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Differential developmental toxicity of naphthoic acid isomers in medaka (*Oryzias latipes*) embryos

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are widespread persistent pollutants that readily undergo biotic and abiotic conversion to numerous transformation products in rivers, lakes and estuarine sediments. Here we characterize the developmental toxicity of four PAH transformation products each structural isomers of hydroxynaphthoic acid: 1H2NA, 2H1NA, 2H3NA, and 6H2NA. Medaka fish (*Oryzias latipes*) embryos and eleutheroembryos were used to determine toxicity. A 96-well micro-plate format was used to establish a robust, statistically significant platform for assessment of early life stages. Individual naphthoic acid isomers demonstrated a rank order of toxicity with 1H2NA > 2H1NA > 2H3NA > 6H2NA being more toxic.

Abnormalities of circulatory system were most pronounced including pericardial edema and tube heart. To determine if HNA isomers were AhR ligands, spatial-temporal expression and activity of CYP1A was measured via in vivo EROD assessments. qPCR measurement of CYP1A induction proved different between isomers dosed at respective concentrations affecting 50% of exposed individuals (EC50s). In vitro, all ANH isomers transactivated mouse AhR using a medaka CYP1A promoter specific reporter assay. Circulatory abnormalities followed P450 induction and response was consistent with PAH toxicity. A 96-well micro-plates proved suitable as exposure chambers and provided statistically sound evaluations as well as efficient toxicity screens. Our results demonstrate the use of medaka embryos for toxicity analysis thereby achieving REACH objectives for the reduction of adult animal testing in toxicity evaluations.

Keywords

Naphthoic acid; PAH metabolites; Medaka; Embryo toxicity; Tube heart; EROD; Eleutheroembryos

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants found in soils, estuaries, and sediments. Most are lipophilic compounds that accumulate in the environment and pose a health threat to fishes and aquatic invertebrates. To date, extensive literature exists on effects of PAHs on adult or juvenile animals, but few studies have addressed effects of PAHs on embryos (Rhodes et al., 2005; Incardona et al., 2004). Lack of ecological data on embryonic toxicity of environmentally relevant compounds has complicated ecological risk assessment and management of PAH-polluted aquatic environments. Risk assessments are further complicated when possible PAH transformation products (biotic and abiotic) are considered.

There is little information available regarding environmental fate, bioavailability, and mechanisms of toxicity for most PAH transformation products. Characterizing the toxicity of these environmental contaminants can be important in some ecosystems where relatively inert parent compounds are metabolized by biotic and abiotic factors with resultant additions of reactive functional groups (Parikha et al., 2004). In this study we have characterized the toxicity of four common PAH metabolites found in PAH contaminated sediments (Parikha et al., 2004). Compounds of interest are bacterial metabolites of phenanthrene, anthracene, and 2-methyl-naphthalene including: 1-hydroxy-2-naphthoic acid (1H2NA), 2-hydroxy-1-naphthoic acid (2H1NA), 2-hydroxy-3-naphthoic acid (2H3NA), and 6-hydroxy-2-naphthoic acid (6H2NA), respectively (Rogoff and Wender, 1957; Cerniglia et al., 1984; Guerin and Jones, 1988; Menn et al., 1993; Machate et al., 1997). These compounds are structural isomers and represent a unique perspective on structure function relationships with regard to observed toxicities.

The objective of this study was to determine embryo toxicity for selected PAH metabolites. Use of medaka (*Oryzias latipes*) embryos and eleutheroembryos in developmental toxicity is well established (Solomon and Weis, 1979; Weis and Weis, 1987; Marty et al., 1990; Oxendine et al., 2006a,b). Medaka embryos have many advantages for establishing toxicant effects profiles. Stages of normal embryo development have been precisely determined and illustrated (Iwamatsu, 1994). Their transparent chorion enables direct observation, with standard stereoscopic dissecting microscopes, of all stages of embryonic development. Lack of pigments in embryonic orange red medaka proves beneficial when identifying internal organ orientation, structure, and in vivo function. These attributes guided use of medaka embryo and eleutheroembryo to determine the effect profiles of each naphthoic acid (NA) metabolite.

Preliminary toxicity studies of the four NA isomers, 1H2NA, 2H1NA, 2H3NA, and 6H2NA, in our lab showed evidence of differential embryo toxicity leading to specific morbidity such as tube heart formation. Tube heart (TH) is a condition in which the formation of chambers via septation is arrested. The resultant heart is an elongated tube, usually in an expanded pericardial cavity (i.e. edematous) and showing irregular contraction (Marty et al., 1990; Incardona et al., 2004). TH is associated with blue sac syndrome (Hornung et al., 1999; Brinkworth et al., 2003), a condition found in freshwater fish populations exposed to planar PAHs activating aryl hydrocarbon receptor (AhR), expression of CYP1A, and possible

generation of reactive oxygen species (Hahn et al., 1997; Safe et al., 2000). However the four NA isomers used in this study are not typical AhR ligands, which raises an interesting question regarding the role of the AhR in TH formation.

To establish a robust statistically significant platform for assessment of ELS toxicity to environmentally relevant compounds, we used a 96-well micro-plate format. This format improves statistical power, widens our scope of inference, and reduces chance of embryo mortality due direct contact with other dead embryos (Oxendine et al., 2006a,b). A 96-well micro-plates have been successfully used as exposure chambers to test copepod *Amphiascus* sp. life-cycle toxicity (Chandler et al., 2004; Cary et al., 2004) and have American society for testing and materials certification (Active Standard #E2317-04) for this use. Additionally, in an earlier study we demonstrated ethanol induced embryo toxicity using a 96-well format (Oxendine et al., 2006b). We thus asked if this format could be used to test embryo toxicity of medaka to our compounds of interest.

2. Materials and methods

2.1. Embryo collection

Both orange red (OR) and see-through (STII) (Wakamatsu et al., 2001) medaka embryos and eleutheroembryos were used in this study. Embryos were collected from our colony maintained at the Duke Forest Aquatic Research Facility. Brood fish used for egg production were 4–6 months old, on a 16 and 8 light/dark cycle, at 25 °C and fed Otohime B1 (Otohime Beta, Nisshin Feed Co. Ltd., Tokyo) with live brine shrimp nauplii (*Artemia* sp.) as supplements. To achieve a sufficient number of synchronized-aged embryonated eggs, all eggs were collected at 3–5 h after initiation of spawning. Attachment filaments of egg masses were disrupted by manipulation with moistened finger tips and individual embryonated eggs were washed in 20 ppm Instant Ocean (Marineland, Moorpark, CA). Embryos were examined under a dissecting microscope, and abnormal or dead individuals were discarded. This process yielded 800–900, multicellular OR embryos within a 3–4-h range (stages 3–8) of development and these were used for each assay.

2.2. Embryonic assessment

Glass coated flat bottom 96-well micro-plates (SUN-Sri, Duluth, GA) were used as experimental exposure vessels. Individual embryos (stages 3–8, Iwamatsu, 1994) were loaded into each well of the micro-plate using forceps. Stock solutions of DMSO vehicle and pure compound were prepared so that each well was spiked with 3 µl of test solution in 297 µl of embryo rearing medium (ERM) (Yamamoto, 1975) to achieve a 1% vehicle concentration. ERM/compound solution was carefully changed daily to maintain appropriate DO and chemical concentrations. Chemical concentration remained constant throughout exposure period. Plates were covered with a breathable membrane (Cole-Parmer, Vernon Hills, Illinois) to limit volatilization of compound and evaporation of water. Dissolved oxygen (DO) was monitored in test solutions when changing solutions using a YSI DO Meter, Model 57 (Yellow Springs Instrument Co., Inc., Ohio). Spent test solution DO was at least greater than 6.5 mg/l or approximately 85% saturation throughout experimental exposures. Incubation temperature was maintained at 26.0 °C with a 16/8 h light/dark cycle.

Five treatment levels were selected for each metabolite from an initial range-finding study that employed three independent experimental replicates to determine LC50 values. Treatment levels were as follows 1H2NA: 5, 10, 20, 30, and 40 μ M; 2H1NA: 10, 20, 30, 40, and 50 μ M; 2H3NA: 30, 40, 50, 60, and 70 μ M; 6H2NA: 100, 200, and 300 μ M. A DMSO control was analyzed concurrently with the five experimental treatments for each metabolite. Each treatment level consisted of an entire 96-well plate with one embryo per well. This format provided a total of 576 independent individual, randomly selected embryos per metabolite. Further randomization was insured by plate and well selection achieving a completely randomized design.

Several end points were concurrently assessed during the exposures. These included: mortality, embryonic development, abnormal heart index, frequency of specifically selected teratogenic effects, behavioral abnormalities, heartbeats per minute, and craniofacial index. Experimental endpoints are summarized below.

2.3. Mortality

Number of dead embryos per treatment group was used to generate LC50 values for each metabolite. Mortality was defined by loss of heartbeat and absence of blood cell circulation. Dead embryos were recorded on a master data sheet that identified well location and then each was removed from chamber with disposable pipette.

2.4. Embryonic development assessment

The medaka specific scoring system, first described by Shi and Faustman (1989) then employed by Marty et al. (1990) and Skinner et al. (1999), was used throughout this study. Embryos were assessed at four time points during development (stages 27, 31, 36, and 2 days after stage 36, i.e., eleutheroembryos). The procedure for embryonic staging was that of Iwamatsu (1994). Thirteen developmental characteristics including cranial (eye, brain, head) development, circulatory system characteristics, relative caudal length (referenced to position of caudal tip over brain sections), pigmentation, liver location/shape, gall bladder size/color, and hatching were scored. Twenty individual embryos were randomly selected from each treatment group and monitored for each of the four time points. For visualization, embryos were sequentially removed from respective wells with a disposable pipette and placed onto a deep well glass slide (Southern Scientific, McKenzie, TN #S1897) for observation under dissecting microscope. Scores were summed for each individual embryo and averaged to generate a mean and standard deviation for each treatment group at each time point. Scores were only determined for live embryos. Embryos were considered viable if active heartbeat and circulation of pigmented blood cells was present in yolk sac vessels. Following individual embryos throughout the exposure provided a unique opportunity to identify sequential aspects of toxicity.

2.5. Abnormal heart index

Classification of developing heart occurred on day seven and utilized an index of 0–4, with 0 representing normal embryos and 4 being those embryos that had a narrow tube heart (TH) with no identifiable chambers. The illustration from Solomon and Weis (1979) was used to establish an index score for 15 randomly selected embryos. Abnormal heart development is

a sensitive end-point for various compounds, including AhR agonists and is a well described teratogenic effect of planar aromatics (Hill et al., 2004; Wolf, 1969). Embryos that scored 3 and 4 were classified as exhibiting tube heart and analyzed accordingly.

2.6. Frequency of teratogenic effects

Malformations included: TH, anisophthalmia (unequal eye size), bent caudal peduncle, pooled blood, microcephaly, pericardial edema (PE), and cranial edema. 15 randomly selected embryos from each treatment were assessed at days 5 and 7 post fertilization (dpf). All embryos were observed under a dissection microscope (Olympus SZH) without removal from test chamber (Oxendine et al., 2006b). To photographically record specific malformations some embryos were placed in a 100 mm by 15 mm Falcon dish (Becton Dickinson, NJ) and imaged at 10× magnification by use of a Nikon SMZ 1500 stereozoom microscope with Nikon DXM 1200 camera operated by Nikon E600 EclipseNet software (Nikon Inc. Torrance, CA).

2.7. Behavioral abnormalities

Behavioral abnormalities in eleutheroembryos included: lack of swim bladder inflation with attendant required movement, incomplete or partial hatching, and hatched but showing reduced response to stimuli, such as lying on bottom of well on day of hatch. These abnormalities were described by Marty et al. (1990) when investigating medaka embryo toxicity. Embryonic observations were extended to eleutheroembryonic stages and total body length and interocular distance (to .001 μM) were measured. At higher treatment levels, when affected embryos did not hatch, a random selection of hatched larvae was used for morphometric assessment to achieve similar sample sizes.

2.8. Heartbeats per minute

Heart rate was recorded for a random sample of 15 individual embryos in each treatment group on 3 and 5 dpf. A single plate was removed from the incubator at a time, immediately placed under a dissecting stereoscope (Olympus SZH, Olympus America, Melville, NY), and heart rates recorded. A glass pipette with nylon monofilament tip of 1 cm was used to position embryos inside each well providing a clear view of the heart. Each beat was counted for 30 s with aid of a digital laboratory timer and doubled to get a heart rate per minute. Embryos were not removed from the 96-well plate test chambers and time for analysis of each plate was identical for each treatment and metabolite. All measurements were randomized and made in triplicate.

2.9. Craniofacial index

One craniofacial developmental parameter, the relative convergence of embryonic eye development leading to cyclopia, has been successfully used as a sensitive endpoint for methylmercury exposures in developing *Fundulus heteroclitus* embryos (Weis and Weis, 1987). Our evaluation followed this procedure. Briefly, a six-part index of 0–6 is used with 0 being normal, 1–5 being degrees of convergence of eyes leading to cyclopia, and 6 to indicate no discernible cranial structure. Observations were made at 7 dpf for 15 randomly selected embryos per treatment group.

2.10. Eleutheroembryonic assessments

To further define toxicity of the four isomers, we performed 96-h LC50s with yolk sac fry (eleutheroembryos). Newly hatched OR medaka were used. These were obtained by incubating embryos in 100 mm by 15 mm Flacon dishes (Becton Dickinson, Franklin Lakes, NJ), containing ERM (Yamamoto, 1975). Eggs were washed and changed daily until hatch. Hatchlings were removed from dishes and larvae from a single day's collection of eggs were randomly selected for exposure. This technique resulted in larvae of 7–8 dpf and restricted from exogenous food. Eleutheroembryos were placed in 20 ml glass scintillation vial containing 14.85 ml of freshly prepared ERM and 150 μ l of compound in DMSO. Dose concentrations between 0.1 and 10 μ M (1H2NA, 2H1NA, 2H3NA) and 0.1–500 μ M (6H2NA) were tested for each HNA isomer. All doses were conducted in triplicate with each exposure consisting of ~250 individual larvae of the same age class brood-stock. Mortalities (absences of heartbeat) were detected using the stereozoom dissection microscope. Dead larvae were removed with disposable pipette at each 24 h inspection.

2.11. PH assessments

Since compounds of interest are acids, pH was evaluated for putative effects on embryo and larval toxicity. To determine pH in ERM and stock solutions at final exposure concentrations, a calibrated Fisher Scientific (Pittsburgh, PA) Accumet AB15 pH meter and litmus paper color change strips (colorpHast, Gibbstown, NJ) were used. After making final working solution, pH was established. The difference between our initial reading and our final reading provided an estimate of resultant pH change with preparation of final exposure solution from ERM stock.

To determine relative pH toxicity, larvae were collected as above and added to 15 ml ERM treatment in 20 ml scintillation vials. Range of treatment levels was pH 7.0, 6.0, 5.0, 4.0, and 3.0 with the stock of ERM used in each. The pH was adjusted from 6.6 to 7.0 using sodium bicarbonate and lowered to each respective pH level using HCl. A 150 μ l sample of DMSO was added to 14.85 ml of pH-adjusted ERM to achieve a similar assay vehicle concentration as described above. Individual vials were monitored (at 24 h intervals) for condition of individual organism and resultant mortalities were removed daily. At termination of 96 h exposure final pH was recorded. Two replicates were performed ($N = 250$ for each pH level). Two additional replicates were performed to test pH toxicity without DMSO.

2.12. Ethoxyresorufin-o-deethylase (EROD) in vivo

STII medaka embryos (5 dpf) were used to assess *in vivo* induction of EROD activity in liver and epithelium using a modification of Nacci et al. (1998) and Meyer et al. (2002) first used on urinary bladders of *Fundulus heteroclitus*. Embryos (~200/HNA isomer) were enzymatically dechorionated using a 0.5 mg/ml protease (Sigma-Aldrich, St. Louis, MO #P-8811) and ERM solution (Smithberg, 1966; Villalobos et al., 2000) up to 3 h. During incubation, embryos were continuously inspected and solution was changed each hour. Upon hatching, single dechorionated embryos were removed from the dish, washed in a 5 mg/ml bovine albumin (Sigma-Aldrich, St. Louis, MO #A-8022) in ERM solution, and transferred to a clean, sterile 100 mm by 15 mm Falcon dish in clean ERM. To effectively normalize each response, exposures were conducted at respective embryo LC₅₀ values or the

eleutheroembryo LC₅₀ value for 6H2NA. After 48 h NA isomer exposure, 2 μ M (final concentration) ethoxyresorufin (Sigma) in DMSO was added to each vial, incubated for 10 min and then washed in fresh ERM. Embryos were subsequently anesthetized with MS-222 (Sigma) and transferred to a deep well glass slide for image analysis. Images were acquired with a Zeiss Axioskop fluorescence compound microscope (Carl Zeiss Inc., Thornwood, NY) and a rhodamine-red filter set with xenon light source (Carl Zeiss Inc., Thornwood, NY #XBO75). All images were recorded using a Photometrics Coolsnap AP FX digital camera (Roper Scientific, Duluth, GA) and IP-Lab Software version 3.5 (Scanalytics, Inc., Fairfax, VA) package.

2.13. CYP1A induction

Quantitative real time PCR (qPCR) was used to quantify relative induction of CYP1A between the four NA isomers. Chorionated OR embryos (5 dpf) were placed into 20 ml glass scintillation vials (5 embryos per vial, 3 replicates/treatment) containing 10 ml of ERM and NA isomers at their respective embryo LC₅₀ values or the larval LC₅₀ value for 6H2NA isomer. Vehicle and 100 nM betanaphthoflavone (BNF)-exposed animals served as controls. After a 48 h exposure, embryos were snap frozen in liquid nitrogen and stored at -80 °C for RNA extraction.

RNA extraction and qPCR were performed on three sets of 5 pooled medaka embryos/treatment. Total RNA was isolated by the RNA-Bee (TelTest Inc., Friendswood, Texas) method followed by on-column-digestion with DNase according to manufacturer's instructions (Qiagen Inc., Valencia, CA, # 79254). RNA concentration and A_{260/280} ratio were verified using a NanoDrop[®] ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). RNA integrity was further verified using an Agilent 2100 Bioanalyzer.

First-strand cDNA was generated from 1.5 μ g total RNA with superscript reverse transcriptase (Invitrogen) and oligo dT(15) primers (500 μ g/ml; Promega) in 20 μ l reactions. Relative levels of CYP1A and β -actin transcripts were measured using real time PCR. The following medaka-specific primers were used for CYP1A (Accession ID: AY297923), forward primer 5'-ACA-TCGGCCTGAACCGAAATCCTA-3', reverse primer 5'-TGCTTCATTGTGAGCCCGTACTCT-3'; and β -actin (Accession ID: D89627), forward primer 5'-ACAACGGATCTGGCATGTGCAAAG-3', reverse primer 5'-AGGGCTGTGATCTCCTTCTGCATT-3'. CYP1A and β -actin cDNAs were PCR-amplified separately in triplicate using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Two microliters of cDNA was PCR amplified in a 25 μ l reaction volume using the QuantiTect SYBR Green PCR Kit (Qiagen). Real time PCR reaction conditions were: 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min. Formation of specific products was verified through melting curve analysis and electrophoresis. Quantification of gene expression was calculated as described in (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Foster City, CA). For each sample, the threshold cycle for reference (β -actin) amplification (Ct, β -actin) was subtracted from the threshold cycle for target amplification (Ct, CYP1A) to yield a Δ Ct. The mean or standard deviation of Δ Ct for DMSO-treated samples was subtracted from the mean or standard deviation of Δ Ct for HNA-treated samples to yield a mean and

standard deviation of C_t for each target. Fold induction relative to DMSO-treated organs and 95% confidence intervals were calculated using 2^{-C_t} .

2.14. AhR transactivation

The aryl hydrocarbon response element (AHRE)-driven luciferase reporter vector was constructed as follows. Primers were designed to amplify a partial fragment (1205 bp) of the medaka CYP1A promoter (936 bp upstream to 210 bp downstream of the transcriptional start site). This promoter sequence (named here as mf1Ap) was identified by mapping medaka CYP1A mRNA (GenBank Accession No. AY297923) to version 200406 of the medaka genome (<http://dolphin.lab.nig.ac.jp/medaka/>) (scaf-fold911:25996-29142), exported and searched manually for AHREs (5'-CACGCA/T-3'). This promoter sequence used for construction of the reporter contained 6 AHREs upstream of the transcriptional start site. mf1Ap was amplified from medaka liver genomic DNA using primers with incorporated 5'-MluI and 3'-SmaI restriction sites, and directionally cloned into pGL3-basic (Promega) vector (mf1Ap-Luc). Constructs were sequenced to verify correct sequence and orientation using pGL3-basic primers RV3 and GL2.

Mutant mouse Hepa-1c1c7 c12 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and used for all reporter assays. These cells have significantly reduced AHR levels (~10% of wild-type Hepa-1c17 cells) but normal ARNT levels (Pollenz et al., 1993; Zhang et al., 1996), resulting in minimal endogenous levels of basal and inducible CYP1A1/1A2 activity (Hankinson, 1979; Miller et al., 1983). c12 cells were maintained at 37 °C under 5% CO₂ in minimal essential media (MEM) (without ribonucleosides and deoxyribonucleosides) supplemented with 2 mM L-glutamine (Invitrogen), 10% heat-inactivated fetal bovine serum (FBS), 50 µg/ml gentamycin, 10 IU/ml penicillin, and 10 µg/ml streptomycin.

Cells were seeded at 4×10^4 cells/well in two 24-well plates. After 24 h, media was aspirated, cells were washed with phosphate-buffered saline (PBS), and 250 µl Opti-MEM® Reduced Serum Medium (Invitrogen) was added to each well. Plasmids 300 ng/well was combined with Lipofectamine™ 2000 (2 µl/well) (Invitrogen) per the manufacturer's instructions. Following a 20-min incubation, 100 µl of DNA containing 250 ng mf1Ap-Luc, 0.05 ng pRL-CMV, and 50 ng pSPORT-mAHR were added to each well and cells transfected for 5 h. *Renilla* luciferase (pRL-CMV) (Promega) was used for transfection normalizations. Control transformations were conducted with pGL-basic and/or empty pSPORT vectors. Following transfection, cells were washed with PBS and immediately dosed with HNAs for 18 h. For each chemical, treatment solutions were made up in MEM and 500 µl spiked media was aliquoted to four replicate wells per treatment. DMSO served as vehicle for all chemicals tested in this study, and the final DMSO concentration in vehicle controls and treatments was 0.5%. Following exposure cells were washed with PBS, lysed for 30 min with 100 µl 1 × Passive Lysis Buffer (Promega) and assayed for firefly and *Renilla* luciferase activity using a Dual Luciferase Assay kit (Promega) and TD 20/20 Luminometer (Turner Designs). Relative luciferase units (RLUs) were expressed as a ratio of firefly to *Renilla* luciferase units. RLU's were tested for significant differences using a two-way analysis of variance (ANOVA) general linear model (GLM) procedure (PROC

GLM) ($\alpha = 0.05$) with Bonferroni-based multiple comparisons in SAS v9.1 (SAS). A two-way GLM ANOVA was used to test for significant differences between cells transfected with empty vector or pSPORT-mAHR, as well as among treatment groups within each transfection regimen.

2.15. Statistical evaluations

Statistical assessment of data was designed to generate mutually accepted differences between treatment level groups of each NA isomer and control groups. Means and 95% CIs, of estimate-biased uncertainties were determined. Since a variety of endpoints are considered, it was necessary to employ a wide variety of parametric and non-parametric tests in order to establish statistical approaches to test HNA isomer toxicity. LC50 and EC50 with respective 95% CIs for each end point was determined using the Trimmed Spearman-Kärber Method 1.5 available from the USEPA Ecological Exposure Research Division (Hamilton et al., 1977).

The morphological score data generated from the embryonic development assessment was displayed similar to Shi and Faustman (1989) results with 95% CI of the mean development score. One-way ANOVA was used to generate statistically significant concentration-morphological score relationships. A modified development assessment model was employed generating a more complete statistical analysis to determine differences in score between concentrations and HNA isomers using analysis of covariance (ANCOVA) described by Skinner et al. (1999). A multiple linear regression model was used to test for differences in development rate (score over time) between each HNA isomer using control, 30 μ M, and 40 μ M treatments. In this assessment a full linear regression model was fitted with two-way interaction terms and subsequently assessed with a sum of squares (ESS) *F*-test to determine interaction term significance. Data were prepared so that time and concentration were treated as continuous variables and the three NA isomers (1H2NA, 2H1NA, 2H3NA) coded with two separate model parameters (Iso1 and Iso2). This resulted in a fully saturated model that contained four interaction terms (time: Iso1, time: Iso2, conc: Iso1, and conc: Iso2). To confirm that concentration and HNA isomer coefficients are significant in the presence of the other, we tested the significance of time in the presence of concentration and time in the presence of Iso1 and Iso2. Once established, slope coefficients were condensed to generate estimates of differences between developmental score over time while holding concentration constant.

Standard two-sample *t*-tests were used to find differences between each HNA isomer treatment concentration and control for total larval length, days to hatch, and heart rate data. All estimates for differences among treatment groups for each HNA isomer and estimates for differences between HNA isomer included a 95% CI of the reported group mean or mean difference. Embryos used for each toxicological/developmental assessments were selected from each plate at random using the with S-Plus 6.2 (Insightful, Seattle, WA) single uniform random number generator-statistical program. All statistical tests and assumption checks were made using S-plus 6.2 and SAS 9.1 (SAS Institute Inc., Cary, NC) software.

3. Results

3.1. Embryo assessments

The ability to follow individual embryos in the micro-plate format and view developmental stages through the transparent chorions were key design characteristics of this experiment. Table 1 shows embryo LC50 results and 95% CIs generated using the Trimmed Spearman-Kärber method. Each metabolite exhibited a significantly different LC50. Based on these values HNA isomer 1H2NA demonstrated the highest degree of lethality with an LC50 of 20.23 μ M (95% CI; 18.9, 21.6). The second most lethal metabolite was 2H1NA with a LC50 of 47.65 μ M (46.3, 49.0), more than two times the concentration of 1H2NA. The LC50 of 2H3NA was slightly greater than 2H1NA at 51.2 μ M (50.1, 52.3). A LC50 value for 6H2NA was not attainable. Although we increased the concentration of 6H2NA several fold, a lethal concentration was not observed within solubility limits. At concentrations, exceeding 300 μ M, embryos developed normally but died immediately after hatching. This partial hatch mortality proved variable during exploratory range finding analysis. No DMSO control mortalities occurred for 1H2NA and a single (1 of 15) DMSO control mortality was observed for 2H1NA and for 2H3NA, respectively.

Fig. 1 illustrates the embryonic development assessment results and 95% CIs of mean treatment time point scores for 1H2NA, 2H1NA, and 2H3NA. Mean development score increased over time in conjunction with embryo development, for each treatment level, and for each HNA isomer with exception of the 1H2NA 40 μ M treatment and 2H3NA 60 μ M and 70 μ M treatment levels. Sample variability of the development score proved very low for control and lower treatment levels while higher treatment levels had greater variability. Results of 1H2NA development assessment (Fig. 1a) show that reduced rate of development occurred between 96 and 144 h pf at 40 μ M and 144–192 h at 30 μ M treatment level, respectively. Embryo exposures for control, 5 μ M, 10 μ M, and 20 μ M treatment levels resulted in similar mean development score increases over time. The 1H2NA treatment level of 10 μ M (0.2, 1.5) was statistically significant from control (ANOVA, Tukey's multiple comparison procedure) as was 20 μ M, 30 μ M, and 40 μ M, 95% CI for the difference in mean scores (0.3, 1.5), (1.6, 2.9) and (4.5, 5.8). 2H1NA results (Fig. 1b) demonstrated a similar decrease in development score between 96 and 144 h but at a greater (50 μ M) treatment level. This treatment level was statistically significant from control (ANOVA, Tukey's multiple comparison procedure), 95% CI for the difference in mean scores (2.2, 3.0) as was 30 μ M (0.03, 0.9) and 40 μ M (0.3, 1.1). Mean score variability was small for all treatment levels and time points when compared to other metabolites. Results of 2H3NA development assessment (Fig. 1c) showed that control, 30 μ M, 40 μ M, and 50 μ M treatment levels mean scores were similar over all time points. The 2H3NA 60 μ M treatment gave a lower development score than other concentrations between 144 and 192 h pf with little change at 192 h pf. At 70 μ M, the mean development score increased until the 96 h time point at which point it remained consistently low (Fig. 1c). Score variability was greatest for the 70 μ M treatment level as indicated by wide confidence intervals of the mean score. This was in part due to the reduced number of surviving embryos available at 192 h ($n = 7$). Treatment levels 50 μ M, 60 μ M, and 70 μ M were statistically different from control

(ANOVA, Tukey's multiple comparison procedure) and 95% CI for the difference in mean scores (0.2, 1.3), (2.9, 4.0), and (8.0, 9.4).

A multiple linear regression model was used to test for differences in development rate (score over time) between each HNA isomer using control, 30 μ M, and 40 μ M treatments. A separate lines model proved to be most appropriate to test the claim that time and HNA isomer interaction coefficients were not equal to zero ($p < 0.001$) (Fig. 2). This regression model explains roughly 93% of the total variation in development scores. There was strong statistical significance that the concentration and specific HNA isomer had an effect on estimated development score after accounting for time. Using the fitted linear regression model parameters we demonstrated that a one-unit increase in time for 1H2NA had an accumulative effect on the average developmental score rate of 1.30 (95% CI 1.0, 1.6) from 2H1NA after accounting for concentration. A one-unit increase in time for 2H1NA had an accumulative effect on the average developmental rate of 0.15 (95% CI .09, 0.4) from 2H3NA, after accounting for concentration. The significance of interaction terms and fitted separate lines multiple linear regression model provided evidence that the three HNA isomers tested have significantly different effects on development rates at 30 μ M, and 40 μ M treatments regimes.

The unique ability to follow individual embryos from a large sample provided insight into individual developmental characteristics over time. Noticeable trends were observed in control embryos which retained a high developmental score for individuals throughout the duration of the experiment. Of those individuals exposed to HNA isomers, a significant dose-response was observed demonstrated by a downward trend in the overall index. This trend was most obvious at higher HNA isomer concentrations. Reduction in index values occurred predominantly later than 60 hpf suggesting early developmental aberrations were not identified. An alternative view is that HNA isomer specific toxicity occurs within a critical window during embryonic development (Oxendine et al., 2006a). In some instances individual embryos scored low between 0 and 60 hpf. These individuals usually retained a low index score throughout the duration of the exposure.

Table 2 reports a selected subset of EC50 values for TH, PE, pooled blood, delayed hatching, and partial hatching developmental toxicity endpoints. TH EC50 values showed a similar trend as LC50 values among the three HNA isomers in developing embryos, with 1H2NA being the lowest at 27.6 μ M followed by 2H1NA 49.3 μ M, then 2H3NA 52.6 μ M. EC50 values for PE were lower than respective LC50 values but still followed a similar increasing trend among compounds. Pooled blood EC50 values for 1H2NA and 2H3NA were very similar to those observed for TH. Trimmed Spearman-Kärber method could not generate an EC50 for 2H1NA pooled blood data because of non-monotonic dose-response. Similarly, an EC50 value could not be generated for one or more of the HNAs with delayed or partial hatch end points. Additional teratogenic or behavioral endpoints that were assessed included anisophthalmia, bent caudal penuncle, microcephaly, cranial edema, lack of swim bladder inflation, and reduced flight response. Slight anisophthalmia was observed in some embryos but did not follow a non-monotonic dose-response needed to conduct an EC50. Anisophthalmia was only observed 5 dpf. Control, low, and medium concentrations had 2–4% incidence whereas high concentration treatments had 5–8% incidence for all HNA

isomers. Individuals were monitored again at 7 dpf and anisophthalmia was reversed even at high concentrations. It is important to note that critical evaluation and identification of anisophthalmia were employed. Any disconformities between each contralateral eye's diameter or shape was needed for positive identification of anisophthalmia. Bent caudal peduncle incidence seen at 7 dpf abnormality assessment followed a non-monotonic dose-response in 1H2NA, 2H1NA, and 2H3NA treatments. Among individual isomers 1H2NA high treatment exhibited greatest prevalence of bent caudal peduncle (25%). 2H1NA and 2H3NA exhibited ~10% incidence rate at the highest treatment. No other cases of bent caudal peduncle occurred in any of the sampled embryos. No cases of microcephaly were identified during the 5 and 7 dpf assessments. Cranial edema was only observed in eleutheroembryos. Individuals receiving the 1H2NA 20 μ M treatment hatched as live embryos with 24% cranial edema incidence rate, 2H1NA had a single case in 30 μ M and one in 40 μ M treatments, and 2H3NA produced no cranial edema. Swim bladder inflation was assessed with eleutheroembryos and was recorded on day of hatch. This behavioral endpoint did not show a dose-response and was not different between HNA isomers. Reduced flight response was assessed in larvae on day of hatch and defined as larvae lying on bottom of well following tactile stimulation. Observations of 1H2NA exposed embryos showed 40% incidence rate at 20 μ M treatment, 2H1NA had 25% at 40 μ M treatment, and 2H3NA had 26% at 40 μ M. No cases of reduced response were recorded for lower treatment levels for each isomer. Larval length for all control groups remained constant for 1H2NA, 2H1NA and 2H3NA. Whereas a significant decrease in mean total length was observed as HNA isomer concentration increased. 1H2NA showed a statistically significant decrease in total length from control at 20 μ M (two-sample t -test, $t_{38} = 11.09$, $p < 0.001$), 95% CI for the difference in means (568.9, 829.2). A statistically significant decrease in total length from control occurred at 30 μ M for 2H1NA (two-sample t -test, $t_{36} = 7.7$, $p < 0.001$), 95% CI for the difference in means (246.6, 422.5), and 30 μ M for 2H3NA (two-sample t -test, $t_{35} = 5.3$, $p < 0.001$), 95% CI for the difference in means (188.7, 425.7). In general, variability increased with increase in concentration. The high variability observed in 40 μ M 2H1NA treatment was in part due to a small resultant sample size ($n = 6$), and lack of variability observed in 50 μ M 2H3NA treatment was due to a small resultant sample size ($n = 1$) due to mortalities.

Mean days required for hatching with respective 95% CI's are illustrated in Fig. 3. A dose-response relationship was observed between days to hatch and concentration for each HNA isomer except for a slight non-monotonic response in 1H2NA between control and 5 μ M and in 2H1NA between 10 μ M and 30 μ M. Concentrations at which mean days to hatch increased proved different among each HNA isomer. A statistically significant increase in mean days to hatching from control occurred at 20 μ M for 1H2NA (two-sample t -test, $p < 0.001$), 95% CI for the difference in means (-3.67, -0.8), and 40 μ M for 2H1NA (two-sample t -test, $p < 0.001$) and 2H3NA (two-sample t -test, $p < 0.001$), 95% CI for the difference in means (-2.0, -1.5) and (-1.4, -0.9). Variation of days to hatching within treatments was similar for each isomer, except for slightly greater variation in the highest treatments for 1H2NA and 2H3NA.

Both 1H2NA and 2H1NA showed an initial increase in heart rate at lower treatment vs that observed in controls in the second heart rate assessment (Fig. 4). This response was

followed by a steady decrease in heart rate as concentration increased with 1H2NA but not 2H1NA. In general, mean treatment level heart rate in 2H1NA varied and showed little positive or negative trend over treatment levels. 2H1NA had a statistically significant greater mean heart rate at 10 μM (two-sample *t*-test, $p < 0.001$), 95% CI for the difference in means ($-18.4, -7.5$). 2H3NA mean heart rate remained steady until 60 μM and continued to drop at 70 μM . A statistically significant decrease in mean heart rate from control occurred at 30 μM for 1H2NA (two-sample *t*-test, $p = 0.016$), 95% CI for the difference in means (1.7, 14.7) and 60 μM for 2H3NA (two-sample *t*-test, $p < 0.014$), 95% CI for the difference in means (7.3, 60.2). The highest treatment levels for 1H2NA and 2H3NA exhibited the greatest variability for this measurement. Mean heart rates measured at the 1st heart beat assessment (3 dpf), did not show any trends or differences within and between metabolites (data not shown).

3.2. Hatchling assessment

The trend in eleutheroembryo toxicity is similar to LC50 values generated using embryo data but greater concentrations were required for the former tests (Table 1). We had sufficient data to estimate 6H2NA toxicity when using the eleutheroembryo model. 6H2NA was the least toxic of all four isomers (227.6 μM) with a eleutheroembryo LC50 value 1.8-fold greater than 2H3NA (123.3 μM). LC50s for all four metabolites were significantly different from each other.

3.3. pH assessments

To determine putative effects of pH on experimental results, change in pH value was determined following addition of HNA isomers and following 96hr eleutheroembryo incubations. Addition of 1% DMSO into ERM resulted in a pH increase of 0.3. Addition of 1H2NA at 20.2 μM and 74.0 μM resulted in a 0.1 and 1.0 decrease in pH, respectively. 2H1NA at 47.7 μM and 102.2 μM resulted in a decrease in pH by 0.3 and 1.6. 2H3NA at 51.2 μM and 123.3 μM resulted in a 0.2 and 1.8 decrease in pH. 6H2NA at 227.6 μM decreased pH by 1.7. The initial ERM pH was 7.2, the greatest decrease in pH was observed with 2H3NA at 123.3 μM resulting in a final pH of 5.4. Eleutheroembryo 96-h pH toxicity assessment was performed to determine if pH effects influenced observed developmental toxicities. No embryo toxicity was observed at pH 7.0, 6.0, and 5.0. Two embryos out of 65 died at pH 4.0. 100% mortality rate was observed at pH 3.0 within the first 24 h.

3.4. EROD assessment

In vivo EROD was conducted with dechorionated STII medaka embryos incubated at the LC50 value for each HNA isomer or larval LC50 for 6H2NA. EROD activity was discernible in liver, gill arches, skin epithelium brain and gall bladder. Bile auto fluorescence was observed in gall bladder. DMSO controls (Fig. 5A) exhibited minimal fluorescence whereas embryos from 1H2NA, 2H1NA, 2H3NA and 6H2NA treatments exhibited qualitatively similar patterns of fluorescence indicative of CYP1A activity. In medaka liver dealkylated resorufin accumulated in biliary passageways, demonstrated by the net-like pattern illustrated in Fig. 5B–F. This pattern has been shown using 3D reconstructions of STII medaka intrahepatic biliary system to represent canaliculi and bile

preductular and ductular passageways (Hardman et al., 2007). In addition, we have imaged cells lining these passageways and shown their responsiveness to reference biliary toxicants (Hardman et al., 2008). Florescence from BNF treated embryos was used as a positive control and exhibited a high degree of EROD activity at 0.1 μ M. Under light microscopy, the gall bladder of non-monotonic embryos was enlarged compared to controls; and, all HNA-treated embryos exhibited PE.

3.5. CYP1A induction

Quantitative real time PCR was used for relative quantification of CYP1A transcripts in RNA isolated from HNA exposed embryos. Relative to non-treated fish, mean CYP1A transcript levels were roughly 562 ($\pm$ 143.3), 343.4 ($\pm$ 85.1), 352.9 ($\pm$ 109.3), 81.6 ($\pm$ 15.2) and 27.9 ($\pm$ 4.1)-fold higher compared to DMSO control for BNF, 1H2NA, 2H1NA, 2H3NA and 6H2NA, respectively, following a 48 h exposure (Fig. 6). Thus, significant differences in CYP1A inducibility were observed at this sampling time and respective concentrations.

3.6. AHR transactivation

A luciferase reporter-based system was developed to determine if HNA isomers initiate AHR-mediated transcription. Transactivation assays incorporated the mouse AhR (mAhR) and a medaka CYP1A promoter reporter vector (mf1Ap-Luc) containing 6 putative AHRE's. Mutant mouse hepatoma cells (Hepa-1c1c7 c12 cells) with significantly reduced constitutive AHR activity were used in order to prevent high background levels of reporter activity and to definitively identify AHR-specific ligands. Dioxin (TCDD) was used a positive control for reporter assay development and optimization. Prior to assay optimization, mAHR was transfection into Hepa-1c1c7 c12 cells and evaluated via PCR to ensure constitutive expression. Transactivation of the mouse AhR demonstrated a dose-response relationship with all HNAs tested (Fig. 7). Relative to vehicle-treated cells, significantly induced reporter activity was detected at 1–100 μ M for 1H2NA and 2H3NA and 10, and 100 μ M for 2H1NA and 6H2NA. Interestingly 2H3NA demonstrated the most responsive dose-response across concentrations tested and all HNA isomers demonstrated comparable activity at 100 μ M.

4. Discussion

The approach used in this set of studies included molecular-through individual levels of biological organization and provided putative evidence for possible mechanisms of toxicity while establishing a strong foundation in effects profile for specific PAH (HNAs) that are found in contaminated soils and sediments (Parikha et al., 2004). The study was restricted to embryos and eleutheroembryos of medaka illustrating that with these metabolites embryos can be used rather than juvenile and adult fish for ectotoxicological research purposes. Results in medaka embryos characterized developmental toxicity of each HNA metabolite, further illustrating the potential importance of embryos in environmental risk characterizations.

Using published and original methods for investigating early life stage (ELS) toxicity in medaka, we illustrate that each of the four metabolites exhibited specific PAH like toxicity at varying concentrations (Hardman et al., 2008; Oxendine et al., 2006b). Differential toxicity was observed in rate of embryonic development, EC50, and LC50 values in

embryos, *in vivo* EROD activity, CYP1A induction and mouse AhR transactivation. Embryonic assessment was extended to eleutheroembryos and the latter confirmed that differential toxicity occurred in the absence of a chorionic semipermeable barrier. It was initially proposed that the chorion might serve as a protective barrier thereby limiting uptake and exposure of embryos (Villalobos et al., 2000). Results were opposite of those expected. Eleutheroembryos or yolk sac larvae showed decreased susceptibility to HNA toxicity as evident by an increase in LC50 values approximately 2-fold greater than those of embryos (Table 1) except for the 6H2NA which proved to be non-toxic to embryos. This finding suggests that the window of susceptibility for these compounds is during embryogenesis. Interestingly, eleutheroembryo toxicity followed the same rank order of metabolite toxicity observed in developing embryos.

The 96-well plate format again proved to be a useful and efficient way to assess multiple endpoints with a single exposure population (refer to study by Oxendine et al., 2006b). The ability to follow individual embryo development throughout the metabolite exposures was essential for development of an effect profile and aided in performing statistical assessments. Tracking embryo development was crucial when satisfying statistical criteria. Particularly endpoints that required larger sample sizes to achieve desirable power, such as mean days to hatch, LC50s, determinations. The ability to follow single individuals is unique to micro-plate test chambers and allowed for identification of very sensitive endpoint evaluations such as natural sample variation, where trends in individual development scores compared to sample scores revealed sample population characteristics over time. Additionally, anisophthalmia, observed early, and followed serially in individuals, was shown to undergo correction in later stages of development. Without the ability to follow and track progression of this abnormality in affected individuals, this information would likely have been lost.

Multiple linear regression, proved extremely useful for the fitted regression model (Fig. 2) which demonstrated how interpretation of model selection tests (ESS *F*-test) and fitted model parameters could be adapted to identify significant differences in target endpoints. Model parameter manipulation can be used for estimating responses, inverse prediction of indicators, and to perform effect estimations. It is important to note that experimental design characteristics must be considered in order to use this method. Assumption of normality of errors exists with any regression modeling technique requiring adequate sample sizes and/or transformations. Alternative transformations such as quadratic functions could prove useful if a non-linear response occurs but model interpretation could be difficult. In the present analysis only three common treatment levels could be compared; control, 30 μ M, and 40 μ M. Thus a preliminary screening was conducted to find overlapping dosing. By randomizing individuals, the scope of inference enabled extrapolation to brood stock populations. And, since prescribed treatment levels were randomized, it can be determined that HNA metabolites are part of the causative pathway to observed toxic effects.

During HNA toxicity effects profiling, we observed specific circulatory abnormalities in exposed embryos including TH, PE, and pooled blood. TH has been associated with blue sac syndrome described in great lake salmonids (Wolf, 1969). We observed a progression of effects that led to tube heart formation following exposure to HNAs. As embryo

development progressed, blood circulation began and followed stages of development as outlined by Iwamatsu (1994). Little if any circulatory abnormalities were observed in our development assay at first heart rate assessment (day 3). At this stage in development the heart, sinus venosus, atrium, ventricle, and bulbous arteriosus were differentiated. Initial toxic response was observed as a decrease in heart rate identified in our second heart rate assessment at 5 dpf. PE followed these initial responses in affected embryos. Severe PE was associated with elongated TH phenotype. It is important to note that there was a continuum of TH phenotype that was objectively scored using illustrations provided by Solomon and Weis (1979). Pooled blood was associated with severe high scoring phenotypes; individuals in this condition showed weak heart contractions, suppressed blood circulation, PE and TH. Although, PE was often seen in individuals with TH it was not a complete association. PE occurred at lower concentrations than did TH also reported by Marty et al. (1990).

Interestingly, cardiovascular toxicity was not observed until 96 hpf when substantial liver circulation, gall bladder pigmentation and vascular rearrangement are occurring throughout the embryo. Several mechanisms of PAH-induced cardiovascular toxicity have been proposed, including: activation of the AhR, induction of CYP1A, and generation of ROS. Teraoka et al. (2003) demonstrated reduced TCDD induced cardiovascular toxicity following morpholino knock down of the AhR during zebrafish embryo development. Later, Carney et al. (2004) additionally confirmed protection of TCDD induced PE by blocking AhR gene expression. This effect proved isoform specific with AhR2 but not AhR1 antagonizing effects of TCDD. This was further confirmed by Billiard et al. (2006) who demonstrated that AhR2 knockdowns reduced the synergistic cardiovascular toxicity in zebrafish embryos co-treated with BNF and aphanaphthoflavone (ANF). AhR is known to regulate numerous genes associated with xenobiotic metabolism, cell proliferation, maintenance of ROS, and additional cellular functions suggesting that multiple putative AhR dependent mechanisms of toxicity might be involved (Cantrell et al., 1996; Dong et al., 2002; Fisher et al., 1990; Puga et al., 2000; Frueh et al., 2001). Surprisingly however, morpholinos targeting CYP1A did not result in attenuation of cardiovascular effects suggesting that CYP1A is not associated with observed cardiovascular toxicities. The lack of attenuation by CYP1A interferences suggests that production of ROS may not be a significant mechanism of cardiovascular toxicity (Schleizinger and Stegeman, 2001). Recently microarray studies demonstrated that in addition to classical AhR known gene targets, TCDD exposure in zebrafish heart resulted in expression of genes associated with cardiac development, contractility, growth and development (Carney et al., 2004). While classical AhR mediated gene expression figures prominently as mechanism of action, non-classical activities including initiation of specific signal transduction pathways have also been demonstrated (Matsumura, 2003).

In this study we observed cardiovascular toxicity such as PE and TH, highly similar to those demonstrated with TCDD and other co-planar aromatics that are known to bind the AhR. This led us to the hypothesis that HNA metabolites may be AhR ligands. To further investigate this mechanism we employed molecular assays to detect and quantify P450 induction. We used an *in vivo* EROD activity assay to qualitatively evaluate temporal and spatial localization of CYP1A expression. From our effects data we consistently

demonstrate an order of toxicity based on evaluations of LD50, development, PE and TH as 1H2NA > 2H1NA > 2H3NA > 6H2NA. For our molecular assessments all embryos were exposed at respective LC50 concentrations to effectively normalize CYP1A expression and associated toxic response across each metabolite. Using this approach we anticipated all metabolites would result in a similar CYP1A and/or dose–response. Maximal CYP1A induction was observed at 48 h post exposure throughout the outer integument, gills, and liver (left longitudinal leaflet, Hinton et al., 2004) for 1H2NA, 2H1NA, and 2H3NA in, dechorionated STII embryos. Control embryos showed very little fluorescence in the outer integument and no fluorescence in the liver. It is important to note however that auto fluorescence in the gall bladder was observed in all embryos and was thus not considered in assessment of CYP1A activity. Qualitatively, activity for 1H2NA, 2H1NA and 2H3NA appeared similar in both temporal and spatial expression. Lower levels of in vivo EROD activity for 6H2NA however was counterfactual considering that embryos had severe pericardial edema, again providing some evidence that HNA induced cardiovascular abnormalities may not be linked to CYP1A activity. To further this evaluation we conducted qPCR to measure CYP1A transcript (mRNA) levels in whole homogenates of HNA exposed embryos. Interestingly, results for qPCR followed the same rank order of toxicity demonstrated for LC50, PE, TH, or development even when normalized by exposing at relative LC50s. While 1H2NA and 2H1NA were highly similar (343.4 and 352.9-fold induction), 2H3NA and 6H2NA were significantly lower by comparison (81.6 and 27.9, respectively). Both in vivo EROD and qPCR data suggest that the four HNA isomers activate medaka CYP1A and thus are putative ligands for the AhR with associated induction of CYP1A.

To confirm AHR binding, we conducted transient transfections assays with the mouse AhR and a medaka specific CYP1A promoter reporter construct. Assays were conducted in mouse c12 cells deficient in endogenous AhR. Dose–response experiments demonstrated that at concentrations between 1 and 100 μ M selective activation of AhR occurs with 1H2NA and 2H3NA demonstrating the highest activity at lower doses. At 100 μ M, transactivation of mouse AhR by all four of the HNA isomers was equivalent. Rank activation of the AhR by PAHs has been demonstrated previously (Barron et al., 2004). While typically weaker than TCDD or co-planar-PCBs, PAHs with common structural features including 4–6 rings containing fluoranthene or phenanthrene structures and an exposed bay region possess AhR binding activities. Interestingly, two and three rings unsubstituted PAHs are generally inactive unlike our observations with the HNA tested. While it appears that HNAs are AhR ligands and do result in AhR mediated gene transcription, the exact mechanism associated with their developmental toxicities is still to be determined. There is recent evidence however that low molecular weight PAHs may result in developmental effects through AhR independent mechanisms and that AhR may in fact play an adaptive or protective role against PAH induced cardiovascular and developmental toxicity (Incardona et al., 2005, 2006). In these studies knockdown of AhR1, AhR2 and CYP1A does not rescue toxicities associated with low molecular weight weathered crude oil or tricyclic PAHs. As for the HNAs, further experimentation using molecular, genomic and biochemical approaches will provide a clearer picture of the molecular connection between HNA exposure and observed cardiovascular phenotypes.

Considering that some PAHs are environmental pollutants that are readily metabolized in contaminated environments, our finding that 3 out of 4 HNA metabolites are toxic is of great importance in risk assessment formulation. In light of this we suggest that hazard ID and risk determinations during formal risk assessment preparation should include environmental fate and toxicity information regarding PAH metabolites. Parikha et al. (2004) reported that 1H2NA demonstrated less sorption and more desorption to organic estuarine sediments at environmentally relevant temperature and pH than did the parent compound, phenanthrene. It was postulated that lower sorption of 1H2NA reflects its greater polarity and water solubility. Thus PAH metabolites could be more bio-available and biologically active than parent compounds. In addition, concentrations of phenanthrene required to produce cardiovascular toxicity in developing zebrafish exceeded 56 μM . This concentration is above the 1H2NA EC50 value for PE (19.1 μM). Assuming that most fish embryos respond similarly, we can postulate that 1H2NA is/will be more toxic than its parent compound, phenanthrene. If risk assessments are conducted using information on parent compounds only, where most of the toxicity information is available, risk characterizations will not reflect real-world scenarios in which parent compounds are metabolized. Results demonstrated in this report will aid risk assessors in providing information on the four NA metabolites characterized herein.

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Fig. 1.

Embryonic development assessment results with respective 95% confidence intervals for each metabolite. (a) 1H2NA, (b) 2H1NA, and (c) 2H3NA. Development scores were summed for each individual embryo and averaged to generate a mean and standard deviation for each treatment group at each time point. Scores were only determined for live embryos. Embryos were considered viable if active heartbeat and circulation of pigmented blood cells was present in yolk sac vessels.

Fig. 2.

Separate lines multiple linear regression modeled development score for 40 μ M treatment after accounting for combinations of interactions. Estimated mean score for 1H2NA (solid), 2H1NA (dotted), and 2H3NA (dashed).

Fig. 3. Mean days to hatch post fertilization for dose (5–50 μ M) and time (8.5–12 days) for 1H2NA, 2H1NA, 2H3NA isomer with respective 95% confidence intervals.

Fig. 4. Mean heartbeats per minute for 1H2NA, 2H1NA, 2H3NA between 5 and 70 μ M measured during 2nd heartbeat assessment with respective 95% confidence intervals.

Fig. 5.

In vivo imaging of hepatobiliary metabolism of ethoxyresorufin: widefield fluorescence microscopy. widefield fluorescence (TRITC) image capture of in vivo ethoxyresorufin for detection of CYP1A metabolic activity. Dechorionated embryos exposed (aqueous bath) to the HNAs at respective EC50s and CYP1A substrate ER exhibited ethoxyresorufin-*O*-dealkylation (EROD) activity, which resulted in the generation of the fluorescent metabolite resorufin (red, C2). Transport of the metabolite through the intrahepatic biliary passageways of the embryonic liver and concentration in the biliary tubules, was imaged in vivo in living dechorionated embryos 6 dpf. Resorufin fluorescence is distinct and limited to the intrahepatic biliary passageways and gall bladder. (A) DMSO control, (B) BNF, (0.1 μ M) (C) 1H2HA (20.23 μ M), (D) 2H1NA (47.65 μ M), (E) 2H3NA (51.17 μ M), and (F) 6H2NA (227.63 μ M).

Fig. 6.

Fold change of medaka cytochrome P450 1A (CYP1A) mRNA in HNA-treated liver.

Results relative to CYP1A induction over DMSO controls and normalized to expression of β -actin following a 48 h exposure. (a) BNF (0.1 μ M) (b) 1H2HA (20.23 μ M), (c) 2H1NA (47.65 μ M), (d) 2H3NA (51.17 μ M), and (e) 6H2NA (227.63 μ M).

Fig. 7.

Transactivation of mouse AhR. AHR:AHRE-driven luciferase reporter assay system for mouse AhR and medaka CYP1A promoter in mutant Hepa-1c1c7 c12 cells. Cells were transfected with 250 ng mf1Ap-Luc, 0.05 ng pRL-CMV, and 50 ng pSPORT-mAHR for 5 h. *Renilla* luciferase (pRL-CMV) was used for transfection normalizations and pGL-basic and/or empty pSPORT vectors were used for controls. HNAs (0.1 and 100 μM) were added immediately post transfection for a total of 18 h following cell harvest and assay for firefly and *Renilla* luciferase activity.

Table 1
Embryo and larval toxicity assessment LC50 results with respective 95% confidence

Isomer	LC50 (μM)	Lower CI (μM)	Upper CI (μM)
<i>Embryo</i>			
1H2NA	20.2	18.9	21.6
2H1NA	47.7	46.3	49.0
2H3NA	51.3	50.1	52.3
6H2NA	na	na	na
<i>Larval</i>			
1H2NA	74.0	70.9	77.3
2H1NA	102.2	98.8	105.7
2H3NA	123.3	119.3	127.4
6H2NA	227.6	219.9	235.7

na – no mortality observed at maximal concentration within solubility limits. Highest concentration tested 300 μM.

Table 2
Embryonic development teratogenic effects and behavioral abnormality EC50 values for
HNA isomers with respective 95% confidence intervals in parentheses

	1H2NA (μM)	2H1NA (μM)	2H3NA (μM)
Tube heart	27.6 (22.4, 34.0)	49.3 (na)	52.6 (50.4, 54.9)
Pericardial edema	19.1 (15.3, 24.0)	40.5 (37.18, 44.13)	48.6 (46.2, 51.2)
Pooled blood	27.7 (24.5, 31.4)	na	54.0 (52.6, 55.5)
Delayed hatch	na	44.0 (41.52, 46.65)	48.0 (46.0, 50.2)
Partial hatch	22.0 (20.0, 24.3)	na	51.3 (49.9, 53.0)