HPLC-grade MeOH at a dilution of 7 ml/g for body tissue, and 15 ml/g for liver and sac-fry tissue. Tissues were homogenized and sonicated for 25 min below 30°C, after which the homogenate was forced through a 0.45-μm nylon filter (Nalgene, Fisher Scientific) and the filtrate was passed through a 1-ml C18 cartridge (International Sorbent Technologies). Bile was transferred into 7-ml glass scintillation vials (Fisher Scientific), weighed, and diluted 2,500 times (w/v) with a 1:1 (v/v) water:ethanol solution (adapted from Fragoso et al. [7]).

**Fluorescence analysis**

Fluorescence analysis of a 3-ml aliquot of each sample was measured in a 1-cm quartz cuvette (NSG Precision Cells, Farmingdale, NY, USA) with a Quanta-Master fluorescence spectrometer (PTI, London, ON, Canada) at a 2-nm slit width. Metabolite emission spectra were collected at a 254-nm excitation wavelength. Concentrations of parent compounds were determined with synchronous fluorescence scanning [14], with 50- and 51-nm offsets for retene and phenanthrene, respectively. In both cases, the concentrations were determined by measuring the areas under the spectra and comparing these areas to a linear standard curve comparing retene concentrations to the area under the curve (eight points per curve; r > 0.99).

**Statistical analyses**

The EROD activity, PAH tissue concentration, toxicity, and mortality data were log transformed and compared by using analysis of variance followed by a pairwise Student–Newman–Keuls test (SigmaStat Ver 1.0, Jandel Scientific, Chicago, IL, USA; p < 0.05). Log transformations were needed to correct for the nonhomogenous variance typical of data showing more than a 10-fold variation in average values [23], as indicated by tests for normality (SigmaStat, Jandel Scientific).

**RESULTS**

**Phenanthrene**

Parent phenanthrene exposure did not increase EROD activity (Fig. 2). The EROD activities among the PBO, phen-
Figure 2. Rainbow trout hepatic ethoxysresorain- O-deethylase (EROD) activity after a 96-h exposure to methanol (MeOH), β-naphthoflavone (bNF), piperonyl butoxide (PBO), phenanthrene (PHN), retene (RET), or to combinations of these chemicals. Treatments sharing the same letters were not significantly different from each other (p > 0.05). Error bars represent 95% confidence limits. The values presented within the treatment bars represent the total number of samples across all replicates.


table

Table 1. Mean (±95% confidence limits [CL]) tissue polycyclic aromatic hydrocarbon (PAH) concentrations (mg PAH/g wet wt tissue)*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Juvenile body</th>
<th>Juvenile liver</th>
<th>Sac fry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>33.8 (36.4)</td>
<td>0.226 (0.232)</td>
<td>316 (243)</td>
</tr>
<tr>
<td>Phenanthrene–bNF</td>
<td>0.661 (0.930)</td>
<td>&lt;LOD</td>
<td>105 (81)</td>
</tr>
<tr>
<td>Phenanthrene–PBO</td>
<td>50.1 (35.5)</td>
<td>0.117 (0.085)</td>
<td>338 (261)</td>
</tr>
<tr>
<td>Retene</td>
<td>0.117 (0.26)</td>
<td>&lt;LOD</td>
<td>&lt;0.54</td>
</tr>
<tr>
<td>Retene–bNF</td>
<td>0.170 (0.182)</td>
<td>&lt;LOD</td>
<td>&lt;0.54</td>
</tr>
<tr>
<td>Retene–PBO</td>
<td>4.57 (4.91)</td>
<td>&lt;LOQ</td>
<td>&lt;0.54–&lt;LOQ</td>
</tr>
</tbody>
</table>

* bNF = β-naphthoflavone; PBO = piperonyl butoxide; LOD = limit of detection; LOQ = limit of quantification.

a A denotes significant difference from single compound exposure (p < 0.05).
increased significantly above MeOH control levels (Table 2). Increases in the prevalence of BSD symptoms observed in the coexposure treatments were accompanied by a significant increase in ELS mortality (Fig. 4B and Table 2).

Treatment controls

Exposure of fish to βNF or PBO, alone or in combination, significantly induced EROD activity above that of MeOH control fish (Fig. 2).

Early life stages of trout exposed to PBO alone did not show increased mortality (Fig. 4) or toxicity (Table 2) above rates observed for MeOH control fish. The ELS exposure to βNF also did not increase mortality above rates for MeOH control levels, but at the end of the experiment, βNF-exposed fish had a significantly higher prevalence of yolk-sac edema, craniofacial abnormalities, and hemorrhaging in the yolk sac, head, and eye than observed in control fish (Table 2).

DISCUSSION

The results of these experiments support a role for CYP1A metabolism in the excretion and toxicity of phenanthrene and retene. Metabolism was required for biliary PAH excretion, because neither parent phenanthrene nor retene was observed in the bile (Fig. 3B and E). In the absence of induced CYP1A activity, examination of the results from the phenanthrene tests suggested that PAH metabolism was mediated by endogenous CYP1A activity. Inhibition of endogenous activity decreased the excretion of biliary PAH metabolites (Fig. 3B), and in some instances resulted in lethargy and loss of equilibrium in juvenile fish. For phenanthrene-exposed fish, induction of CYP1A (Fig. 2) was accompanied by decreased tissue phenanthrene concentrations (Table 1), increased phenanthrene metabolite concentrations in juvenile liver tissue and bile and in sac-fry tissue (Fig. 3A to C), and increased ELS toxicity and mortality (Fig. 4A and Table 2). Conversely, inhibition of CYP1A by PBO increased tissue retene concentrations (Table 1) and altered the proportions of the retene metabolites, generating a distinct set of tissue metabolites (Fig. 3D and F) that were excreted in the bile (Fig. 3E); these were presumed to be primary metabolites. Altered retene metabolism was accompanied by increased toxicity and mortality to trout ELSs (Fig. 4B and Table 2), suggesting that it was not the absolute rate of PAH metabolism that mediated toxicity, but rather the abundance and persistence of specific metabolites.

Phenanthrene metabolism and toxicity

Endogenous CYP1A activity was not sufficient to prevent phenanthrene accumulation in hepatic and extrahepatic tissues (Table 1). With PBO-mediated inhibition of endogenous activity, phenanthrene tissue concentrations increased slightly (Table 1), the relative concentration of biliary phenanthrene metabolites decreased (Fig. 3B), and more than 70% of juvenile fish displayed a loss of equilibrium or lethargy, both of which are symptoms of narcosis. None of the juvenile trout exposed to phenanthrene alone displayed signs of narcosis, although narcosis was evident in larval trout at higher exposure concentrations. Therefore, decreasing the rate of endogenous phenanthrene metabolism and excretion apparently has biological consequences. After CYP1A induction with βNF coexposure, phenanthrene metabolism and excretion were greatly increased (Fig. 3A and B and Table 1). This suggested that the long half-life [17] and accumulation of phenanthrene in trout tissues (Table 1) was a consequence of low AhR binding affinity [25] and consequent inability to induce CYP1A activity [8], and not because of a low affinity of the CYP1A enzyme for phenanthrene as a substrate.

The increased conversion of parent phenanthrene to metabolites with βNF coexposure (Fig. 3C and Table 1) resulted...
in a definitive increase in phenanthrene toxicity and mortality to trout ELSs (Table 2 and Fig. 4A). The increase in mortality may be a consequence of either the higher rate of conversion and abundance of toxic phenanthrene metabolites (Fig. 3C), or of oxidative stress resulting from a higher rate of metabolism of the phenanthrene substrate [19,15].

Phenanthrene toxicity did not decrease with CYP1A inhibition (Table 2), as might be anticipated if phenanthrene toxicity was the consequence of endogenous metabolism to toxic intermediates. Sac fry in the phenanthrene and phenanthrene–PBO treatments exhibited lethargy, and had a significant increase in craniofacial abnormalities and yolk-sac edema relative to solvent control fish (Table 2). Phenanthrene is directly cytotoxic to rainbow trout cells through accumulation in, and disruption of, the membrane [26]. Therefore phenanthrene, in the absence of an inducer, may cause toxicity through narcosis, because edema would be consistent with the cell death and membrane deterioration associated with narcotic membrane disruption [2]. Craniofacial abnormalities could be simply jaw gape resulting from arrested development or decreased perfusion [27]. Relative to fish in the phenanthrene or solvent control treatments, the significant increase in mortality observed in ELSs coexposed with phenanthrene and a CYP1A inhibitor (Fig. 4A and Table 2) supports the interpretation of narcosis toxicity.

Retene metabolism and toxicity

Increased concentrations of tissue retene after PBO coexposure (Table 1) indicated that retene metabolism and excretion were inhibited, despite the apparent increases in EROD activity (Fig. 2). A similar increase in activity was observed after intraperitoneal injection of retene and isosafrol, a PBO analogue [28]. A follow up EROD assay with α-naphthoflavone (αNF), a CYP1A inhibitor, demonstrated that the CYP1A activity induced by PBO could be competitively inhibited in vitro (data not shown). Therefore, CYP1A enzyme activity was likely inhibited in vivo, yet functional in the in vitro EROD assay, as indicated by the high activity that could be inhibited by αNF. The apparent increase in hepatic retene concentrations

Table 2. Prevalence of blue-sac disease (BSD) symptoms in early life stages of rainbow trout exposed to polycyclic aromatic hydrocarbons and cytochrome P4501A modulators

<table>
<thead>
<tr>
<th></th>
<th>MeOH</th>
<th>βNF</th>
<th>PBO</th>
<th>PHEN</th>
<th>PHEN–βNF</th>
<th>PHEN–PBO</th>
<th>RET</th>
<th>RET–βNF</th>
<th>RET–PBO</th>
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<tr>
<td>n At start</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>n Hatched and alive at 12 days</td>
<td>41</td>
<td>41</td>
<td>39</td>
<td>28</td>
<td>0</td>
<td>18</td>
<td>43</td>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>n At end</td>
<td>41</td>
<td>37</td>
<td>40</td>
<td>32</td>
<td>0</td>
<td>26</td>
<td>40</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>% Alive at end</td>
<td>82</td>
<td>74</td>
<td>80</td>
<td>64</td>
<td>52AB</td>
<td>80</td>
<td>58A</td>
<td>28AB</td>
<td></td>
</tr>
<tr>
<td>% Hatched and alive at 12 d</td>
<td>80</td>
<td>55</td>
<td>53</td>
<td>56A</td>
<td>0AB</td>
<td>36AB</td>
<td>86</td>
<td>86</td>
<td>78</td>
</tr>
<tr>
<td>n Normal at hatch</td>
<td>35</td>
<td>29</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>22</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>% Normal hatch</td>
<td>85</td>
<td>71</td>
<td>79</td>
<td>0</td>
<td>0</td>
<td>44</td>
<td>51</td>
<td>33</td>
<td>33</td>
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<tr>
<td>% Abnormal at hatch</td>
<td>15</td>
<td>29</td>
<td>21</td>
<td>100A</td>
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<td>56A</td>
<td>49</td>
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<td>n Normal at end</td>
<td>37</td>
<td>11</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>1</td>
<td>0</td>
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<tr>
<td>% Normal at end</td>
<td>90</td>
<td>30</td>
<td>78</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>3</td>
<td>0</td>
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<tr>
<td>% of Sac fry remaining at end with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Head or eye hemorrhaging</td>
<td>0</td>
<td>32A</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>23A</td>
<td>48</td>
<td>79AB</td>
<td></td>
</tr>
<tr>
<td>Yolk-sac hemorrhaging</td>
<td>2</td>
<td>16A</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>6</td>
<td>34A</td>
<td>26A</td>
<td></td>
</tr>
<tr>
<td>Craniofacial abnormalities</td>
<td>0</td>
<td>34A</td>
<td>2</td>
<td>100A</td>
<td>96A</td>
<td>5A</td>
<td>91AB</td>
<td>87AB</td>
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<tr>
<td>Yolk-sac edema</td>
<td>0</td>
<td>25A</td>
<td>2</td>
<td>9A</td>
<td>11A</td>
<td>23A</td>
<td>61AB</td>
<td>91AB</td>
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<tr>
<td>Pericardial edema</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>41AB</td>
<td>34AB</td>
<td></td>
</tr>
<tr>
<td>% Abnormal at end</td>
<td>10</td>
<td>70A</td>
<td>23A</td>
<td>100A</td>
<td>100A</td>
<td>53A</td>
<td>97A</td>
<td>100A</td>
<td></td>
</tr>
</tbody>
</table>

*MeOH = methanol; βNF = β-naphthoflavone; PBO = piperonyl butoxide; PHEN = phenanthrene; RET = retene.

A denotes significant difference from MeOH control (p < 0.05); B denotes significant difference from single compound exposure (p < 0.05).

Totals include non-BSD symptoms, and represent percentage of fish with one or more abnormalities.
in PBO coexposed fish (Table 1) may be the cause of the increased CYP1A induction relative to fish exposed to retene or PBO alone [28]. By inhibiting CYP1A activity and retene excretion in vivo, PBO may have indirectly increased CYP1A activity by causing the retention of retene.

Despite PBO inhibition, a distinct set of retene metabolites was produced and excreted by juvenile trout (Fig. 3D and E). The spectrum of these metabolites was similar to that first identified as biliary parent retene [7]. However, further studies have determined that parent retene is not present in bile [29]. This metabolite spectrum seems to be the result of preferential metabolite formation, because corresponding shoulders are present in the broad retene metabolite spectrum (Fig. 3E). The distinct emission spectrum suggests that the metabolites are not hydroxylated or conjugated retene metabolites, and are likely initial phase I metabolites. This interpretation is congruent with their increased excretion as CYP1A activity increases [7].

Two explanations are possible for the accumulation of the distinct retene metabolites. The PBO forms two different complexes dependent on the state of the CYP heme. The binding of the PBO metabolite–intermediate (MI) complex with the ferrous heme is not readily displaced by alternate CYP substrates [22]. However, the PBO MI bound to the ferrous heme state can be displaced by hydrophobic CYP substrates, restoring oxidative metabolic activity [22]. Therefore, the structure and hydrophobicity of retene may be sufficient to outcompete ferric-bound PBO MI complexes and undergo an initial oxygenation cycle. However, the resulting metabolite structure may lack the hydrophobicity or CYP binding affinity to undergo subsequent MI displacement reactions and metabolism. Consequently, the initial retene metabolites would be the only metabolites produced and subsequently excreted. The MI complex was dissociated in the EROD assay, as reflected by the high EROD activity (Fig. 2), and despite the increased retene tissue concentrations (Table 1) providing evidence for in vivo complexation. However, the EROD data do not provide any information regarding the state of the heme–MI complex in vivo because the ferrous–ferrous state is altered by both the addition of reduced nicotinamide adenine dinucleotide phosphate and the fluorescence wavelengths used in the EROD assay.

Alternatively, the distinct metabolites could be the result of metabolic differences due to different isoforms of CYP proteins. In addition to the CYP1A1 isofrom, trout produce a second isofrom, CYP1A3 [31,32]. Preliminary research indicates that the two isoforms do not differ in substrate affinity or metabolic products [33]. However, differences in \(^{13}C\) retene metabolism between PBO- and \(\beta\)NF-induced trout [30,34], and between \(\beta\)NF and isosafrole (a PBO analog) in EROD, CYP1A protein, and conjugation enzyme induction [35], support the hypothesis that two different CYP1A xenobiotic response elements [31], and two AhR isoforms play different roles in CYP1A gene activation [36]. Although proposed to occur through an alternate mechanism, evidence also has been gathered for independent CYP1A1 and CYP1A3 mRNA induction [37], with CYP1A protein and EROD activity increases correlating only with CYP1A1 mRNA [38].

Compared to fish exposed to retene alone, retene coexposure with \(\beta\)NF decreased CYP1A activity (Fig. 2), as well as retene metabolism (Table 1) and excretion (Fig. 3D and E). The decrease in CYP1A activity was unexpected, because these two compounds cause additive EROD activities after waterborne exposure or intraperitoneal injection [11,28]. The decrease in retene metabolism may result from \(\beta\)NF competition for CYP1A metabolic sites, potentially coupled with the decrease in CYP1A induction.

Retene toxicity to trout ELSs has been characterized previously [16]. For sac fry coexposed to retene and PBO, tissue concentrations of retene increased above detection limits (Table 1), but did not approach the molar concentrations associated with narcosis (2–8 mmol/kg [18]). Additive toxicity of retene and PBO was unlikely, because PBO alone did not cause the BSD symptoms typical of retene toxicity, and the prevalence of BSD symptoms increased significantly with retene and PBO coexposure (Table 2). The changes in retene metabolism suggest that toxicity was not mediated by the absolute rate of retene metabolism and excretion, but rather by the abundance or persistence of the metabolites. Whether this mechanism is due to direct metabolite toxicity, or to the toxicity associated with oxidative stress [15,19], pathology coincides with the site of CYP1A induction [39,40], and follows the pattern of AhR binding and membrane deterioration [41].

No indications were found from the juvenile or ELS assays to suggest that the increase in toxicity associated with retene and \(\beta\)NF coexposure was the consequence of altered retene metabolism or excretion. The prevalence of each of the BSD symptoms in the retene and \(\beta\)NF treatments suggests that the significant increase in toxicity observed with retene and \(\beta\)NF coexposure was the consequence of the additive toxicity of these two compounds. Whether the \(\beta\)NF metabolite was itself toxic, or if \(\beta\)NF metabolism contributed to another toxic mechanism, is not known.

**Importance of findings**

Phenanthrene does not accumulate to high concentrations in tissues of fish collected from contaminated sites [10], yet it accumulated in tissues of sac fry and juvenile trout exposed only to phenanthrene. Because phenanthrene from environmental sources (i.e., crude oil) is unlikely to occur in the absence of other CYP1A-inducing PAHs (e.g., benzo[a]pyrene), phenanthrene will likely be metabolized and excreted quickly, and bioaccumulation will be a poor indicator of phenanthrene exposure. Similarly, LC50s of pure phenanthrene will be poor predictors of environmental phenanthrene toxicity, because the mechanism of toxicity will differ in environmental circumstances when phenanthrene is part of complex mixtures containing CYP1A inducers. Increased CYP1A activity increases the toxicity of phenanthrene, likely by switching its mechanism of toxicity from nonspecific narcosis associated with high tissue concentrations of phenanthrene to a more specific syndrome of BSD. Examination of these data indicates that BSD is associated with reduced tissue concentrations, increased synthesis of metabolites, and increased rates of excretion.

Modulating CYP1A activity also changed retene toxicity, although in this case, the mechanism (BSD) appeared unchanged but the intensity varied with the apparent rate of excretion of initial metabolites. The increase in retene toxicity associated with the accumulation of a subset of metabolites suggests that the toxicity may be tied to specific stages of retene metabolism or to specific metabolites, and that the absolute rates of substrate metabolism and excretion do not correlate directly with toxicity.

For both phenanthrene and retene, the effect of a CYP1A inhibitor (PBO) and inducer (\(\beta\)NF) caused marked changes in metabolism and excretion, but the changes in their toxicities
were obscured somewhat by the chronic, interactive toxicities of PBO and βNF. However, the failure of the interactive effect of each compound to remain uniform across treatments supports the interpretation that the mechanism of toxicity was different among treatments. Future studies with compounds that reduce CYP1A activity by downregulating the CYP1A gene (e.g., β-estradiol) will help determine if the toxicity in the coexposure treatments was the result of PBO interactive effects.

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CYP1A activity in PAH metabolism and toxicity


